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Genetic and Molecular Analysis of Transgenic Rice cv. Rojolele Expressing Lactoferrin

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Abstract

In a previous study, human lactoferrin gene have introduced into Javanica rice cv. Rojolele by *Agrobacterium*-mediated transformation. Lactoferrin (LF) is an 80 kDa iron-binding glycoprotein that has been proposed to have many biological roles such as protection against microbial and virus infection. This study aims to analyze the integration and level of lactoferrin gene expression of transgenic rice cv. Rojolele. The study also aims to examine the genetic character of transgenic rice expressing recombinant lactoferrin. Stability expression of recombinant lactoferrin transgenic rice seeds over generations were analyzed by ELISA, while the integration stability of recombinant hLF gene in transgenic plants performed by PCR. The mitotic time, cell cycle and chromosome characterization of transgenic and non-transgenic rice cv. Rojolele were determined. Chromosome characterization of the trangenic and non transgenic rice cv. Rojolele was investigated to determine the genetic variation. All of the above efforts were aimed to evaluate the genetically engineered rice containing recombinant lactoferrin as a nutraceutical food. The results showed that the expression was stable through three consecutive generations. The expression of the *hLF* gene increased during grain-filling period. The active time of mitotic cells of transgenic rice rojolele was longer than the cells of non-transgenic rice. In addition, the cycle cell of transgenic and non-transgenic rojolele contained prophase, prometaphase, metaphase, anaphase, telophase and interphase. The result showed that all of the transgenic lines had diploid (2n) chromosome number = 24.

Keywords: lactoferrin, transgenic rice cv. rojolele, karyotype, mitotic time

1. Introduction

Rice is one of the very few major food sources of the world. Rojolele is a famous of Javanica rice cultivar that is cultivated commercially in Indonesia. Rojolele has become a popular local rice variety in Central Java and is distributed throughout Indonesia because it has an aromatic fragrance and a delicious taste (Mudjisihono et al., 2002). Rachmawati et al. (2005) have been introduced the lactoferrin gene into Javanica rice cv. Rojolele. The transgenic plants expressed recombinant lactoferrin in the grain. Further studies are needed to determine the usefulness of engineered rice as a nutraceutical food.

Human milk proteins are considered to play many biological activities that are beneficial to the newborn infants, such as protection against infection. Lactoferrin (LF) is an 80-kDa iron-binding glycoprotein present of high concentrations (average 1-2 g/l) in human milk (Nandi et al., 2002). It is a bioactive milk protein playing an important role in the immune system response and contributing to the protection of the body against infections (Brock et al., 2000). In addition to the stimulation of the immune system, scientific studies have revealed that lactoferrin also prevents the growth of pathogens, exhibits antibacterial and antiviral properties, controls cell and tissue damage caused by oxidation, and facilitates iron transport (Lönnerdal, 2000; Tomita et al., 2000; Brock, 2002). Moreover, it has been reported that lactoferrin is a protein contributing to the growth promotion of Bifidobacteria (Kim et al., 2004).

We have expressed the human lactoferrin protein (hLF) in rice cultivar Rojolele under the control of the promoter ubiquitin. This protein was genetically fused to the signal peptide from rice glutelin. The expression levels of rhLF significantly increased in the mature seeds. A considerable amount of recombinant hLF (rhLF) in seeds were approximately 15% of the total soluble protein (Rachmawati et al., 2005). However, protein expression level depends on both production and stability. The factors that control high-level accumulation of recombinant proteins in seed include promoters and enhancers, subcellular trafficking and targeting of the desired polypeptides or proteins (Boothe et al., 2010). Subcellular targeting plays an important role in determining the yield of recombinant proteins because the compartment where a protein accumulates can strongly influence the interrelated processes of folding, assembly, and post-translational modification (Fischer et al., 2004). Many factors contribute to variation in transgene expression, including the position of transgene integration, copy number of transgene and configuration of transgenic locus, as well as mechanisms of epigenetic silencing (Iglesias et al., 1997; Fagard & Vaucheret, 2000; Ma et al., 2003). When transgene integrate into genomic DNA, the expression level is often influenced by the surrounding chromatin (Gelvin, 2003). Transgene copy number can greatly affect the expression level and genetic stability of the target gene (Donnarumma et al., 2011).

In contrast to the rapid progress of molecular analysis of the rice genome, only limited success has been achieved toward a cytogenetic characterization of the rice genome. Karyotypes of japonica and indica rice has been reported by Kurata and Omura (1978), while javanica rice has not been reported yet.

The aim of this study was to analyze the stability of recombinant human lactoferrin expressed in rice seeds. The study also aims to examine genetic character of transgenic rice expressing recombinant lactoferrin. All of the above efforts aimed to evaluate the genetically engineered rice containing recombinant lactoferrin as a nutraceutical food.

2. Methods

2.1 Plant Materials

Javanica rice cv. Rojolele was obtained from Yogyakarta Assessment Institute for Agricultural Technology (DIY AIAT), Indonesia. Transgenic rice cv. Rojolele expressing recombinant human lactoferrin gene was obtained through Agrobacterium-mediated transformation (Rachmawati et al., 2005). Transgenic lines TR-7, TR-8, and TR-10 carried mature human lactoferrin gene was fused to the DNA sequence encoding the rice glutelin signal peptide with the constitutive maize ubiquitin-1 promoter. The transgenic lines TR-7, TR-8, and TR-10 contained 2, 1 and 3 copies of hLF gene. Transgenic and non transgenic were analyzed for cell cycle and karyotype, stability of expression and integration of recombinant human lactoferrin.

2.2 Expression of Recombinant Human Lactoferrin (rhLF)

The expression levels of rhLF in the transgenic plants were quantified by sandwich ELISA. A 96-well microtiter plate (Nunc-Immunoplate C96 Maxisorp, Denmark) was coated with 100 µl of rabbit anti-hLF (1 µg/ml) in PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl) at 4°C overnight. After the plate was equilibrated at room temperature for 1 h, the coating solution was removed and the plate was washed with PBS-T [PBS containing 0.05% (v/v) Tween-20]. Then 200 µl of blocking solution [1% (w/v) non-fat dry milk (Nacalai Tesque), 0.1% Tween-20, 0.05% (w/v) Merthiolate (SIGMA) in PBS] was added to the well. The plate was incubated at 4°C overnight and washed three times with PBS-T. Protein samples (100 µl) at an appropriate dilution and standard LF were prepared in the blocking solution and then added to the wells. The plate was incubated at 37°C for 1 h and washed with PBS-T. Immunodetection was performed at 37°C using rabbit anti-hLF antibody (Fab') and goat anti-rabbit IgG coupled to HRP at 1:1000 dilutions in PBS-T. One hundred microliters of the diluted HRP-conjugated secondary antibody were added into each well and the plate was incubated at 37°C for 1 h. After three times washing with PBS-T, staining was initiated by adding 100 µl of substrate orthophenyldiamine dichloride in 0.05 M Phosphate-Citrate buffer and the plate was incubated at room temperature for 10 min. The reactions were stopped with 100 µl of 1 M H_2SO_4 per well. The plates were read at 490 nm. Raw ELISA data were converted to nanogram/milliliter of rhLF of total soluble protein by reference to an ELISA standard curve constructed using native hLF. The rhLF expression over generations were statistically analyzed using T test at significancy level of 95%.

2.3 Integration and Segregation of hLF Gene

Integration of the hLF gene under the control of a constitutive promoter over generations was performed by PCR analysis. Genomic DNA was isolated from mature leaves according to the method of Murray and Thomson (1980). Briefly, one gram of fresh leaf tissue was homogenized to a powder in liquid nitrogen by a mortar and a

pestle. The tissue powder obtained was suspended in 5 ml CTAB extraction buffer [2% CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl (pH 8.0), 0.2% 2-mercaptoethanol] and incubated at 60°C for 30 min. The suspension was then purified by phenol:chloroform:isoamylalcohol (25:24:1) extraction and precipitated by 2-propanol. The DNA was dissolved in TE buffer [10 mM Tris-HCl (8.0), 1 mM EDTA].

PCR analysis was carried out in a 20 µl reaction mixture containing 200 µM of dNTPs (1 µl), 0.5 µM of each primer (1 µl), 0.02 unit Taq DNA Polymerase (0.2 µL) (Takara Bio Inc), 1x Taq Buffer (2 µl), genome DNA (1 µl), and ddH_2O (15.8 µL). The amplification reaction was 1 cycle of 95°C for 2 min followed by 30 cycles of 95°C for 1 min, 54°C for 2 min and 72°C for 3 min, and one cycle of 8 min at 72°C. This reaction was carried out in an eppendorf Mastercycler personal. Primers used for amplification of the hLF gene were LF8: 5'-TCACTGCCATCCAGAACTTG and LF9: 5'-TCTGATCTCCTAACCACCGC. The primers amplified an internal hLF sequence of 356 bp. The amplification products from transgenic and non-transgenic plants were separated on a 1.5% agarose gel and visualized by UV fluorescence of the ethidium bromide-stained DNA. Segregation of hLF gene on transgenic rice was determined based on PCR analysis followed by chi-square test.

2.4 Expression of rhLF During Rice Endosperm Developments

Expression of rhLF during endosperm development of the rice was monitored at 7, 14, 21, 28, 35, and 42 DAP (Day After Pollination). Immature spikelets were harvested at 7, 14, 21, 28, 35, and 42 DAP (Day After Pollination) and analyzed for the rhLF expression by ELISA. Expression of rhLF in transgenic rice during endosperm development was confirmed by SDS-PAGE and Western blot analysis .

The extracted proteins from transgenic rice seeds (5 µg) were treated with sample buffer [60 mM Tris-HCl (pH 6.8), 10% (w/v) glycerol, 2% (w/v) SDS, and 0.01% bromophenol blue with 5% (v/v) 2-mercaptoethanol] and were boiled for 5 min at 99°C. SDS-PAGE was performed according to the method of Laemmli (1970) using a 10% polyacrylamide gel. Electrophoresis was performed in 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS buffer at 20 mA for 30 min. The proteins in the gel were stained with Coomassie Brilliant Blue (Rapid Stain CBB Kit, Nacalai Tesque). For western blot analysis, the separated proteins in the polyacrylamide gel were transferred to transfer buffer [12.5 mM Tris-HCl (pH 8.8), 86mM glycine, 10% MetOH] and transblotted onto a 0.45 µm PVDF membrane (Nihon Eido, Japan) using a semidry-blotting apparatus (Nihon Eido, Japan) for 1 hour at 140mA/gel. The blot was blocked with 5% (w/w) skimmed milk in PBS-T (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, 0.01% Tween-20) for 1 h followed by three washes with PBS-T for 10 min each times. PBS-T solution containing 5% skimmed milk was used to block nonspecific binding of primary antibody. The blot was incubated in the primary antibody [rabbit anti-hLF antiserum was diluted at 1:1000 in PBS-T] for 1 h followed by three washes in PBS-T. The blot was incubated in the secondary antibody solutions [goat anti-rabbit IgG HRP-conjugate (BIORAD) was diluted in PBS-T at a 1:1000 in PBS-T] for 1 h at room temperature. After three washes, the blot was stained with HRP staining solution using Konika immunostaining HRP1000 until the signal of the positive control was clearly visible. The staining was stopped by distilled water.

2.5 Studies on Cell Cycle and Karyotype of Transgenic and Non Transgenic Rice

Chromosomes were prepared according to the methods described by Jashier and Tanguy (1996). Seeds of transgenic and non transgenic rice were sown and germinated in petridish. The root were cut about 3-5 mm and used as sample for chromosome preparation. Chromosome preparation was conducted from 08.00 a.m. to 10.00 a.m. with 15 minute intervals. Fresh root tips of germinated seeds were fixed in 45% acetic acid at 4°C for 15 minutes. Fixed root tips were then macerated in 1 N hydrochloric acid for about 11 minutes at 55°C. The root tips were stained in 1% aceto-orcein for about 24 hours before they squashed. The slides were then photographed using Olympus C-35-AD-4 and Fuji film ASA 200.

Characterization of chromosomes and karyotype formulation based on the number of chromosomes, length of the short arm (p) and long arm (q), the absolute length of chromosomes (p + q) and centromere index. The centromere index was calculated using the following formula: Centromere Index = (length of the long arm/ the absolute length of chromosomes) x 100.

The measurement of chromosome size was made on the chromosomes observed at prometaphase using Adobe Photoshop CS2 for Windows program. Centromere position of chromosome was classified by centromeric index calculated by short arm/ total length following Levan et al. (1964). Metacentric chromosome with centromeric index of 37.50-50.00; submetacentric chromosome with centromeric index of 25.00-37.49; subtelocentric chromosome with centromeric index of 12.5-24.99; and telocentric chromosome with centromeric index of 0-12.49. Data of chromosome size and centromere position of chromosomes were then arranged to construct karyogram using Adobe Photoshop CS2 for Windows program, and idiogram using CorelDRAW Graphic Suite

X3. Characterization of the genetic trait of transgenic and non transgenic rice including chromosome number, mitotic time, cell cycle and karyotype.

3. Results

3.1 Expression of Recombinant Human Lactoferrin

In this study, the T2 and T3 seeds of transgenic rice expressed recombinant human lactoferrin were analyzed to determine their stability. The stability of the rhLF expression under the control of a constitutive promoter of the three transgenic lines TR-7, TR-8 and TR-10 were examined using ELISA. The rhLF expression levels in the T1 seeds varied among transgenic lines, such as TR-7, TR-8 and TR-10 were 1.6 mg/g DW of dehusked seeds, 2.0 mg/g DW of dehusked seeds and 1.0 mg/g DW of dehusked seeds. The rhLF expression levels in the T1 seeds were almost identical with those in the T2 seeds as well as T3 seeds for the same transgenic lines (Figure 1). The results indicated that recombinant lactoferrin in mature seeds was stable through three consecutive generations.

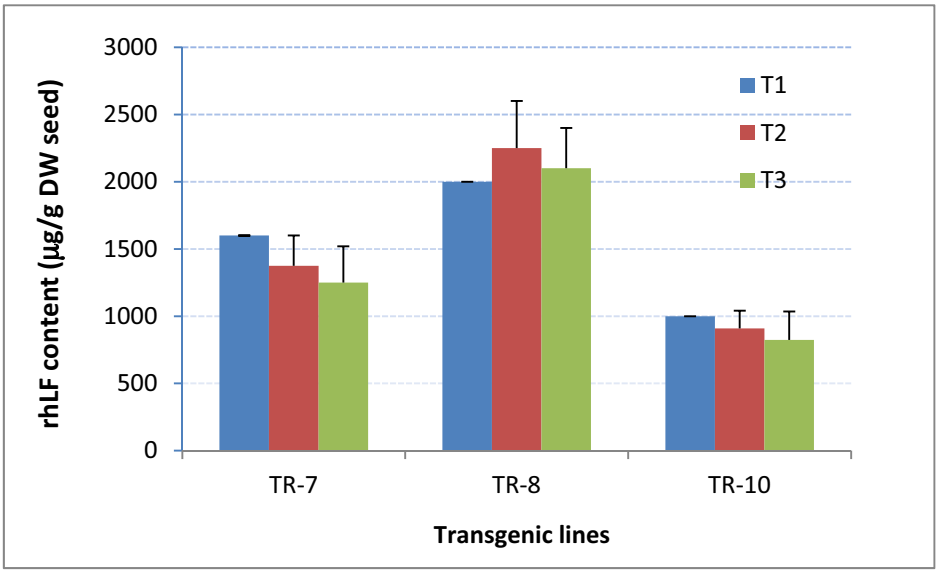

Figure 1. Expression levels of rhLF in T_1, T_2 and T_3 seeds

3.2 Integration and Segregation of hLF Gene

Segregation of hLF on transgenic rice was determined based on PCR analysis followed by chi-square test (Table 1). From the calculation of chi-square test on transgenic lines TR-7 and TR-8 showed the value of $\chi 2 = 1.333$ and 2.557. From table $\chi 2$ with degrees of freedom 1, the P-value lies between 0.1 and 0.3. Because P-values are higher than 0.05, therefore data meet the 3:1 ratio and can be said that the segregation of these transgenic lines followed the Mendelian patterns. Meanwhile, for TR-10 showed the 15:1 ratio with the $\chi 2 = 0.938$. Based on the table $\chi 2$ with degrees of freedom 1, the P-value lies between 0.3 and 0.5 and can be said that segregation of transgenic line TR-10 also followed the Mendelian patterns.

Table 1. Segregation of hLF gene in seeds of transgenic rice Rojolele in 3rd generation

Transgenic line	*hLF* copy number in T_0 generation[1)]	Number of seeds analyzed		$\chi 2$
		Total	LF^+	
TR-7	2	100	80	1.333
TR-8	1	95	78	2.557
TR-10	3	92	84	0.938

[1)] The copy number in the T_0 generation was determined by Southern blot analysis with a *hLF* probe. (Rachmawati et al., 2005).

3.3 Expression of rhLF During Rice Endosperm Developments

The rhLF expression during endosperm development of the rice transgenic lines were monitored in TR-7. Immature spikelets were harvested at 7, 14, 21, 28, 35, and 42 DAP (Day After Pollination) and analyzed for the rhLF expression by ELISA. The rhLF expression in seed of transgenic lines TR-7 were detected at 7 DAP (still in milky stage) and dramatically increased at 21 DAP, thereafter the rhLF content slightly increased through seed maturation (Figure 2A). These results were consistent as reported by Bechtel and Juliano (1980) that protein body deposition begins about 7 days after pollination and gradually increased during rice endosperm development. Based on SDS-PAGE and western blot analysis (Figures 2B-C), high level of rhLF was detected on mature seeds of transgenic rice cv. Rojolele.

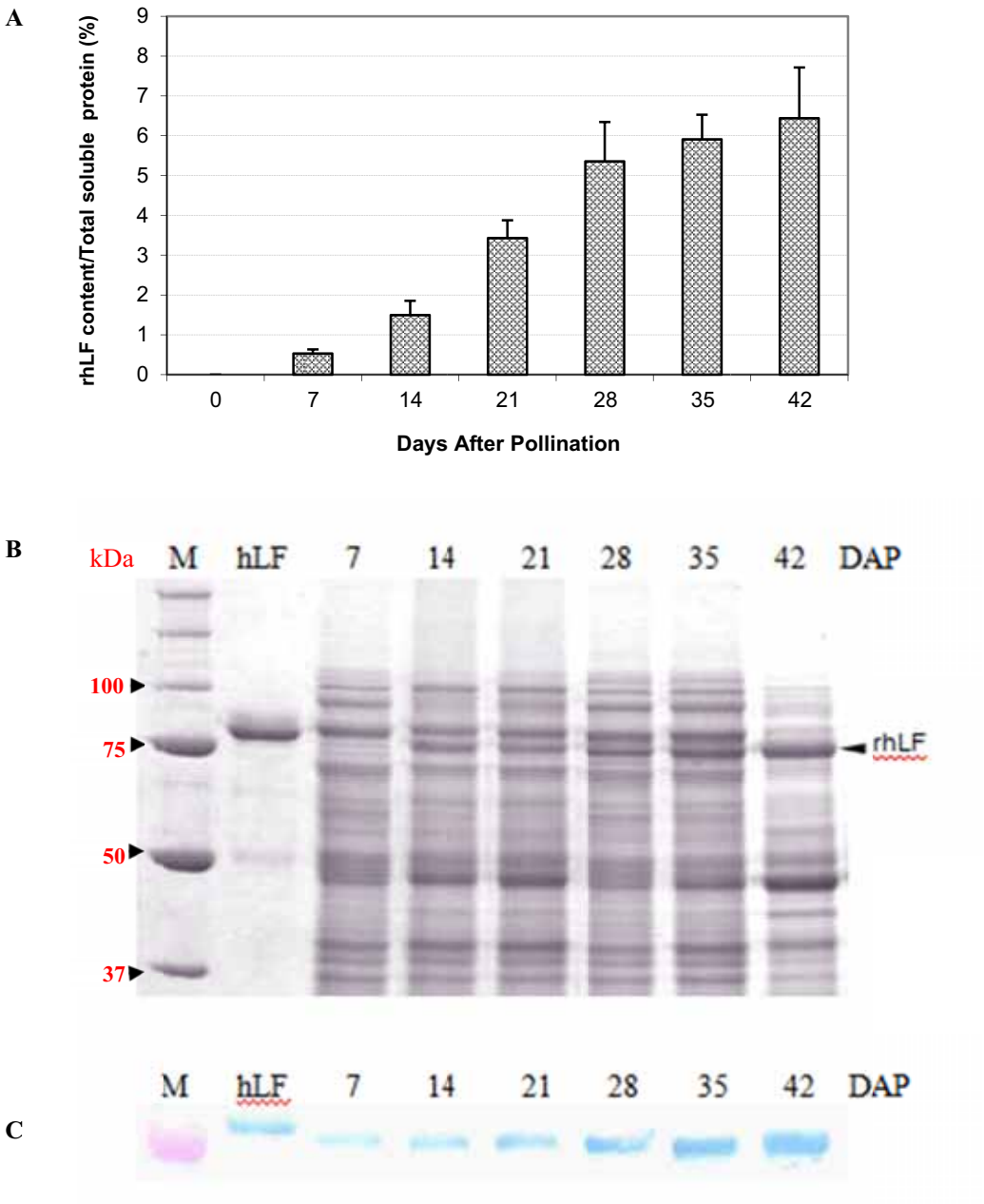

Figure 2. Expression of rhLF during rice endosperm developments. Immature spikelets at 7, 14, 21, 28, 35, and 42 DAP (Day After Pollination) were analyzed for rhLF expression by ELISA (A), SDS-PAGE staining with CBB (B) and Western blot analysis (C)

As shown in figure 2B-C, SDS-PAGE staining with CBB and western blot analysis demonstrated that the molecular weight of rhLF in transgenic rice was slightly smaller than that of native hLF. Based on MALDI-TOF Mass spectrometry analysis the molecular weight of rhLF was 77801 Da, while the molecular weight of native hLF was 79827 Da.

3.4 Studies on Cell Cycle and Karyotipe of Transgenic and Non Transgenic Rice

In this research, transgenic rice expressing recombinant human lactoferrin were used as material. Studies of the cell cycle obtained active time of mitotic cells in transgenic rice cv. Rojolele TR-7, TR-8 and TR-10 were longer than the cells of non-transgenic rice. At that time there were many cells in a state prometaphase. In prometaphase, the chromosomes are in the form of the most solid and well dispersed, therefore easy to observe.

Based on observation, the number of chromosomes of rojolele transgenic rice and non-transgenic are 2n = 24 (Figures 3A, 3B, 3C and 3D). Three lines of transgenic rice investigated in this study had similar chromosome number (2n = 24) to the non-transgenic rice. This amount is in accordance with the research on the rice chromosome number of Cianjur 2n = 24 (Daryono & Sumardi, 1996). The findings of the research are expected to enrich valuable information concerning the genetic identity and diversity of rice.

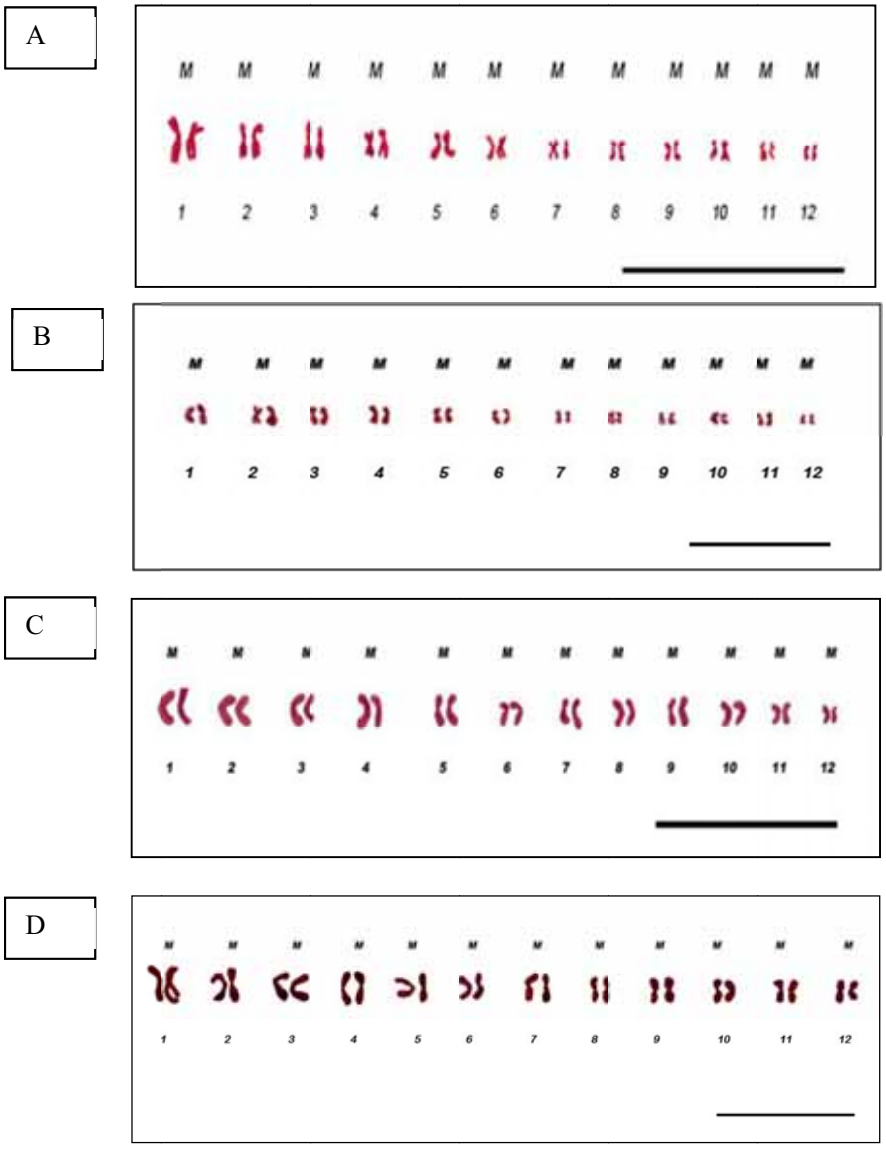

Figure 3. Karyotype of transgenic rice expressing recombinant lactorferrin. A: non-transgenic B:TR-7, C:TR-8, and D:TR-10. Scale bar correspond to 0,5 μm. (m = metacentric chromosome; number under the karyotype correspond to chromosome pairs)

From the result showed in Figure 3, we can note that there is only one group karyotipe symmetry group (metacentric). According to Singh (1999), karyotipe symmetry is considered more conservative when compared with karyotipe asymmetry in its evolution. Thus, it is known that rice cultivars of Rojolele that have chromosomal symmetry shows the evolution of a more conservative level or Rojolele rive cultivar has not been crossed with another rice cultivar.

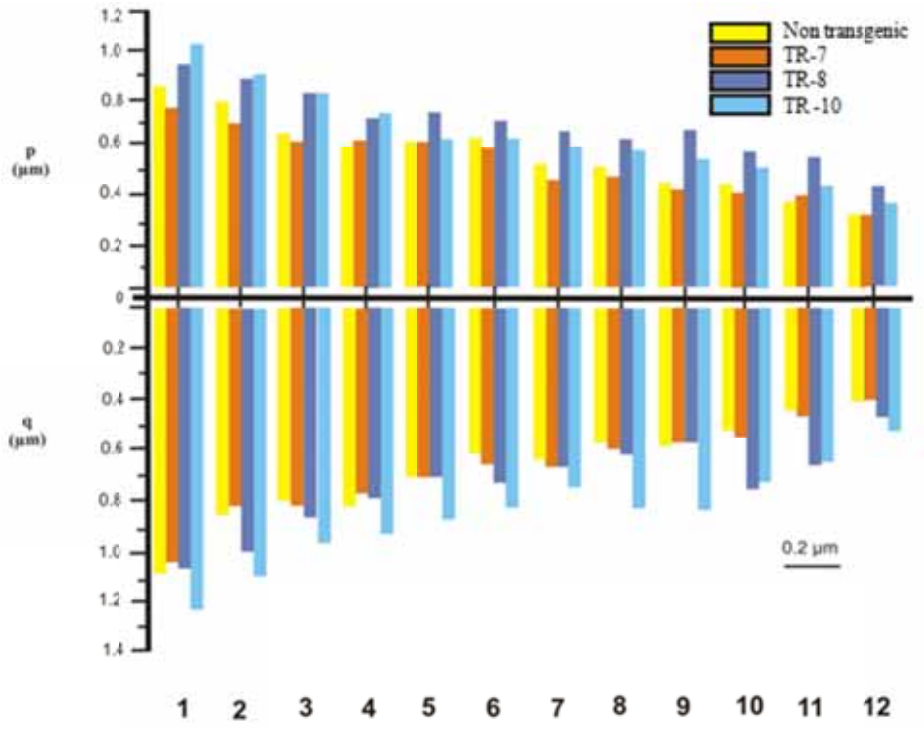

Figure 4. Idiogram showing comparison chromosome size among non-transgenic and transgenic rice expressing recombinant lactoferrin TR-7, TR-8 and TR-10. Number below the figure showed chromosome pairs. Scale bar correspond to 0.2 µm

The diploid chromosome number of *Oryza sativa* L. cv. Rojolele was 2n=24 consisted 12 pairs of metacentric chromosomes displaying karyotype formula 2n=2x=24 m (Figure 3 and Table 2). Eventhough karyotype formula of transgenic was similar to non transgenic rice, there were differences in chromosome size (Table 2). The R value showed proportion of largest chromosomes total length with the smallest chromosome total length. The R results of transgenic lines TR-7, TR-8, TR-10 and non transgenic were 2.841, 2.421, 3.174 and 2.743 respectively. The findings of the research are expected to enrich valuable information concerning the genetic identity of transgenic rice expressing recombinant human lactoferrin gene.

Table 2. Chromosome length, karyotype and R value of non-transgenic and transgenic rice TR-7, TR-8 and TR-10

Characters	Non-transgenic Rojolele	Transgenic TR-7	Transgenic TR-8	Transgenic TR-10
Short arm (p)	0.314-0.969 µm	0.318-0.783 µm	0.437-0.969 µm	0.355-1.132 µm
Long arm (q)	0.402-1.090 µm	0.414-1.071 µm	0.585-1.056 µm	0.465-1.253 µm
Total length (p+q)	0.717-1.967 µm	0.732-1.456 µm	1.021-2.024 µm	0.820-2.384 µm
Karyotipe	2n = 2x = 24 = 24m	2n = 2x = 24 = 24m	2n = 2x = 24 = 24m	2n = 2x = 24 = 24m
R value	2.743	2.841	2.421	3.174

Three lines of transgenic rice expressing rhLF investigated in this study had chromosome number (2n = 24). This count was similar from the previous studies reported by Kurata et al. (1981), who recorded that the diploid chromosome number of indica or japonica rice. Therefore, it can be concluded that insertion of hLF in Rojolele rice did not change the chromosome number and their karyotipe.

4. Discussion

We have analyzed the stability expression of recombinant human lactoferrin transgenic rice seeds over generations. Transgenic rice expressing hLF with the signal peptide from rice glutelin under the control of the maize ubiquitin-1 promoter were obtained by *Agrobacterium*-mediated transformation. This promoter has been shown to be constitutive in rice, conferring high-level transgene expression in the endosperm of seeds.

Recombinant human lactoferrin expression under the control of the constitutive maize ubiquitin-1 promoter significantly increased in the seeds, but not in the vegetative tissues of the transgenic plants. The rhLF was produced in rice seeds at levels exceeding 15% of the total soluble protein. In the vegetative tissues of transgenic plants, these proteins are expressed at low levels, typically less than 0.8% of the total soluble protein (Rachmawati et al., 2005). Huang (2004) reported the expression level of human lysozyme under the control of storage protein promoters has resulted in an average expression level of 13%-14% of total soluble protein. Werner at al. (2011) also reported the expression of recombinant protein expression (GFP) at at different tissues in transgenic *Nicotiana benthamiana*. No fluorescence could be detected in the stem, whereas leaf and stem tissue of an induced plant gave approximately the same relative expression levels.

We found that the highest level of rhLF expression with the rice glutelin signal peptide was 2.0 mg/g dry weight of dehusked seeds (TR-8). The stability of the hLF gene expression under the control of a constitutive promoter over generations, we carried out further analysis of three transgenic lines i.e. TR-7, TR-8 and TR-10. These lines were chosen based on ELISA results showing high expression levels of rhLF. The rhLF expression level varied among independent transgenic lines. The expression level of rhLF in transgenic line TR-8 was higher that in transgenic line TR-7 and TR-10. Based on southern blot analysis, transgenic lines TR-8 contained one copy of hLF gene while TR-7 and TR-10 contained 2 and 3 copies. As reported by Ma et al. (2003), the expression level of recombinant protein depends on various factors, including the gene expression construct, the position of transgene integration, the structure of the transgenic locus, copy number of transgene, and the presence of truncated or rearranged transgene copies. Transgene copy number can greatly affect the expression level and genetic stability of the target gene (Donnarumma et al., 2011). Multiple gene copies might suppress the expression of foreign gen in transgenic plants, therefore important to screen transformants with single transgene copy to avoid repeat-induced silenced (Li Xu-Gang et al., 2002). The rhLF expression levels in the T1 seeds were almost identical with those in the T3 seeds for the same transgenic line (Figure 1). The results indicated that recombinant lactoferrin in mature seeds was stable through three consecutive generations.

Recombinant hLF in transgenic rice showed a slightly smaller molecular weight than that of native hLF. We assumed that the size difference between rice rhLF and native hLF from human milk might be due to the modification of the sugar chain or glycosylation. Glycosylation systems in plants are different from those in mammals (Palacpac et al., 1999; Rayon et al., 1998; Spik & Theisen, 2000). Plant-derived recombinant proteins tend to have carbohydrate groups $\beta(1,2)$-xylose and $\alpha(1,3)$-fucose, which are absent in mammals, but lack the terminal galactose and sialic acid residues found on many native human glycoproteins (Ma et al., 2003; Twyman et al., 2003). Spik and Theisen (2000) suggested that the difference in molecular weight between recombinant hLF and native hLF might be associated with the carbohydrate moiety, either the polypeptide chain or the glycan moiety. The difference in the protein size could be ascribed to the fact that the plant protein is not phosphorylated or is incompletely phosphorylated (Herman & Larkins, 1999).

The results of cytological characterization exhibited that the time of mitotic cells of transgenic rice cv. Rojolele longer than that of non-transgenic, thus suspected to have influence lactoferrin gene insertion with respect to active time of mitosis. The finding of mitosis period of transgenic rice investigated in this study is useful to obtain prometaphase stages used to examine chromosome characters. Three lines of transgenic rice were investigated in this study had similar chromosome number (2n = 24) to the non-transgenic rice. In addition all chromosome of transgenic rice and non ttransgenic rice also appeared to have the centromere in the median region and were thus classified as metacentric (Figure 3) and according to Levan et al. (1964) displaying similar karyotipe formula (2n = 2x = 24 = 24m). The result showed that both transgenic and non-transgenic had chromosome metasentris (Figure 4). According to Singh (1999) plants that have a symmetrical karyotype (metasentris) is a primitive species. The finding of metacentric chromosomes in rojolele rice revealed that

Rojolele investigated in this study have symmetry karyotypes indicating that Rojolele have not been cultivated for breeding program.

The diploid chromosome number of *Oryza sativa* L. cv. Rojolele was 2n=24 consisted 12 pairs of metacentric chromosomes displaying karyotype formula 2n=2x=24 m (Figure 1 and Table 2). Eventhough karyotype formula of transgenic was similar to karyotype formula of non transgenic rice, there were differences in chromosome size. R value is comparison of the absolute length of the longest chromosome to the absolute length of the shortest chromosome. This value indicates the presence of chromosomal size variation. If a higher variation of chromosome size, then the R value obtained is also getting bigger. Based on the calculations shown in Table 2, the R values obtained for the non-transgenic rice Rojolele of 2.743, while for transgenic Rojolele TR-7 = 2.841; TR-8 = 2.182 and TR-10 = 3.174. The difference between the R value of Rojolele non-transgenic rice and transgenic rice is 0.011. This value showed that both transgenic and non transgenic rice Rojolele was the same parent of *Oryza sativa* L. cv. Rojolele. Therefore, the insertion of lactoferrin genes in Rojolele rice did not cause changes in chromosome size and their karyotipe. On the basis of the difference of R value, all types of transgenic rice have close genetic relationship and considered to be cultivated from same species or variety.

Expression of recombinant lactoferrin increased with seed ripening process. High expression of recombinant lactoferrin in mature seeds was stable through three consecutive generations. All types of transgenic rice had diploid chromosome number and karyotypes composed of 24 pairs of metacentric chromosomes. Formula karyotipe Rojolele rice cultivars is 2n = 2x = 24 = 24m.

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References

Bechtel, D. R., & Juliano, B. O. (1980). Formation of protein bodies in the starchy endosperm of rice (*Oryza sativa* L.): a reinvestigation. *Ann. Bot., 45*, 503-509.

Boothe, J., Nykiforuk, C., Shen, Y., Zaplachinski, S., Szarka, S., Kuhlman, P., ... Moloney, M. M. (2010). Seed-based expression systems for plant molecular farming. *Plant Biotechnology Journal., 8*, 588-606. http://dx.doi.org/10.1111/j.1467-7652.2010.00511.

Brock, J. H. (2002) .The Physiology of lactoferrin. *Biochem. Cell Biol, 80*, 1-6. http://dx.doi.org/10.1139/o01-212

Brock, J. H., Guillen, C., & Thompson, C. (2000). Anti-inflammatory and immunoregulatory properties of lactoferrin. In K. Shimazaki et al. (Eds), *Proceedings of the 4th International Conference on Lactoferrin: Structure, Function and Applications* (pp. 119-128). Elsevier Science B.V. The Netherlands.

Cheng, Z., Buell, C. R., Wing, R. A., Gu, M., & Jiang, J. (2001). Toward a cytological characterization of the rice genome. *Genome Res., 11*, 2133-2141. http://dx.doi.org/10.1101/gr.194601

Daryono, B. S., & Sumardi, I. (1996). The growth and ploidy levels of callus cells from rice anther culture (Oryza sativa L. cv. Cianjur) by 2,4-D treatment. *Biology, 2*(1), 11-18.

Donnarumma, F., Paffetti, D., Fladung, M., Biricolti, S., Dieter, E., Altosaar, I., & Vettori, C. (2011). Transgene copy number estimation and analysis of gene expression levels in *Populus* spp. transgenic lines. *BMC Proc., 5*(Suppl 7), 152. http://dx.doi.org/10.1186/1753-6561-5-S7-P152

Fagard, M., & Vaucheret, H. (2000). (Trans)gene silencing in plants: How many mechanisms? *Annu. Rev. Plant Physiol. Plant Mol. Biol., 51*, 223-256. http://dx.doi.org/10.1146/annurev.arplant.51.1.167

Fischer, R., Stoger, E., Schillberg, S., Christou, P., & Twyman, R. M. (2004). Plant-based production of biopharmaceuticals. *Curr Opin Plant Biol., 7*(2), 152-8. http://dx.doi.org/10.1016/j.pbi.2004.01.007

Gelvin, S. B. (2003). Improving plant genetic engineering by manipulating the host. *TRENDS in Biotech., 21*, 95-98. http://dx.doi.org/10.1016/S0167-7799(03)00005-2

Herman, E. M., & Larkins, B. A. (1999). Protein Storage Bodies and Vacuoles. *The Plant Cell, 11*, 601-613.

Huang, N. (2004). High-level protein expression system uses selfpollinating crops as hosts. *BioProcess International., 2*, 54-59.

Iglesias, V. A., Moscone, E. A., Papp, I., Neuhuber, F., Michalowski, S., Phelan, T., … Matzke, A. J. M. (1997) Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. *Plant Cell, 9*, 1251-1264.

Jahier, J., & Tanguy, A. M. (1996). *Observation of Chromosomes: Basic Techniques (Mitosis) of Plants Cytogenetics*. Science Publishers Inc. New Hampshire. USA.

Kim, W. S., Ohashi, M., Tanaka, T., Kumura, H., Kim, G. Y., Kwon, I. K., … Shimazaki, K. (2004) Growth-promoting effects of lactoferrin on *L. acidophilus* and Bifidobacterium ssp. *Biometals, 17*, 279-283. http://dx.doi.org/10.1023/B:BIOM.0000027705.57430.f1

Kurata, N., & Omura, T. (1978). Karyotype analysis in rice. I. A new method for identifying all chromosomes pairs. *Japan. J. Genetics, 53*(4), 251-255.

Kurata, N., Omura, T., & Iwata, N. (1981). Studies on centromere, chromomere and nucleolus in pachytene nuclei of rice, *Oryza sativa* L., microsporocytes. *Cytologia, 46*, 791-800. http://dx.doi.org/10.1266/jjg.53.251

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680-685. http://dx.doi.org/10.1508/cytologia.46.791

Levan, A., Fredga, K., & Sanberg, A. A. (1964). Nomenclature for centromeric position on chromosomes. *Hereditas, 52*, 201-220. http://dx.doi.org/10.1111/j.1601-5223.1964.tb01953.x

Lönnerdal, B. (2000). *Clinical studies on lactoferrin added to infant formula*. In K. Shimazaki et al. (Eds.), *Proceedings of the 4th International Conference on Lactoferrin: Structure, Function and Applications* (pp. 291-300). Elsevier Science B.V. The Netherlands.

Ma, J. K. C., Drake, P. M. W., & Christou, P. (2003). The production of recombinant pharmaceutical proteins in plants. Nature Rev. *Genetics, 4*, 794-805. http://dx.doi.org/10.1038/nrg1177

Mudjisihono, R., Santosa, T., & Hendrata, R. (2002). *Report of assessment on Rojolele rice variety in Klaten*. Research Agency for Agriculture Technology, Yogyakarta, Indonesia.

Murray, M. G., & Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Research, 8*(19), 4321-4326. http://dx.doi.org/10.1093/nar/8.19.4321

Nandi, S., Suzuki, Y. A., Huang, J., Yalda, D., Pham, P., Wu, L., … Lönnerdal, B. (2002). Expression of human lactoferrin in transgenic rice grains for the application in infant formula. *Plant Sci., 163*, 713-722. http://dx.doi.org/10.1016/S0168-9452(02)00165-6

Palacpac, N. Q., Yoshida, S., Sakai, H., Kimura, Y., Fujiyama, K., Yoshida, T., & Seki, T. (1999). Stable expression of human β1,4-galactosyltransferase in plant cells modifies N-linked glycosylation patterns. *Proc. Natl. Acad. Sci. USA, 96*, 4692-4697. http://dx.doi.org/10.1073/pnas.96.8.4692

Rachmawati, D., Mori, T., Hosaka, T., Takaiwa, F., Inoue, E., & Anzai, H. (2005). Production and Characterization of Recombinant Human Lactoferrin in Transgenic Javanica Rice. *Breeding Science, 55*, 213-222. http://dx.doi.org/10.1270/jsbbs.55.213

Rayon, C., Lerouge, P., & Paye, L. (1998). The protein N-glycosylation in plants. *J. Exp. Bot., 49*, 1463-1472.

Singh, G. (1999). *Plant Systematics* (pp. 176-180). Science Publishers, Inc. USA.

Spik, G., & Theisen, M. (2000). Characterization of the post-translational biochemical processing of human lactoferrin expressed in transgenic tobacco. *Bundesgesundheitsbl-Gesundheitsforch-Gesundheitsschutz, 43*, 104-109. http://dx.doi.org/10.1007/s001030050019

Tomita, M., Wakabayashi, H., Teraguchi, S., & Hayasawa, H. (2000). *Importance of pathogen-eliminating systems in host protection by lactoferrin feeding*. In K. Shimazaki et al. (Eds), *Proceedings of the 4th International Conference on Lactoferrin: Structure, Function and Applications* (pp. 175-180). The Netherlands: Elsevier Science B.V.

Twyman, R. M., Stöger, E., Schillberg, S., Christou, P., & Fischer, R. (2003). Molecular farming in plants: host system and expression technology. *TRENDS in Biotech, 21*, 570-578. http://dx.doi.org/10.1016/j.tibtech.2003.10.002

Werner, S., Breus, O., Symonenko, Y., Marillonnet, S., & Gleba, Y. (2011). High-level recombinant protein expression in transgenic plants by using a double-inducible viral vector. *PNAS, 108*(34), 14061-14066. http://dx.doi.org/10.1073/pnas.1102928108

Xu-Gang, L., Song-Biao, C., Zi-Xian, L., Tuan-Jie, C., Qian-Chun, Z., & Zhen, Z. (2002). Impact of copy number on transgene expression in tobacco. *Acta Bot Sin, 44*, 120-123.

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Increasing Extender Viscosity Improves the Quality of Cooled Boar Semen

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Abstract

The use of several types of gelling extenders for the storage of semen from several domestic species in the solid state has been shown to have beneficial effects on some semen quality parameters. The objective of this study was to evaluate the effect of a new high-viscosity semen extender, Zoosperm ND-5 3D® (Import-Vet, Centelles, Spain), on the the quality of boar spermatozoa at preserved at 17ºC for 7 days. Sodium alginate was used for the first time to increase the viscosity of the extender for the liquid storage of boar semen. The same extender, but without increased viscosity, was used as a control extender (Zoosperm ND-5®, Import-Vet, Centelles, Spain). Sixteen ejaculates from four Pietrain boars were evaluated for motility (by the CASA system), and for viability, acrosome status, plasma membrane fluidity, externalization of phosphatidylserine at the plasma membrane of the spermatozoa and mitochondrial membrane potential (by flow cytometry). In samples diluted with the Zoosperm ND-5 3D® viscous extender, the STR (straightness) parameter and the number of progressively motile spermatozoa were higher compared to those of the non-viscous extender ($p < 0.05$). In addition, the number of spermatozoa with damaged acrosomes, an unstable sperm plasma membrane and externalization of phosphatidylserine at the plasma membrane was lower in samples treated with the viscous extender ($p < 0.05$). In conclusion, an increase in extender viscosity improves quality of boar spermatozoa following long-term storage.

Keywords: Zoosperm ND-5 3D®, boar, extender, storage, viscosity

1. Introduction

The role that artificial insemination (IA) based on cooled semen technology has played in global boar production is widely acknowledged. Among other advantages, this technology allows for maximizing the genetic potential of high-quality breeding stock. Among the facts that have contributed to the advances in this technology are better knowledge of sow management and physiology, improvements in semen processing and design of better extenders.

Because seminal plasma alone does not allow for long-lasting semen preservation, the addition of an extender permits an increase in the ejaculate volume, extends the average life span of spermatozoa and maintains an adequate fertility level in the sow herd (Gadea, 2003). Because boar spermatozoa are highly susceptible to cold shock, they need to be stored at temperatures of 15-20ºC, which limits storage period.

During the storage of extended boar semen, a further decrease in the fertility potential as a result of the natural ageing process of spermatozoa cannot be prevented. In several studies, functional and structural changes of spermatozoa during storage have been measured. The data include changes in motility, viability, pH, DNA integrity, acrosome intactness, mitochondrial activity, bacterial contamination, tyrosine phosphorylation and more recently, screening for the sperm proteome, cytome and lipidome (De Ambrogi et al., 2006; Dubé, Beaulieu, Reyes-Moreno, Guillemette, & Bailey, 2004; Huo, Ma, & Yang, 2002; Martín-Hidalgo et al., 2013; Waberski, Henning, & Petrunkina, 2011).

Extenders are a factor influencing the cold shock tolerance of spermatozoa. Over the last 10 years, new extenders for boar semen, both for short-term (1-3 days) and long-term preservation (more than 4 days), have been designed (Riesenbeck, 2011). The use of long-term extenders is suitable in situations where work overload is expected at the AI center, when large sow herds are involved or when seminal doses are prepared from low demand or rarely used boars. They also allow for long-distance transportation of the semen. Artificial insemination with cooled semen is likely to remain the most widely used sperm preservation technology in swine rearing programs, given the excellent fertility results achieved until now (Roca et al., 2006).

An increase in extender viscosity has been shown to improve the quality of stored semen. However, research in boars about this subject is scarce. Corcini et al. (2011) observed that adding gelatin to the short-term Beltsville Thawing Solution (BTS) extender for boar semen produced a less marked fall in the percentage of motile and morphologically normal spermatozoa during the preservation period compared to the samples to which no gelatin was added, although no significant differences between extenders were observed in either parameter (Corcini et al., 2011). Coy, Gadea, Rath and Hunter (2009) showed that the re-suspension of boar spermatozoa in a viscous medium increased the stability of the sperm membrane, decreased reactive oxygen species production and increased the capacity of spermatozoa to penetrate oocytes *in vitro*. The use of gelling extenders, mostly by the use of gelatin, has proven to be effective in prolonging the preservation of rabbit (López-Gatius et al., 2005; Nagy, Sinkovics, & Kovacs, 2002; Rosato & Iaffaldano, 2011), ovine (Yániz et al., 2005) and caprine (Salvador, Yániz, Viudes-de-Castro, Gómez, & Silvestre, 2006) sperm in a solid state. However, it remains to be seen whether the reported benefits are also applicable to semen stored in a liquid form. Those benefits were attributed to possible effects on sperm sedimentation and the reduction of metabolic activity due to the reduced sperm motility. Alginate or alginic acid is an anionic polysaccharide distributed widely in the cell walls of brown algae. It has a large gelling capacity and is widely used for the microencapsulation of spermatozoa before storing. Microencapsulation in barium alginate membranes has been found to protect sperm cells during storage, preserving morpho-functional characteristics (Spinaci et al., 2013; Torre et al., 2000; Vigo et al., 2002). We could find no reference in the literature to the use of alginate in long-term viscous extenders for the long-term preservation of boar semen in liquid form. For this reason, Import-Vet, S. A. (Centelles, Spain) has designed Zoosperm ND-5 3D® (hereafter 3D), a viscous extender for long-term preservation. This study evaluates for the first time the effectiveness of this new viscous extender in terms of its capacity to preserve the sperm quality of boar semen aliquots after 7 days at 17ºC. In addition, the same extender but without increased viscosity was used as a control extender (Zoosperm ND-5®, Import-Vet, S. A., hereafter ND-5). On the basis of previous studies of viscous extenders for semen storage in the solid state, our hypothesis is that 3D will improve the sperm quality of the liquid stored semen.

2. Materials and Methods

2.1 Chemicals and Sources

ND-5® and ND-5 3D® were provided by Import-Vet, S. A. (Centelles, Spain). Live/dead spermatozoa viability kits including propidium iodine (PI) and SYBR-14, M540 and YoPro-1 probes were purchased from Molecular Probes (Leiden, The Netherlands). Annexin-V-FITC was obtained from Immnostep (Salamanca, Spain). FITC-PNA was purchased from Sigma-Aldrich® (St. Louis, MO, USA). The coulter isoton II diluent was obtained from Beckman Coulter Inc. (Brea, CA, USA) and the JC-1 (5,5′,6,6′–tetrachloro-1,1′,3,3′ tetraethylbenzymidazolyl carbocyanine iodine) probe was purchased from Life Technologies Ltd. (Grand Island, NY, USA).

2.2 Sample Preparation and Assessment of Sperm Quality Parameters

2.2.1 Sample Preparation

Sixteen ejaculates from four healthy, sexually mature (2-3 years old) and fertile Pietrain boars were analyzed. The animals belonged to an AI center in Segovia (Spain). The boars were subjected to regular semen collection three times every two weeks for commercial use. All the males were kept in individual pens in a controlled environment (15-20ºC) and all were fed the same diet. The ejaculate was collected using the "gloved hand" technique and was then gently homogenized and divided in two equal parts. After an initial assessment of motility (assessed subjectively by light microscopy at 10X), morpho-anomalies and concentration (both by light microscopy in a Bürker chamber after dilution in 10% formaldehyde-saline solution at 40X), one of the fractions was diluted to a 33 x 10^6 cells/mL final concentration with ND-5 extender and the other fraction, at the same concentration level, was diluted with 3D viscous extender. The composition of the extenders differed only in the hydrocolloid compound (sodium alginate) added to the 3D extender in order to increase viscosity to a 13 centipoises value at 17ºC. Viscosity measurement was performed by a specialized laboratory (Brookfield viscometer, CETAEX, Badajoz, Spain). The quantitative composition is unknown because of commercial interests, so only qualitative composition is done (glucose, sodium citrate, EDTA, sodium bicarbonate, potassium chloride, acetylcysteine,

MOPS and antibiotics). Both extenders were prepared following the manufacturer's instructions. Dilutions of both ejaculate fractions were carried out within 15 min after they were obtained. Only those ejaculates containing at least 70% of motile spermatozoa and less than 20% of morphologically abnormal spermatozoa were selected for the trial. The diluted semen was then bottled in 90 ml plastic bottles with clip-tops (Import-Vet, S. A., Centelles, Spain).After lowering the temperature of the semen doses to 20-22ºC over approximately 2 h at room temperature, 3 bottles of each ejaculate diluted in ND-5 or 3D were stored at 16-17ºC until their transport to the laboratory of the Veterinary Faculty of Cáceres. This journey, which took 12 h, occurred at night. The samples were carried in isothermal boxes by a courier with no special precautions. On arrival at the laboratory, the semen doses were stored in a refrigerated incubator (FOC 225 I, VELP Scientifica, Usmate, Italy) at 16-17ºC for 7 days (collection day = day 0). On days 1, 4 and 7, two aliquots were extracted from one bottle of each extender. A total of 192 semen samples were analyzed. The following sperm quality parameters were measured in each of the aliquots: motility, viability, acrosome status, plasma membrane fluidity, phosphatidylserine externalization at the plasma membrane of spermatozoa and mitochondrial membrane potential. The motility analysis was carried out by a CASA (Computer Assisted Sperm Analysis) system and the other parameters were measured by flow cytometry.

2.2.2 Assessment of Sperm Motility

Immediately after gentle mixing, 1mL of semen was taken from each bottle and examined for motility pattern using the CASA system (ISAS® program, Proiser R+D, Paterna, Valencia, Spain). Before the motility analysis, the seminal samples were incubated at 38ºC for 1 h (Mini Galaxy A, RS Biotech, United Kingdom). A total of 2 µl of sample was placed in a pre-warmed counting chamber (Leja®, Luzernestraat, The Netherlands). Sperm motility analysis was based on the examination of 25 consecutive digitalized images obtained from several fields using a 10X negative-phase contrast objective and a heated stage at 38ºC. At least 300 spermatozoa per sample were analyzed. Images were taken with a time lapse of 1s. The following sperm motility parameters were recorded: total motile spermatozoa (% TMS, spermatozoa with an average path velocity, VAP > 10µm/s), progressively motile spermatozoa (% PMS, spermatozoa with a straightness coefficient > 0.8, 80%), VCL (curvilinear velocity in µm/s), VSL (straight-line velocity in µm/s), VAP (average path velocity in µm/s), STR (straightness coefficient in %), WOB (wobble coefficient in %) and ALH (amplitude of lateral head displacement in µm). Spermatozoa with an average path velocity (VAP) < 10 µm/s were considered immotile. Spermatozoa deviating < 10% from a straight line were designated as linearly motile and spermatozoa with a radius < 25 µm were classified as circularly motile (Saravia et al., 2005).

2.2.3 Flow Cytometry Analyses

Flow cytometry analyses were performed using a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter Ltd.) The fluorophores were excited by a 200 mV argon ion laser operating at 488 nm. A total of 10,000 gated events based on the forward scatter and side scatter of the sperm population recorded in the linear mode were collected per sample with a running rate of approximately 500 events/s. The fluorescence data were collected in the logarithmic mode and analyzed using a FACStationTM and EXPOTM 32 ADC software (Beckman Coulter, Inc.).

2.2.3.1 Assessment of Sperm Viability

Fluorescent staining using the LIVE/DEAD Sperm Viability Kit was performed to assess porcine spermatozoa viability (Aparicio et al., 2007). Briefly, 5 µl of SYBR-14 (2 µM) and 10µl of propidium iodide (PI 5 µM) were added to 500 µL of diluted semen sample (33 x 10^6 cells/mL) in isotonic buffered diluent Coulter Isoton II and incubated for 20 min at room temperature in the dark. After incubation, the cells were analyzed and the percentage of viable spermatozoa was expressed as the percentage of SYBR 14 - positive and propidium iodide - negative spermatozoa.

2.2.3.2 Assessment of Acrosome Status of Spermatozoa

The acrosome integrity of the spermatozoa was assessed after staining the spermatozoa with fluorescein isothiocyanate - peanut agglutinin (FITC-PNA), as a marker for acrosomal status, and PI (Waterhouse et al., 2004). Aliquots of 100 µL of each semen sample (33 x 10^6cells/mL) were incubated at room temperature in the dark for 5 min with 5 µL (1 µg/mL) PNA-FITC and 5 µL (6µmol/L) PI. Just prior to flow cytometry, 400 µl of isotonic buffered diluents was added to each sample and remixed. The cells were analyzed and the percentage of spermatozoa with acrosomes that were damaged or reacted was expressed as the percentage of PNA - positive and PI - negative spermatozoa.

2.2.3.3 Assessment of Plasma Membrane Fluidity

The plasma membrane fluidity of the spermatozoa was assessed by staining the sperm with merocyanine 540 (M540) and plasma membrane permeability was assessed by staining with YoPro-1 (Harrison, Ashworth, & Miller,

1996). Aliquots of 100 µl of each semen sample (33 x 10^6 cells/mL) were diluted in 400 µl of isotonic buffered diluent containing 75 nmol/L YoPro-1. The samples were then mixed and incubated at 38ºC for 15 min. Just before flow cytometry, M540 was added to each sample to a final concentration of 2 µmol/L, incubated for 2 min and remixed. After incubation, the cells were analyzed and the results were expressed as the percentage of viable sperm with an unstable plasma membrane (YoPro-1-negative/M-540 - positive).

2.2.3.4 Evaluation of the Phosphatidylserine Externalization at the Plasma Membrane of Spermatozoa

The study of phosphatidylserine (PS) externalization in plasma membrane spermatozoa was performed using Annexin-V-FITC to specifically detect PS translocation from the inner to the outer leaflet of the sperm plasma membrane.

Aliquots of 300 µl of each semen sample (33 x 10^6 cells/mL) were diluted in 200 µl of the following buffer: 96 mmol/l NaCl, 4.7 mmol/l KCl, 0.4 mmol/l $MgSO_4$, 0.3 mmol/l NaH_2PO_4, 5.5 mmol/l glucose, 1 mmol/l sodium pyruvate, 21.6 mmol/l sodium lactate, 20 mmol/l HEPES (pH 7.45), and 2.5 mmol/l $CaCl_2$. Then, a 100µl aliquot was transferred to a 5 ml tube and stained with 5 µl AnnexinV-FITC and 4µl propidium iodide (PI) by incubation for 15 min in the dark at room temperature. Finally, 400 µl of isotonic buffered diluents was added to each sample and mixed before flow cytometry analysis. For statistical analysis, the results were expressed as the percentage of viable sperm with PS externalization (AnnexinV-FITC - positive/PI - negative).

2.2.3.5 Assessment of Mitochondrial Membrane Potential Status

Mitochondrial membrane potential was evaluated using the specific probe JC-1. JC-1 reversibly changes its fluorescence from green (monomeric status) to orange (multimeric status, formation of aggregates, J_{agg}) when the mitochondrial membrane potential is high (Amaral & Ramalho-Santos, 2010). Because J_{agg} formation depends on the chemical environment (Reers, Smith, & Chen, 1991), seminal samples (1.2 mL) were centrifuged at 1500 g for 1.5 min and the pellet was resuspended in 0.8 mL of ND-5 extender. From each sperm sample, 100 µL (30 × 10^6 cells/mL) was diluted in 400 µL of isotonic buffered diluent containing 0.15 mmol/L JC-1 and then mixed and incubated at 38ºC for 30 min. The samples were remixed before flow cytometry analysis. The percentage of orange stained cells was recorded; these cells were defined as the cells with a high mitochondrial membrane potential (hMMP).

2.3 Statistical Analysis

The mean and the standard error of the mean were calculated for descriptive analysis. Q-Q plots were used to check for departures from the normal distribution. The effects of extender (ND-5 and 3D) and storage time (1, 4 and 7 days) on seminal characteristics were assessed using a General Linear Model for Repeated Measures. A mixed-effects model (with boars and ejaculates within boars as random effects and extender and storage time as fixed effects) was applied to the experimental design.

All statistical analyses were performed using the libraries Linear and Nonlinear Mixed Effects Models from the statistical package R 3.0.1 (Pinheiro, Bates, Debroy, Sarker, & The R Development Core Team, 2013). Statistical significance was defined as $p < 0.05$, 0.001.

3. Results

Table 1 shows the pH, osmolarity and viscosity characteristics of both extenders.

Table 1. Characteristics of ND-5® and ND-5 3D® extenders

Extender	pH	Osmolarity (mOsm/L)	Viscosity at 16ºC (cP)	Viscosity at 38ºC (cP)
ND-5	6.9	295	10	8
ND-5 3D	6.9	295	13	11

cP: centipoise.

Table 2 shows the results of the motility analysis. The increase in viscosity did not modify the percentage of motile spermatozoa (TMS). For the rest of the parameters, the values were significantly different between the extenders for some or all preservation days.

Table 2. Effects of extender on motility characteristics on specific days after collection

Parameter	Extender	Day 1	Day 4	Day 7	p value (time)	Interaction (extender x time)
VCL (μm/s)	ND-5	71.19±1.90	74.05±1.89	78.88±1.89	<0.001	n.s.
	ND-5 3D	55.60±1.90	62.11±1.89	65.13±1.93		
	p value (extender)	<0.001	<0.001	<0.001		
VSL (μm/s)	ND-5	49.94±2.17	49.27±2.16	48.07±2.16	n.s.	n.s.
	ND-5 3D	37.00±2.17	38.41±2.16	37.50±2.18	n.s.	
	p value (extender)	<0.001	<0.001	<0.001		
VAP (μm/s)	ND-5	61.82±2.00	63.04±1.99	65.57±1.99	<0.001	n.s.
	ND-5 3D	45.01±2.00	48.19±1.99	48.37±2.02		
	p value (extender)	<0.001	<0.001	<0.001		
STR	ND-5	0.79±0.01	0.77±0.01	0.72±0.01	<0.001	0.052
	ND-5 3D	0.81±0.01	0.79±0.01	0.77±0.01	<0.001	
	p value (extender)	0.018	0.017	<0.001		
WOB	ND-5	0.86±0.01	0.84±0.01	0.82±0.01	<0.001	0.017
	ND-5 3D	0.82±0.01	0.78±0.01	0.75±0.01	<0.001	
	p value (extender)	<0.001	<0.001	<0.001		
ALH (μm)	ND-5	2.14±0.04	2.29±0.04	2.55±0.04	<0.001	n.s.
	ND-5 3D	1.99±0.04	2.26±0.04	2.40±0.04		
	p value (extender)	0.005	n.s.	0.008		
BCF (μm)	ND-5	8.09±0.11	7.86±0.11	7.76±0.11	0.019	<0.001
	ND-5 3D	8.21±0.11	8.92±0.11	9.33±0.11	<0.001	
	p value (extender)	n.s.	<0.001	<0.001		
TMS (%)	ND-5	87.3±0.02	85.8±0.02	82.0±0.02	<0.001	n.s.
	ND-5 3D	87.8±0.02	86.7±0.02	82.5±0.02		
	p value (extender)	n.s.	n.s.	n.s.		
PMS (%)	ND-5	56.1±0.03	48.7±0.03	40.3±0.03	<0.001	n.s.
	ND-5 3D	59.1±0.03	53.1±0.03	47.8±0.03		
	p value (extender)	n.s.	0.012	0.001		

TMS indicates percentage of total motile sperm; PMS, percentage of progressively motile sperm.
Values are means ± standard error of the mean (SEM).
n. s.: not significant ($p > 0.05$).

It is worth noting that both the STR, which indicates the straightness of the sperm trajectory, and the percentage of spermatozoa with progressive motility were higher in 3D. The differences between extenders were not the same throughout the preservation period; the difference was higher on day 7 in relation to days 1 and 4. On day 7, STR

increased 6.9% on average in 3D in relation to the ND-5 extender, whereas on days 1 and 4, a 2.5% and 2.6% increase was observed, respectively. Regarding the percentage of spermatozoa with progressive motility, a 9% increase (day 4) and an 18.6% increase (day 7) was obtained with the 3D extender.

It can also be seen that values for the velocity parameters VCL, VSL and VAP were higher in the ND-5 non-viscous extender; however, for these variables, the behavior of each extender through the storage period was no different, with no effect of storage time in the case of VSL.

The amplitude of lateral head displacement (ALH) was lower in the 3D extender, whereas flagellar beat cross frequency (BCF) was higher, given the higher viscosity of the medium. In addition, for BCF, the behavior throughout the storage time was different for each extender: there was a decrease over time in the ND-5 extender and an increase in the 3D extender.

Table 3 shows the results for the sperm quality variables in both extenders as measured by flow cytometry.

Table 3. Effects of extender on flow cytometry characteristics on specific days after collection

Parameter	Extender	Day 1	Day 4	Day 7	p value (time)	Interaction (extender x time)
VS	ND-5	92.59±0.46	93.32±0.45	93.34±0.45	n.s.	n.s.
	ND-5 3D	93.75±0.46	93.27±0.45	93.79±0.45		
	p value (extender)	n.s.	n.s.	n.s.		
DAS	ND-5	6.93±0.96	7.60±0.95	7.73±0.95	n.s.	n.s.
	ND-5 3D	3.24±0.96	3.40±0.95	3.47±0.95		
	p value (extender)	<0.001	<0.001	<0.001		
VUM	ND-5	6.80±0.43	9.11±0.42	8.67±0.42	0.001	0.028
	ND-5 3D	5.86±0.43	6.24±0.42	6.74±0.44	n.s.	
	p value (extender)	n.s.	<0.001	<0.001		
EPS	ND-5	8.93±0.64	10.45±0.78	11.27±0.87	n.s.	n.s.
	ND-5 3D	5.77±0.62	6.04±0.78	7,74±1.00		
	p value (extender)	<0.001	<0.001	<0.001		
hMMP	ND-5	76.95±3,94	73.36±5,52	79.93±3,39	n.s.	n.s.
	ND-5 3D	81.87±3,67	79.77±3,21	80.54±2,61		
	p value (extender)	<0.001	n.s.	n.s.		

VS indicates viable spermatozoa with an intact plasma membrane (SYBR-14+/PI -); DAS, viable spermatozoa with a damaged acrosome (PNA+/PI-); VUM, viable spermatozoa with an unstable plasma membrane; EPS, viable spermatozoa with phosphatidylserine externalization in plasma membrane; hMMP, spermatozoa with high mitochondrial membrane potential; MF, mean fluorescence intensity (JC-1).

Values are means ± standard error of the mean (SEM).

n. s.: not significant ($p > 0.05$).

There were no differences between the extenders in the percentage of viable spermatozoa with an intact plasma membrane (VS). The percentage of spermatozoa with a high mitochondrial membrane potential (hMMP) was only different on day 1 ($p < 0.001$), with higher values for the 3D extender. Indeed, for the live spermatozoa population, a 4 point average reduction in the percentage of spermatozoa with damaged acrosomes (DAS) (reduction of 54.5%) ($p < 0.001$) and in the percentage of spermatozoa with PS externalization at the plasma membrane (EPS)

(reduction of 36.2%) was obtained with the 3D extender, a decrease that remained constant throughout the 7 preservation days. The use of this extender also resulted in a significant decrease ($p < 0.001$) in the percentage of spermatozoa with an unstable plasma membrane (VUM) on days 4 and 7 (M540 - negative/YoPro-1- negative spermatozoa).

4. Discussion

The sperm velocity parameters were different for the two extenders. The most likely reason for this is their different densities. It has been shown that the viscosity of the medium surrounding the spermatozoon influences its movement (Hirai et al., 1997; Hunter, Coy, Gadea, & Rath, 2011; Kirkman-Brown & Smith 2011; Smith, Gaffney, Gadelha, Kapur, & Kirkman-Brown, 2009). Thus, in the case of a bull spermatozoon, the flagellar beat frequency decreases almost exactly with the square root of the viscosity (Rikmenspoel, 1984). While the hyperactive movement of a mouse spermatozoon is not linear in a low viscosity medium, it becomes more linear in a highly viscous or viscoelastic solution (Suárez & Dai, 1992). Our results are a further contribution to the study of the significant differences in the motility of spermatozoa in media of differing viscosity, and the greater consideration that this fact is being given in the measurement of *in vitro* motility, with a view to their subsequently being correlated to *in vivo* motility. Our study shows that an increase in the viscosity of the extender produced a reduction in the VCL, VSL and VAP velocity parameters. These results are similar to those reported by Suárez and Dai (1992) for mouse spermatozoa.

The number of motile spermatozoa, in particular those with progressive motility, is one of the most frequent measures used to estimate the quality of an ejaculate, given its importance for sperm migration through the female genital tract and penetration of the oocyte membrane. Thus, it is considered to be one of the factors determining *in vitro* fertilization rates (Simon & Lewis, 2011; Turner, 2006). In this study, the 3D viscous extender produced an increase both in the straightness of the trajectory of the spermatozoa and in the percentage with progressive motility. In addition, the difference between the extenders was greater at the end of the preservation period. Coy et al. (2009) used a plant extract to increase the viscosity of a medium for swine spermatozoa and observed an increase in the STR index and the percentage of progressively motile spermatozoa, although they also reported lower VCL, VSL and VAP values in comparison to the control medium. Corcini et al. (2011) found an increase in the percentage of motile spermatozoa after adding gelatin to the BTS extender for short term (three days) preserved swine semen. In our study, this parameter was not different between the extenders. The literature suggests that changes to motility parameters are dependent on the agent used to increase the viscosity (because it determines whether liquefaction will occur at the temperature at which motility is measured), as well as the degree of viscosity (Corcini et al., 2011; Coy et al., 2009; Salvador et al., 2006; Yániz et al., 2005). Different viscous media, therefore, influence the motility characteristics of the spermatozoa.

Various authors have indicated the importance of *in vitro* evaluation of sperm motility in viscous media. During transit through the sow's genital tract, the spermatozoon is surrounded by fluids that are not only of different chemical composition but also of different viscosity. The oviduct secretes a viscous glycoprotein (Hunter, 2002; Hunter, 2005; Jansen, 1978; Jansen, 1980). In experimental *in vitro* conditions, however, the level of viscosity is very low, close to that of water, which is very much lower than that of the oviduct. According to Coy et al. (2009), a certain degree of viscosity is desirable in order for the spermatozoon to interact with the oocyte in *vitro* (culture medium) or *in vivo* (oviduct) once it is no longer in contact with seminal plasma components. For this reason, they suggest that the viscosity of the medium is a parameter that must be taken into account when *in vitro* or *in vivo* experiments are being designed. Smith et al. (2009) also noted the need to measure sperm motility in media with viscosity similar to that found in uterine physiological conditions. In their study, they note how viscosity significantly affects sperm characteristics, such as planarity, torsion, wave form, trajectory and sperm progression and beat cross frequency, modifications that were observed in our study (BCF, ALH and WOB parameters). Those authors conclude that observations carried out in low viscosity liquids, which is the case in the majority of *in vitro* experiments, can provide little information with regard to motility *in vivo*. Furthermore, the fact that a viscous medium reduces the rolling rate allows the full waveform to be captured in a precise way for a period of one or more beats, which is convenient for bi-dimensional techniques for measuring flagellar movement. In the view of these researchers, given the important advances which are being made in the digital capture and processing of high-speed images, the use of a viscous medium has the advantage of allowing a more detailed analysis of the development and propagation of the flagellar wave and so establishes a better correlation between cellular progression in liquids with viscosities similar to physiological ones. These are matters that will surely be taken into consideration in the development of the new generation of CASA systems.

Various studies have attempted to relate the CASA parameters with *in vivo* fertility results. The results are contradictory (Broekhuijse, Sostaric, Feitsma, & Gadella, 2012; Budworth, Amann, & Chapman, 1988; Didion,

2008; Liu, Clake, & Baker, 1991). Because spermatozoa exposed to a viscous medium have a greater capacity to move, unite with and penetrate the oocyte under *in vitro* conditions (Coy et al., 2009), and their movements are more similar to those that occur in the oviduct, it would be interesting if future studies were to correlate the motility in viscous solutions with fertility results.

The 3D viscous extender produced a reduction in the number of spermatozoa with damaged acrosomes and an unstable plasma membrane. These are standard parameters for measuring semen quality. This favorable effect on semen quality was observed by Nagy et al. (2002) in rabbit spermatozoa suspended in an extender with gelatin. Coy et al. (2009) also found greater viability and less generation of reactive oxygen species in swine spermatozoa extended in Androhep® (Minitube, Germany) to which a powdered plant extract was added to increase its viscosity, compared with the viscosity achieved using Androhep® on its own. Unlike other studies (Coy et al., 2009; Nagy et al., 2002; Yániz et al., 2005), we did not find an increase in sperm viability with the viscous extender.

The liquid preservation of boar semen is associated with apoptotic-like changes in the sperm, such as a decrease in the mitochondrial membrane potential and externalization of phosphatidylserine from the inner to the outer leaflet of the sperm membrane (Trzcinska, Bryla, & Smorag, 2011). A decrease in the mitochondrial membrane potential of spermatozoa has frequently been associated with an increase in the number of abnormalities in the semen (Espinoza, Schulz, Sánchez, & Villegas, 2009) and a reduction in fertility (Gallon, C. Marchetti, Jouy, &P. Marchetti, 2006; Grunewald, Said, Paasch, Glander, & Agarwal, 2008). The percentage of spermatozoa with high mitochondrial potential did not differ significantly between extenders except on day 1, when the value for the viscous extender was higher, although there was a tendency towards greater values in the samples diluted in the viscous extender through the storage period.

The assessment of PS externalization is included in the evaluation of cooling - induced damage to spermatozoa. The number of spermatozoa with these early apoptotic changes has been correlated with poor fertility in breeding bulls (Anzar, He, Buhr, Kroestsch, & Pauls, 2002) and with male infertility in humans (P. Marchetti & C. Marchetti, 2007). In this study, the 3D viscous extender was associated with a reduction in the number of spermatozoa with PS externalization at the plasma membrane of spermatozoa. We cannot compare our results with other authors because this parameter is not reported in other studies.

The mechanism by which the 3D extender improves sperm quality remains unclear. López-Gatius et al. (2005) suggest that a viscous medium, because it limits the movement of the spermatozoa during storage, also probably causes a reduction in the metabolic demands made on them. According to Nagy et al. (2002), even when buffers are added to the extenders to minimize pH fluctuations, the sedimentation which inevitably occurs during the preservation probably leads to a lowering of the pH in the region of the sedimented cells as a result of the accumulation of toxic metabolites. Because the gelling extender (solid storage) avoids sedimentation, there is a more homogenous distribution of the spermatozoa, which in turn allows the buffer to act more efficiently. The spermatozoa also benefit from a more homogenous distribution of the various components of the suspension. However, in this study, the slight increase in 3D extender viscosity was not enough to maintain the spermatozoa in suspension through the storage period, and sedimentation was inevitable. The proposed justification is, in this case, not applicable. On the other hand, sperm microencapsulation in barium alginate membranes protects sperm cells during storage. This procedure has been shown to preserve morpho-functional characteristics such as motility, *in situ* enzymatic activity and acrosome integrity (Spinaci et al., 2013; Torre et al., 2000; Vigo et al., 2002). An explanation is that the “dilution shock” (Watson, 1995), with its damaging effects, that spermatozoa suffer when they are stored under refrigeration in extender is avoided. In our study, the fact that sedimentation and dilution were not avoided during storage suggests that some protective effect of the viscous medium is present. More research needs to be done to confirm this statement, including field fertility trials.

5. Conclusion

The use of the Zoosperm ND-5 3D® viscous extender for the long-term storage of swine semen improves sperm quality in terms of a lower percentage of spermatozoa with unstable plasma membranes, externalization of PS and damaged acrosomes. These results introduce a new possibility in the design of new boar extenders for different storage conditions.

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References

Amaral, A., & Ramalho-Santos, J. (2010). Assessment of mitochondrial potential: implications for the correct monitoring of human sperm function. *International Journal of Andrology, 33*(1), e180-e186. http://dx.doi.org/10.1111/j.1365-2605.2009.00987.x

Anzar, M., He, L., Buhr, M. M., Kroetsch, T. G., & Pauls, K. P. (2002). Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. *Biology of Reproduction, 66*(2), 354-360. http://dx.doi.org/10.1095/biolreprod66.2.354

Aparicio, I. M., Bragado, M. J., Gil, M. C., García-Herreros, M., González-Fernández, L., Tapia, J. A., & García-Marín, L. J. (2007). Phosphatidylinositol 3-kinase pathway regulates sperm viability but not capacitation on boar spermatozoa. *Molecular Reproduction and Development, 74*(8), 1035-1042. http://dx.doi.org/10.1002/mrd.20663

Boe-Hansen, G. B., Ersboll, A. K., Greve, T., & Christensen, P. (2005). Increasing storage time of extended boar semen reduces sperm DNA integrity. *Theriogenology, 63*(7), 2006-2019. http://dx.doi.org/10.1016/j.theriogenology.2004.09.006

Broekhuijse, M. L., Sostaric, E., Feitsma, H., & Gadella, B. M. (2012). Application of computer-assisted semen analysis to explain variations in pig fertility. *Journal of Animal Science, 90*(3), 779-789. http://dx.doi.org/10.2527/jas.2011-4311

Budworth, P. R., Amann, R. P., & Chapman, P. L. (1988). Relationships between computerized measurements of motion of frozen-thawed bull spermatozoa and fertility. *Journal of Andrology, 9*(1), 41-54.

Conejo-Nava, J., Fierro, R., Gutiérrez, C. G., & Betancourt, M. (2003). Membrane status and in vitro capacitation of porcine sperm preserved in long-term extender at 16 degrees C. *Archives of Andrology, 49*(4), 287-295. http://dx.doi.org/10.1080/01485010390204931

Corcini, C. D., Moreira, F., Pigozzo, R., Varela, A. S., Torres, N. U., & Lucia, T. (2011). Semen quality and reproductive performance after artificial insemination with boar sperm stored in a gelatin-suplemented extender. *Livestock Sciences, 138*(1), 289-292. http://dx.doi.org/10.1016/j.livsci.2010.11.019

Coy, P., Gadea, J., Rath, D., & Hunter, R. H. (2009). Differing sperm ability to penetrate the oocyte in vivo and in vitro as revealed using colloidal preparations. *Theriogenology, 72*(9), 1171-1179. http://dx.doi.org/10.1016/j.theriogenology.2009.07.011

De Ambrogi. M., Ballester, J., Saravia, F., Caballero, I., Johannisson, A., Wallgren, M., Andersson, M., & Rodríguez-Martínez, H. (2006). Effect of storage in short-and long-term commercial semen extenders on the motility, plasma membrane and chromatin integrity of boar spermatozoa. *International Journal of Andrology, 29*(5), 543-552. http://dx.doi.org/10.1111/j.1365-2605.2006.00694.x

Didion, B. A. (2008). Computer-assisted semen analysis and its utility for profiling boar semen samples. *Theriogenology, 70*(8), 1374-1376. http://dx.doi.org/10.1016/j.theriogenology.2008.07.014

Dubé, C., Beaulieu, M., Reyes-Moreno, C., Guillemette, C., & Bailey, J. L. (2004). Boar sperm storage capacity of BTS and Androhep Plus: viability, motility, capacitation, and tyrosine phosphorylation. *Theriogenology, 62*(5), 874-886. http://dx.doi.org/10.1016/j.theriogenology.2003.12.006

Espinoza, J. A., Schulz, M. A., Sánchez, R., & Villegas, J. V. (2009). Integrity of mitochondrial membrane potential reflects human sperm quality. *Andrologia, 41*(1), 51-54. http://dx.doi.org/10.1111/j.1439-0272.2008.00878.x

Gadea, J. (2003). Review: semen extenders used in the artificial insemination of swine. *Journal of Agricultural Research, 1*, 17-27. http://dx.doi.org/10.1016/j.fertnstert.2006.03.055

Gallon, F., Marchetti, C., Jouy, N., & Marchetti, P. (2006). The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability. *Fertility and Sterility, 86*(5), 1526-1530.

Grunewald, S., Said, T. M., Paasch, U., Glander, H. J., & Agarwal, A. (2008). Relationship between sperm apoptosis signalling and oocyte penetration capacity. *International Journal of Andrology, 31*(3), 325-330. http://dx.doi.org/10.1111/j.1365-2605.2007.00768.x

Harrison, R. A., Ashworth, P. J., & Miller, N. G. (1996). Bicarbonate/CO_2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Molecular Reproduction and Development, 45*(3), 378-391. http://dx.doi.org/10.1002/(SICI)1098-2795(199611)45:3<378::AID-MRD16>3.0.CO;2-V

Hirai, M., Cerbito, W. A., Wijayagunawardane, M. P., Braun, J., Leidl, W., Ohosaki, K., Matsuzawa, T., Miyazawa, K., & Sato, K. (1997). The effect of viscosity of semen diluents on motility of bull spermatozoa. *Theriogenology, 47*(7), 1463-1478.http://dx.doi.org/10.1016/S0093-691X(97)00136-2

Hunter, R. H. (2002). Vital aspects of Fallopian tube physiology in pigs. *Reproduction in Domestic Animals, 37*(4), 186-190. http://dx.doi.org/10.1046/j.1439-0531.2002.00376.x

Hunter, R. H. (2005). The Fallopian tubes in domestic mammals: how vital is their physiological activity? *Reproduction, Nutrition, Development, 45*(3), 281-290. http://dx.doi.org/10.1007/s10815-010-9531-3

Hunter, R. H., Coy, P., Gadea, J., & Rath, D. (2011). Considerations of viscosity in the preliminaries to mammalian fertilisation. *Journal of Assisted Reproduction and Genetic, 28*(3), 191-197.

Huo, L. J., Ma, X. H., &Yang, Z. M. (2002). Assessment of sperm viability, mitochondrial activity, capacitation and acrosome intactness in extended boar semen during long-term storage. *Theriogenology, 58*(7), 1349-1360. http://dx.doi.org/10.1016/S0093-691X(02)00953-6

Jansen, R. P. (1978). Fallopian tube isthmic mucus and ovum transport. *Science, 201*(4353), 349-351.

Jansen, R. P. (1980). Cyclic changes in the human fallopian tube isthmus and their functional importance. *American Journal of Obstetric and Gynecology, 136*(3), 292-308.

Johnson, L. A., Weitze, K. F., Fiser, P., & Maxwell, W. M. (2000). Storage of boar semen. *Animal Reproduction Science, 62*(1-3), 143-172. http://dx.doi.org/10.1016/S0378-4320(00)00157-3

Kirkman-Brown, J. C., & Smith, D. J. (2011). Sperm motility: is viscosity fundamental to progress? *Molecular Human Reproduction, 17*(8), 539-544. http://dx.doi.org/10.1093/molehr/gar043

Kuster, C. E., & Althouse, G. C. (1999). The fecundity of porcine semen stored for 2 to 6 days in Androhep and X-CELL extenders. *Theriogenology, 52*(3), 365-376. http://dx.doi.org/10.1016/S0093-691X(99)00135-1

Liu, D. Y., Clarke, G. N., & Baker, H. W. (1991). Relationship between sperm motility assessed with the Hamilton-Thorn motility analyzer and fertilization rates in vitro. *J Androl, 12*(4), 231-239.

López-Gatius, F., Sances, G., Sancho, M., Yániz, J., Santolaria, P., Gutiérrez, R., ... Soler, C. (2005). Effect of solid storage at 15 degrees C on the subsequent motility and fertility of rabbit semen. *Theriogenology, 64*(2), 252-260. http://dx.doi.org/10.1016/j.theriogenology.2004.11.015

Marchetti, P., & Marchetti, C. (2007). *Impact and significance of apotosis in human ejaculated spermatozoa*. C.R. Kettleworth: Nova Publishers.

Martín-Hidalgo, D., Barón, F. J., Robina, A., Bragado, M. J., Llera, A. H., García-Marín, L. J., & Gil, M. C. (2013). Inter- and intra-breed comparative study of sperm motility and viability in Iberian and Duroc boar semen during long-term storage in MR-A and XCell extenders. *Animal Reproduction Science, 139*(1-4), 109-114. http://dx.doi.org/10.1016/j.anireprosci.2013.04.001

Nagy, S., Sinkovics, G., & Kovacs, A. (2002). Viability and acrosome integrity of rabbit spermatozoa processed in a gelatin-supplemented extender. *Animal Reproduction Science, 70*(3-4), 283-286. http://dx.doi.org/10.1016/S0378-4320(01)00189-0

Pinheiro, J., Bates, D., Debroy, S., Sarker, D., & The R Development Core Team (2013). nlme: Linear and Nonlinear Mixed Effects Model. *R package version, 3.1-111* .

Reers, M., Smith, T. W., & Chen, L. B. (1991). J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. *Biochemistry, 30*(18), 4480-4486. http://dx.doi.org/10.1021/bi00232a015

Riesenbeck, A. (2011). Review on international trade with boar semen. *Reproduction in Domestic Animals, 46*(Suppl 2), 1-3. http://dx.doi.org/10.1111/j.1439-0531.2011.01869.x

Rikmenspoel, R. (1984). Movements and active moments of bull sperm flagella as a function of temperature and viscosity. *The Journal of Experimental Biology, 108*, 205-230.

Roca, J., Vázquez, J. M., Gil, M. A., Cuello, C., Parrilla, I., & Martínez, E. A. (2006). Challenges in pig artificial insemination. *Reproduction in Domestic Animals, 41*(Suppl 2), 43-53. http://dx.doi.org/10.1111/j.1439-0531.2006.00768.x

Rosato, M., & Iaffaldano, N. (2011). Effect of chilling temperature on the long-term survival of rabbit spermatozoa held either in a tris-based or a jellified extender. *Reproduction in Domestic Animals, 46*(2), 301-308. http://dx.doi.org/10.1111/j.1439-0531.2010.01667.x

Salvador, I., Yániz, J., Viudes-de-Castro, M. P., Gómez, E. A., & Silvestre, M. A. (2006). Effect of solid storage on caprine semen conservation at 5 degrees C. *Theriogenology, 66*(4), 974-981. http://dx.doi.org/10.1016/j.theriogenology.2006.02.042

Saravia, F., Wallgren, M., Nagy, S., Johannisson, A., & Rodríguez-Martínez, H. (2005). Deep freezing of concentrated boar semen for intra-uterine insemination: effects on sperm viability. *Theriogenology, 63*(5), 1320-1333. http://dx.doi.org/10.1016/j.theriogenology.2004.06.012

Simon, L., & Lewis, S. E. (2011). Sperm DNA damage or progressive motility: which one is the better predictor of fertilization in vitro? *Systems Biology in Reproductive Medicine, 57*(3), 133-138.

Smith, D. J., Gaffney, E. A., Gadelha, H., Kapur, N., & Kirkman-Brown, J. C. (2009). Bend propagation in the flagella of migrating human sperm, and its modulation by viscosity. *Cell Motility and the Cytoskeleton, 66*(4), 220-236. http://dx.doi.org/10.1002/cm.20345

Spinaci, M., Chlapanidas, T., Bucci, D., Vallorani, C., Perteghella, S., Lucconi, G., ... Torre, M. L. (2013). Encapsulation of sex sorted boar semen: sperm membrane status and oocyte penetration parameters. *Theriogenology, 79*(4), 575-581. http://dx.doi.org/10.1016/j.theriogenology.2012.10.021

Suárez, S. S., & Dai, X. (1992). Hyperactivation enhances mouse sperm capacity for penetrating viscoelastic media. *Biology of Reproduction, 46*(4), 686-691. http://dx.doi.org/10.1095/biolreprod46.4.686

Torre, M. L., Maggi, L., Vigo, D., Galli, A., Bornaghi, V., Maffeo, G., & Conte, U. (2000). Controlled release of swine semen encapsulated in calcium alginate beads. *Biomaterials, 21*(14), 1493-1498. http://dx.doi.org/10.1016/S0142-9612(00)00035-1

Trzcinska, M., Bryla, M., & Smorag, Z. (2011). Apoptotic-like changes in the spermatozoa of fresh and stored boar semen and the quality of embryos produced in vivo. *Animal Reproduction Science, 124*(1-2), 90-97. http://dx.doi.org/10.1016/j.anireprosci.2011.02.016

Turner, R. M. (2006). Moving to the beat: a review of mammalian sperm motility regulation. *Reproduction, Fertility and Development, 18*(1-2), 25-38. http://dx.doi.org/10.1071/RD05120

Vigo, D., Faustini, M., Torre, M. L., Pecile, A., Villani, S., Asti, A., … Maffeo, G. (2002). Boar semen controlled-delivery system: morphological investigation and in vitro fertilization test. *Reproduction, Fertility and Development, 14*(5-6), 307-314. http://dx.doi.org/10.1071/RD02004

Waberski, D., Henning, H., & Petrunkina, A. M. (2011). Assessment of storage effects in liquid preserved boar semen. *Reproduction in Domestic Animals, 46*(Suppl 2), 45-48. http://dx.doi.org/10.1111/j.1439-0531.2011.01836.x

Waberski, D., Medings, S., Dirksaen, G., Weitze, K. F., Lewiding, C., & Hahn, R. (1994). Fertility of long term-stored boar semen: influence of extender (Androhep and Kiev), storage time and plasma droplets in the semen. *Animal Reproduction Science, 36*, 145-151. http://dx.doi.org/10.1016/0378-4320(94)90061-2

Waterhouse, K. E., De Angelis, P. M., Haugan, T., Paulenz, H., Hofmo, P. O., & Farstad, W. (2004). Effects of in vitro storage time and semen-extender on membrane quality of boar sperm assessed by flow cytometry. *Theriogenology, 62*(9), 1638-1651. http://dx.doi.org/10.1016/j.theriogenology.2004.03.001

Watson, P. F. (1995). Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reproduction, Fertility and Development, 7*(4), 871-891. http://dx.doi.org/10.1071/RD9950871

Yániz, J., Marti, J. I., Silvestre, M. A., Folch, J., Santolaria, P., Alabart, J. L., & López-Gatius, F. (2005). Effects of solid storage of sheep spermatozoa at 15 degrees C on their survival and penetrating capacity. *Theriogenology, 64*(8), 1844-1851. http://dx.doi.org/10.1016/j.theriogenology.2005.04.012

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Selenium Application Timing: Influence in Wheat Grain and Flour Selenium Accumulation Under Mediterranean Conditions

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Abstract

Millions of people have an inadequate supply of selenium (Se) and Se-biofortified crops could prevent such deficiency. In order to establish an effective Se biofortification program under Mediterranean conditions on wheat, the objective of the present study was to evaluate the effect of the Se application timing on the Se accumulation in the grain, yield and protein content. In a field experiment, ten g ha^{-1} of sodium selenate were foliar-applied at four different growth stages: at 1st node detectable (GS-31); at 5th node detectable (GS-35); at boots just swollen (GS-45); and at 1st spikelet visible (GS-51), in two different growing seasons, 2010-2011 and 2011-2012. The application of Se between GS-35 and GS-45 produced the highest Se accumulation in grain, especially in humid years. The milling process caused Se losses of about 15%. In the special conditions of the Mediterranean area, a proper timing of Se application might have major importance in the Se accumulation in the grain, but due to the rainfall before application, rather than to the plant growth stage.

Keywords: agronomic biofortification, application timing, sodium selenate, cereal, semiarid conditions

1. Introduction

Two billion people in the world are thought to suffer from one or more micronutrient deficiencies (FAO, IFAD & WFP, 2012). Among such deficiencies, an inadequate supply of selenium (Se), micronutrient essential for humans and animals, has been associated to a multitude of health disorders, including oxidative stress-related conditions, reduced fertility and immune function, cardiomyopathy, and an increased risk of cancers (Reid et al., 2008; Zeng & Combs, 2008; Rayman, 2012). This deficiency is supported by the studies carried out in Europe by Roman-Viñas et al. (2011), which showed inadequate intake of Se in more than 20% of the population. In this context, the Se intake should be highly increased to reach the recommended values. The European Recommended Dietary Allowance (RDA) of Se for humans is about 55 μg Se day^{-1} (Elmadfa, 2009). Several authors go further and after carrying out clinical trials, have recommended a regular oral dose of 200 μg Se day^{-1} of Se to reduce the incidence of certain cancers and other diseases (Arthur, 2003; Reid et al., 2008). Because food consumption provides the principal route of Se intake for most of the population, the Se biofortification of crops has demonstrated to increase the Se in common dietary foodstuff (Broadley et al., 2010) and thus to enhance human nutrition. Among various crops, cereals could constitute a major source of Se as they are consumed in large amounts in the human diet.

Among cereals, bread making wheat (*Triticum aestivum* L.) has a great relevance in Mediterranean areas and it is the most consumed cereal by humans in the European countries. The wheat grain has been shown to contain a wide range of Se concentrations depending on the Se concentration in the soil. In Spain, the Se concentration in the wheat grain derived products (white flour and biscuits) ranges between 30 μg kg^{-1} and 60 μg kg^{-1} of Se (Diaz-Alarcon, Navarro-Alarcon, de la Serrana & Lopez-Martinez, 1996); concentrations not enough to accomplish the Se intake recommendations. Most of the Se biofortification studies carried out on wheat have been performed in oceanic or continental areas, with high and regular rainfall and a temperate temperature (Broadley et al., 2010; Stroud et al., 2010). Under semiarid Mediterranean or other similar conditions, characterised by scarce precipitations and irregularity in the rainfall, Se biofortification have shown a different pattern, with a higher accumulation potential, at least in other cereals such as two-rowed barley and hard wheat

(Rodrigo, Santamaria, Lopez-Bellido, & Poblaciones, 2013; Poblaciones, Rodrigo, Santamaria, Chen, & McGrath, 2014). On bread making wheat, although there are studies in Portugal (Galinha et al., 2013), Australia (Lyons et al., 2004) and New Zealand (Curtin, Hanson, & van der Weerden, 2008), the effect of the Se biofortification under Mediterranean conditions is still poorly understood.

Regarding the time of application, many authors (Curtin, Hanson, Lindley, & Butler, 2006; Chu, Yao, Yue, Li, & Zhao, 2013) recommended just one application at stem elongation stage, when the flag leaf ligule/collar is just visible (GS-39 according to the Zadocks scale). That growth stage has been regarded as the most effective one in the later Se accumulation in the grain when several applications moments were evaluated. However most of those studies were performed on pots experiments under glasshouse or in oceanic or continental areas. Due to the different soil, climatic and cropping conditions the application of such experiences under Mediterranean conditions is questionable. Therefore, the main objective of the present study was to evaluate the effect of Se application time on the uptake and later accumulation of Se in grain and flour on bread making wheat, and on the grain yield and protein content, in order to provide the basis for an optimal implementation of a Se biofortification program under Mediterranean conditions. In addition, because in previous studies under Mediterranean conditions, the rainfall occurred during the growing season seemed to play a major role in the accumulation of Se in the grain (Poblaciones, Rodrigo, & Santamaria, 2013), it is also hypothesized and analyzed the importance of the rainfall according to the application time.

2. Materials and Methods

2.1 Study Site

Field experiment was conducted in Badajoz, southern Spain (38º54' N, 6º44' W, 186 m above sea level), in a Xerofluvents soil under rainfed Mediterranean conditions in 2010-2011 and 2011-2012 growing seasons. Weather-related parameters for this area in the study years, as well as in the average over a 30-year period, are shown in Figure 1. All climate data were taken from a weather station located at the study site. The precipitation was much higher (more than double), during the growing period (from late November to July), in 2010-2011 (492 mm) than in 2011-2012 (248 mm). In 2010-2011, at full flowering (between April and May), there was a severe drought period of about 40 days (in April, most of rainfall occurred during the first 10 days of the month, and in May, rainfall occurred in the final days of the month). In 2011-2012, at tillering (between late January and March) a very dry period took place (Figure 1).

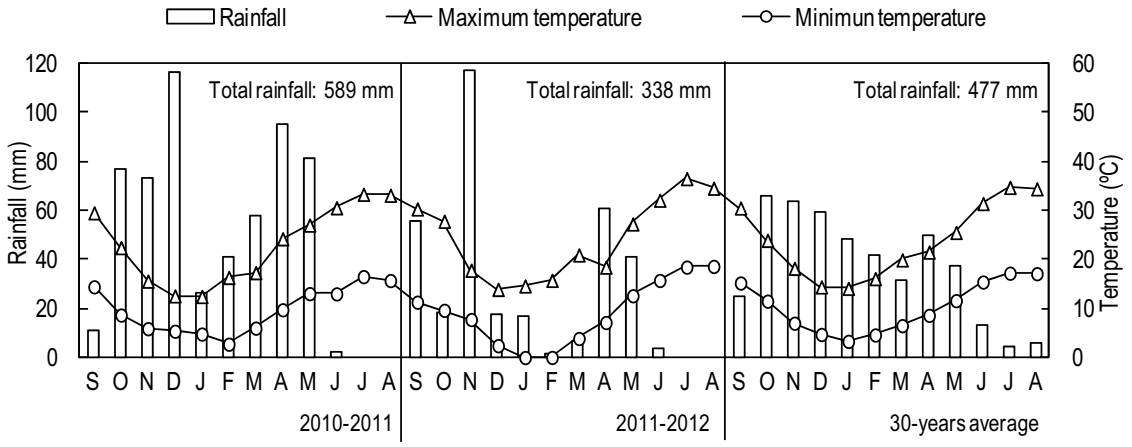

Figure 1. Monthly and annual rainfall and mean maximum and minimum temperatures in 2010-2011, 2011-2012 and in an average year from a 30-year period at Badajoz (Spain)

2.2 Experimental Design and Crop Management

The study area was sown with bread making wheat, cultivar "Roxo". Conventional tillage treatment was used to prepare a proper seedbed before sowing. Sowing was made in late December both study years (2010-2011 and 2011-2012), at a rate of 180 kg ha^{-1} of seeds, in rows of 20 cm. A N-P-K fertilizer (8-15-15) was applied before sowing at a rate of 200 kg ha^{-1} in all plots. The experiment was arranged as a randomized complete block design with four repetitions. In each block, Se was applied foliarly on dry and sunny days at a dose of 10 g ha^{-1} of sodium selenate diluted in 3 L of water in one of the following four different moments: (1) at the stem elongation:

1st node detectable (GS-31); (2) at the stem elongation: 5th node detectable (GS-35); (3) at boots just swollen (GS-45); and (4) at the inflorescence emergence: first spikelet of inflorescence visible (GS-51). Additional plots without any Se application (treatment control) were also performed to be compared with those Se fertilized. The crop area for each Se fertilization treatment and repetition was 15 m^2 (3 m × 5 m). The experimental area used each year had not been previously fertilized with Se, therefore a potential residual effect of Se in the soil can be ruled out.

2.3 Soil Analysis

Each year, before sowing, four representative soil samples to 30 cm depth were taken from the experimental site. Soil samples were air dried and sieved to < 2 mm using a roller mill. Texture was determined gravimetrically; soil pH was measured using a calibrated pH meter (ratio, 10 g soil: 25 ml deionized H_2O), and soil organic matter (SOM) was determined by oxidation by dichromate. Texture, pH and SOM was only determined at the beginning of the experiment.

From these soil samples, total Se was determined as follows: a portion of each soil was finely ground (< 0.45 mm) using an agate ball mill (Retch PM 400 mill); 1 g was digested with ultrapure concentrated nitric acid (2 ml) and 30% w/v hydrogen peroxide (2 ml) using a closed-vessel microwave digestion protocol (Mars X, CEM Corp, Matthews, NC), and diluted to 25 ml with ultrapurified water (Adams, Lombi, Zhao, & McGrath, 2002). Sample vessels were thoroughly washed with acid before use. For quality assurance, a blank and a standard (tomato leaf material, NIST 1573a) were included in each batch of samples. Concentrations of Se were determined using an inductively coupled plasma mass spectrometer (ICP-MS) (Agilent 7500ce, Agilent Technologies, Palo Alto, CA, USA) operating in the hydrogen gas mode. These analyses were developed by the Elemental and Molecular Analysis Service of the University of Extremadura (Spain). All the results were reported on a dry weight basis. Extractable Se in the soil samples was determined by using KH_2PO_4 (0.016 mM, pH 4.8) at a ratio of 10 g dry weight soil: 30 ml KH_2PO_4 w/v (Zhao & McGrath, 1994). The Se concentration in the extracts was determined by ICP-MS, as described above.

2.4 Grain and Flour Analysis

Harvesting took place at crop maturity in early June. Grain yield (expressed as kg ha^{-1} of grain) and grain protein content were determined. Total N content was determined using the Dumas combustion method (Leco FP-428 analyzer, LECO Corp., Saint Joseph, MI U.S). Grain protein was determined by multiplying the total N by 5.7 as a conversion factor. Total Se contained in the milled grain and in the white flour was determined by ICP-MS as described above for soil samples. Grain was milled with a corundum mill (WolfgangMOC, Germany), and white flour was obtained using a Laboratory Mill CD 1 (Chopin, France). All the results were also reported on a dry weight basis.

2.5 Statistical Analysis

The effect of the cropping year (2010-2011 and 2011-2012) on the total and extractable Se into the soil was evaluated by 1-way analysis of variance (ANOVA). Total Se in flour expressed as μg kg^{-1}, Se in grain/Se in flour ratio, grain yield and grain protein were subjected to a 2-way ANOVA, including 'year' (2010-2011 and 2011-2012), 'Se application timing' (Control, GS-31, GS-35, GS-45, GS-51), and their interaction in the model. When significant differences were found in ANOVA, means were compared using Fisher's protected least significant difference (LSD) test at $P < 0.05$. A linear regression was also carried out between the total Se in grain and total Se in flour. In order to evaluate the effect of the weather conditions in the Se accumulation in the grain and flour, Pearson correlation tests, including the data of the two years and of the four application moments, were performed between total Se (in grain and in flour) and the following climate related parameters: (1) the number of days without rain after Se application, (2) the number of days without rain before Se application, (3) the amount of rainfall from seeding to Se fertilization, (4) the amount of rainfall from Se fertilization to harvesting, (5) the amount of rainfall during the 10 days before the Se fertilization, and (6) and the amount of rainfall during the 10 days before and 10 days after the Se fertilization. All these analyses were performed with the Statistix v. 8.10 package.

3. Results

3.1 Soil Properties of the Field Sites

The soil of the experimental area had a loamy texture, with a pH of 7.0 ± 0.13 (mean ± standard error), and a soil organic matter (SOM) of 9.9 ± 0.13 g kg^{-1}. According to ANOVA there was not a significant effect of the year on total Se in the topsoil (degree of freedom (df) = 1, $P = 0.055$), with values of 137.2 ± 6.5 μg kg^{-1} in

2010-2011 and 121.2 ± 6.9 μg kg^{-1} in 2011-2012. Extractable Se, was neither significantly affected by the growing season (df = 1, $P = 0.315$), and was 2.6 ± 0.3 μg kg^{-1} in 2010-2011 and 3.3 ± 0.4 μg kg^{-1} in 2011-2012.

3.2 Effects of Se Application Timing on Grain Yield and Protein Content

Grain yield and grain protein content were significantly affected by the year. Grain protein was also affected by the Se application timing (Table 1). As the interaction between yearxtiming was not significant, the main effects could be analyzed separately. In 2011-2012, it was obtained the highest grain yield and the lowest protein content values (Table 2). Regarding Se timing, when Se was applied at the earliest stages, i.e. at the stem elongation (GS-31 and GS-35), the protein content was higher than when it was applied later, regardless of the growing year (Table 2).

Table 1. ANOVA table showing the effect of the year, Se application timing and their interaction on the total Se in flour (μg kg^{-1}), total Se in grain and flour ratio, grain yield (kg ha^{-1}) and grain protein content (%)

	DF	Total Se flour	Total Se grain/Total Se flour	Grain yield	Grain protein
Year	1	638.46***	0.66	1229.75***	168.25***
Timing	3	55.27***	1.68	1.07	7.73***
Year x Timing	3	13.13***	0.53	0.17	1.72

DF: degree of freedom. *F* values, including the level of significance (*** $P < 0.001$), are shown in the rest of the columns.

Table 2. Mean ± standard error in total Se in flour, grain protein content and grain yield as affected by Se timing (control: without Se fertilization; Se fertilization at growth stages GS-31, GS-35, GS-45, and GS-51 according to Zadocks scale) and year

Timing	Total Se flour (μg kg^{-1})			Grain protein (%)			Grain yield (kg ha^{-1})		
	2010-2011	2011-2012	Mean	2010-2011	2011-2012	Mean	2010-2011	2011-2012	Mean
Control	55 ± 10D	30 ± 5B	42 ± 7D	15.5 ± 0.3	14.1 ± 0.7	14.8 ± 0.4A	1198 ± 44	1967 ± 83	1583 ± 110
GS-31	730 ± 41aB	293 ± 36bA	512 ± 85B	15.6 ± 0.2	13.0 ± 0.1	14.3 ± 0.5A	1274 ± 106	2022 ± 93	1648 ± 113
GS-35	897 ± 60aA	318 ± 46bA	607 ± 94A	15.2 ± 0.1	13.2 ± 0.2	14.2 ± 0.4A	1292 ± 110	1962 ± 86	1627 ± 102
GS-45	860 ± 92aA	326 ± 13bA	593 ± 108AB	14.3 ± 0.4	12.6 ± 0.2	13.4 ± 0.4B	1314 ± 48	2094 ± 70	1704 ± 112
GS-51	519 ± 68aC	257 ± 13bA	388 ± 58C	14.7 ± 0.3	12.1 ± 0.1	13.4 ± 0.5B	1340 ± 81	2082 ± 32	1711 ± 107
Mean	**612 ± 74a**	**245 ± 275b**		**15.1 ± 0.1a**	**13.0 ± 0.2b**		**1284 ± 30b**	**2025 ± 28a**	

For each parameter, averages in the same row, with different lowercase letters mean significantly affected by year ($P < 0.05$) according to LSD test. Averages in the same column, with different uppercase letters are significantly affected by Se application timing ($P < 0.05$) according to LSD test. When letters do not appear, differences were not significant according to ANOVA.

3.3 Total Se Uptake and Accumulation in the Grain and in the Flour

The total Se contained in flour, referred to μg kg^{-1} DW, was significantly affected by the year, application timing and their interaction (Table 1). The Se fertilization, regardless of the application timing and the study year, increased at a great extent the Se concentration in the flour (Table 2) in relation with the control (on average, 525 *vs*. 42 μg kg^{-1} DW). The effect of the Se application timing on the Se accumulation in the flour was regarded to the year. Whilst in the driest year (2011-2012) it was not significantly affected by the application time, in the most humid year (2010-2011), the highest Se accumulation values were obtained when the fertilizer was applied at GS-35 and in GS-45 (Table 2).

The ratio total Se in the grain/total Se in the flour, which indicates the loss of Se during the milling process, was not significantly affected by any of the studied variables (Table 1). The relationship between the total Se in grain and the total Se in flour was linear and highly significant with a Se in grain/Se in flour ratio of 1.15 (Figure 2).

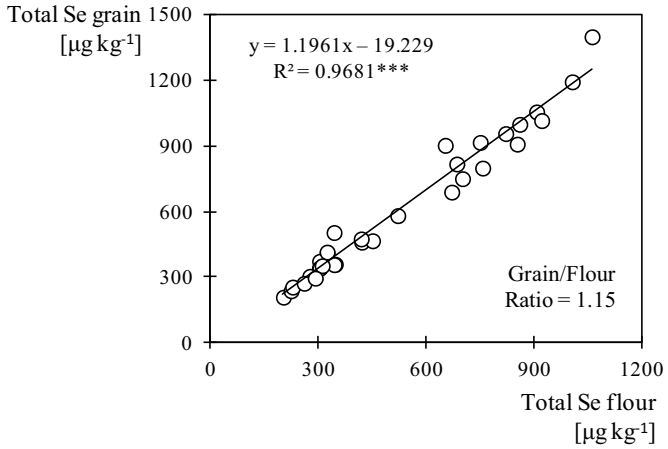

Figure 2. Total Se in grain (µg kg^{-1} DW) expressed as a linear relationship of the total Se in flour (µg kg^{-1} DW). Level of significance: *** $P< 0.001$

3.4 Influence of Rainfall Parameters on Total Se in the Grain and Flour

Considering the pooled data of the two study years and the four application times, both total Se in the flour and total Se in the grain correlated positive (R^2 = 0.63/0.65) and significantly with the number of days before the Se application without rainfall, and negatively (R^2 = -0.76/-0.78) with the amount of rain fallen during the ten days before Se application (Table 3).

Table 3. Correlation coefficients (R^2) obtained in the Pearson correlation tests performed between both the total Se in the flour and the total Se in the grain and each one of several weather-related parameters.

	Days without rain before Se application	Days without rain after Se application	Amount of rain from sowing to Se application	Amount of rain from Se application to harvesting	Amount of rain during the 10 days before application	Amount of rain during the 10 days before and after application
Total Se flour	0.634**	0.083	0.132	0.393	-0.756**	0.145
Total Se grain	0.649**	0.113	0.112	0.437	-0.778**	0.102

** Significant correlation at a level $P< 0.01$.

4. Discussion

4.1 Se Content in Soil

Based on the classification given by Hawkesford and Zhao (2007) the soils of the experimental area could be considered as deficient-marginal in Se, regardless it was obtained as total or extractable Se. These soils might not be able then to provide crop products with enough Se to accomplish the Se intake recommendations, as discussed by Poblaciones et al. (2013) and Rodrigo et al. (2013) in two similar studies conducted on field pea and two-rowed barley in the same area. Under such conditions of deficient Se availability, the introduction of a Se biofortification program might be indicated. The lack of significant effect of the year on total and extractable Se in the topsoil might indicate that further differences in the studied parameters relating to Se content in grain and flour between years could be mainly attributed to climatic variability rather than soil.

4.2 Effects of Treatments on Grain Yield and Grain Protein Content

As it is well known in rainfed conditions, precipitation is a key factor in the crop yield. Although in 2010-2011 the precipitation was higher than in 2011-2012, the severe and prolonged drought occurring during 2010-2011 at full flowering might be the cause of the lower grain production and higher protein content, probably due to a dilution effect. Grain yield was not affected by the Se application timing, in clear agreement with Ducsay and Ložek (2006). In contrast, Chu et al. (2013) found that, when Se was applied at joining-heading and

heading-blooming stages, the grain yield was much higher than it was applied at other stages. However, such study was carried out in much more humid conditions and Se was applied as sodium selenite (instead selenate). Those two relevant differences could explain such disagreement. Regarding protein content, it was higher when Se was applied at the earliest stages, regardless the growing year. It is known the toxicity of Se for the plants at very high concentrations into the soil (Hermosillo-Cereceres et al., 2011). Although in our case a serious toxicity problem might by unlikely due to the initial low Se values into the soil, and the very small dose of Se used in the fertilization, a slight toxicity could affect at some extent the plant protein synthesis or the efficiency of the N absorption by roots. According to the results, as at the earliest stages the protein content was not different than in controls, such slight toxicity only might affect at the latest stages. In any case, the differences in the protein content due to Se timing, although significant, were quite lower than those caused by the climatic conditions. Therefore their relevance could be considered as low in terms of management.

4.3 Total Se in the Grain and in the Flour

Due to the higher grain yield obtained in 2011-2012, a possible dilution effect could be the responsible of the lower Se concentration obtained that year. However, when data were referred to mg ha^{-1} (multiplying the total Se in μg kg^{-1} of Se by the grain yield) to take into account that possible dilution effect, the amount of Se in the flour was also much higher in 2010-2011 than in 2011-2012 (786 *vs*. 496 mg ha^{-1} DW, respectively). Therefore, in this case, rather than a dilution effect, it could be hypothesized that the lower the water availability, the lower the uptake, and consequently the smaller the Se accumulation in the grain. A significant and positive correlation between grain Se concentration and the amount of precipitation during the growing season have been already indicated (Johnson, 1991). Such hypothesis is also in agreement with the stated by Rodrigo et al. (2013) in two-rowed barley and Poblaciones et al. (2013) in field pea, who obtained similar results under similar conditions when Se was also applied as sodium selenate. Hence, the irregular precipitations typical of the Mediterranean conditions may prompt differences in the uptake and accumulation of Se in the grain after fertilization. Consequently, special attention to this irregularity should be paid in order to get a Se biofortification as effective as possible.

In the most humid year, the Se accumulation in the grain was higher when the fertilizer was applied at GS-35 and in GS-45. This result was in line with the general recommendation (application at GS-39 stage, at stem elongation) given for humid regions in temperate climates, such as those in central and northern Europe. In a similar study carried out on winter wheat (Ducsay & Ložek, 2006), when Se fertilizer was applied at the GS-29 stage at a dose of 10 g ha^{-1}, the Se concentration in the grain was much lower than that obtained in the present study. Although several other parameters could have affected such lower Se concentration in the grain, a very early application does not seem to be the most convenient. Other authors have reported a better Se accumulation when Se was applied at flowering stage (Curtin et al., 2006) or even at grain filling (Chu et al., 2013). However, these studies were conducted in more humid regions or in irrigated crops. In our Mediterranean conditions it does not seem convenient such so late applications, as the accumulation of Se in the grain was significantly reduced when applications were performed later than at the boot stage, which is in agreement with the stated by Lyons et al. (2004) in a study on Se biofortification conducted in South Australia on wheat.

The ratio total Se in the grain/total Se in the flour gives information about how much Se is lost during the milling process. Milling is considered as a critical process affecting the concentration of Se on wheat grain (Cubadda, Aureli, Raggi, & Carcea, 2009). Because bran (pericarp, seed coat and aleurone) and germ are removed in the milling process, the value of 1.15 may indicate that about 15% of the selenium in the grain was accumulated in the bran and germ. Similar losses in the milling process were found by Hart et al. (2011) in bread making wheat (Se loss of about 13%) and Cubadda et al. (2009) in durum wheat (Se loss of about 16%). The loss of Se during the milling process was constant regardless the Se application time. This fact could indicate that the distribution of Se within the grain, i.e. in the bran, germ and endosperm, was not affected by the moment in which Se was provided to the plant.

4.4 Influence of Rainfall Parameters on Total Se in the Grain and Flour

According to the results, it seems clear that the rainfall in the previous days before the Se application was the key factor affecting the absorption and later accumulation of Se into the plant, at least when Se was provided as foliar application. In fact, the highest accumulation of Se in the flour during 2010-2011, when Se was applied at the GS-35 and GS-45 stages, agreed precisely to the application carried out with the lowest amount of rainfall during the 10 days before the Se application. This fact could be explained by the high and negative osmotic potential which may occur in the plant under such dry conditions. In this situation, the plant may absorb eagerly and very efficiently through the stomata the Se fertilizer applied, reducing greatly the loss of Se. Therefore, the

importance of the moment of the Se application on the Se accumulation in the grain and flour could be mainly attributed to the rainfall before the application, rather than to the exact growth stage of the plant. A lower amount of rain in this period led to a higher Se accumulation in the grain.

5. Conclusion

The application of Se between the GS-35 and the GS-45 stages according to the Zadocks scale produced the highest accumulation of Se in the grain and in the flour, especially in humid years. However, the rainfall of the growing season seemed to play a very important role in the accumulation of Se in the grain, even more important than the exact growth stage of the plant in the application time. The milling process produced a loss of Se of about 15%, which was not affected by the Se application time. Therefore according to our results, the general recommendations given for the Se biofortification management under humid or temperate climates would be mostly adequate under Mediterranean conditions. However, special attention should be paid to the precipitation as clearly affected the absorption and later accumulation of Se in the grain, especially the rainfall during the ten days before the application. A lower amount of rain in this period led to a higher Se accumulation in the grain. Bread making wheat would be thus a suitable candidate to be included in Se biofortification programs under Mediterranean conditions. The foodstuff derived from its grain could efficiently increase the Se content in the human food chain.

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References

Adams, M. L., Lombi, E., Zhao, F. J., & McGrath, S. P. (2002). Evidence of low selenium concentrations in UK bread-making wheat grain. *Journal of the Science of Food and Agriculture, 82*, 1160-1165. http://dx.doi.org/10.1002/jsfa.1167

Arthur, J. R. (2003). Selenium supplementation: does soil supplementation help and why? *Proccedings of the Nutrition Society, 62*, 393-397. http://dx.doi.org/10.1079/PNS2003254.

Broadley, M. R., Alcock, J., Alford, J., Cartwright, P., Foot, I., Fairweather-Tait, S. J., … Zhao, F. J. (2010). Selenium biofortification of high-yielding winter wheat (*Triticum aestivum* L.) by liquid or granular Se fertilization. *Plant and Soil, 332*, 5-18. http://dx.doi.org/10.1007/s11104-009-0234-4

Chu, J., Yao, X., Yue, Z., Li, J., & Zhao, F. J. (2013). The effects of selenium on physiological traits, grain selenium content and yield of winter wheat at different development stages. *Biological Trace Element Research, 151*, 434-440. http://dx.doi.org/ 10.1007/s12011-012-9575-6.

Cubadda, F., Aureli, F., Raggi, A., & Carcea, M. (2009). Effect of milling, pasta making and cooking on minerals in durum wheat. *Journal of Cereal Science, 49*, 92-97. http://dx.doi.org/10.1016/j.jcs.2008.07.008

Curtin, D., Hanson, R., Lindley, T. N., & Butler, R. C. (2006). Selenium concentration in wheat (*Triticum aestivum* L.) grain as influenced by method, rate, and timing of sodium selenate application. *New Zealand Journal of Crop and Horticultural Science, 34*, 329-339. http://dx.doi.org/10.1080/01140671.2006.9514423

Curtin, D., Hanson, R., & van der Weerden, T. J. (2008). Effect of selenium fertilizer formulation and rate of application on selenium concentrations in irrigated and dryland wheat (*Triticum aestivum* L.). *New Zealand Journal of Crop and Horticultural Science, 36*, 1-7. http://dx.doi.org/10.1080/01140670809510216

Diaz-Alarcon, J. P., Navarro-Alarcon. M., de la Serrana, H. L. G., & Lopez-Martinez, M. C. (1996). Determination of selenium in cereals, legumes and dry fruits from southeastern Spain for calculation of daily dietary intake. *Science of Total Environment, 184*, 183-189. http://dx.doi.org/10.1016/0048-9697(96)05079-6.

Ducsay, L., & Ložek, O. (2006). Effect of selenium foliar application on its content in winter wheat grain. *Plant, Soil and Environment, 52*, 78-82.

Elmadfa, I. (2009). *The European Nutrition and Health Report.* Forum of Nutrition, Vienna, 62.

FAO, IFAD, & WFP. (2012). *The State of Food Insecurity in the World: Economic growth is necessary but not sufficient to accelerate reduction of hunger and malnutrition.* The Food and Agriculture Organization of the United Nations, Rome.

Galinha, C., Freitas, M. do C., Pacheco, A. M. G., Coutinho, J., Macas, B., & Almeida, A. S. (2013). Selenium supplementation of Portuguese wheat cultivars through foliar treatment in actual field conditions. *Journal of Radioanalytical and Nuclear Chemistry, 297*, 227-231. http://dx.doi.org/10.1007/s10967-012-2372-z

Hart, D. J., Fairweather-Tait, S. J., Broadley, M. R., Dickinson, S. J., Foot, I., Knott, P., ... Hurst, R. (2011). Selenium concentration and speciation in biofortified flour and bread: Retention of selenium during grain biofortification, processing and production of Se-enriched food. *Food Chemistry, 126*, 1771-1778. http://dx.doi.org/ 10.1016/j.foodchem.2010.12.079

Hawkesford, M. J., & Zhao, F. J. (2007). Strategies for increasing the selenium content of wheat. *Journal of Cereal Science, 46*, 282-292. http://dx.doi.org/10.1016/j.jcs.2007.02.006.

Hermosillo-Cereceres, M. A., Sanchez-Chavez, E., Balandran-Quintana, R. R., Mendoza-Wilson, A. M., Guevara-Aguilar, A., ... Garcia-Banuelos, M. L. (2011). Ionome variation in bean plant growth under different Se forms and application rates. *Journal of Food Agriculture and Environment, 9*, 374-378.

Johnson, L. (1991). Trends and annual fluctuations in selenium concentrations in wheat grain. *Plant and Soil, 138*, 67-73. http://dx.doi.org/10.1007/BF00011809

Lyons, G., Lewis, J., Lorimer M. F., Holloway, R. E., Brace, D. M., Stangoulis, J. C., & Graham, R. D. (2004). High-selenium wheat: agronomic biofortification strategies to improve human nutrition. *Food, Agriculture & Environment, 2*, 171-178.

Poblaciones, M. J., Rodrigo. S. M., & Santamaria, O. (2013). Evaluation of the potential of peas (*Pisum sativum* L.) to be used in Selenium biofortification programs under Mediterranean conditions. *Biological Trace Element Research, 151*, 132-137. http://dx.doi.org/10.1007/s12011-012-9539-x

Poblaciones, M. J., Rodrigo, S. M., Santamaria, O., Chen. Y., & McGrath. S. P. (2014). Agronomic selenium biofortification in Triticum durum under Mediterranean conditions: From grain to cooked pasta. *Food Chemistry, 146*, 378-384. http://dx.doi.org/10.1016/j.foodchem.2013.09.070.

Rayman, M. P. (2012). Selenium and human health. *The Lancet, 379*, 1256-1268. http://dx.doi.org/10.1016/S0140-6736(11)61452-9

Reid, M. E., Duffield-Lillico, A. J., Slate, E., Natarajan, N., Turnbull, B., Jacobs, E., … Marshall, J. R. (2008). The nutritional prevention of cancer: 400 Mcg per day selenium treatment. *Nutrition and Cancer, 60*(2), 155-163. http://dx.doi.org/10.1080/01635580701684856.

Rodrigo, S., Santamaria, O., Lopez-Bellido, F. J., & Poblaciones, M. J. (2013). Agronomic selenium biofortification of two-rowed barley under Mediterranean conditions. *Plant, Soil and Environment, 59*, 115-120.

Roman-Viñas, B., Ribas-Barba, L., Ngo J., Gurinovic, M., Novakovic, R., Cavelaars, A., … Serra-Majem. L. (2011). Projected Prevalence of Inadequate Nutrient Intakes in Europe. *Annuals of Nutrition and Metabolism, 59*, 84-95. http://dx.doi.org/10.1159/000332762

Stroud, J. L., Li, H. F., Lopez-Bellido, F. J., Broadley, M. R., Foot, I., Fairweather-Tait, S. J., ... Zhao, F. J. (2010). Impact of sulphur fertilisation on crop response to selenium fertilisation. *Plant and Soil, 332*, 31-40. http://dx.doi.org/10.1007/s11104-009-0230-8.

Zeng, H., & Combs, G. F. (2008). Selenium as an anticancer nutrient: roles in cell proliferation and tumor cell invasion. *Journal of Nutrition Biochemistry, 19*, 1-7. http://dx.doi.org/10.1016/j.jnutbio.2007.02.005

Zhao, F. J., & McGrath, S. P. (1994). Extractable sulphate and organic sulphur in soils and their availability to plants. *Plant and Soil, 164*, 243-250. http://dx.doi.org/10.1007/BF00010076

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Evaluation of Selected Methods in the Control of Plant Parasitic Nematodes Infecting Carnation

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Abstract

Plant parasitic nematodes, whose control options are restricted, continue to cause enormous losses in crop production systems including carnations. This study was carried out with the aim of developing environmentally sound approaches for adoption in the management of phytonematodes affecting carnations. Various organic amendments were evaluated namely sugarcane bagasse, tea and assorted flower composts and molasses, in addition nematophagous fungus *Paecilomyces lilacinus* (PL plus®) and neem (Achook®). Meanwhile, a standard chemical nematicide, fenamiphos (Nemacur®), was used next to untreated plots were as control. The experiments were carried out under greenhouse conditions. Soil samples were collected before application of the amendments and at 90 and 180 days after treatment. Parasitic nematodes belonging to 16 genera were detected in plots where carnation had been produced under monoculture over several years. The most predominant of the nematodes detected were in the genera *Scutellonema, Helicotylenchus* and *Meloidogyne,* with 100, 82 and 100% frequencies of occurrence, respectively. Treatments effects caused a reduction in numbers of plant parasitic nematodes, with the exception of nematodes in the genera *Helicotylenchus, Criconema* and *Longidorus.* Galling due to root-knot nematode was reduced by between 53%, in plots treated with sugarcane bagasse, and 69% in those treated with neem. This study has established that application of organic substrates, neem and *P. lilacinus* reduced plant parasitic nematodes. The materials can be recommended for use in sustainable carnation production systems.

Keywords: Dianthus caryophyllus, Meloidogyne spp., neem, organic substrates and Paecilomyces lilacinus

1. Introduction

Carnation (*Dianthus caryophyllus* L.) is estimated to be grown on more than 500 ha mainly under greenhouse conditions in Kenya (HCDA, 2005). Production of the crop has been increasing gradually with expanding market demand. Despite the steady increase in carnation production, there are a number of limitations to the exploitation of the full potential of this industry. These include pests and diseases, excess pesticide usage, low quality produce and low yields (HCDA, 2000). Plant parasitic nematodes have been ranked among the principal constraints limiting carnation production in Kenya. The nematodes are known to damage roots individually but they also form complexes with other organisms which severely disrupts the ability of plants to take up water or nutrients from soil (Back et al., 2002; Masse et al., 2002). Yield loss on carnations due to nematodes alone is estimated at 10% - 20% on a worldwide scale (Phyllis, 1997).

A number of strategies have been developed for the management of plant parasitic nematodes. These strategies include chemical nematicides, fallowing, crop rotation, biological control, host resistance and organic soil amendments (Sikora & Fernandez, 2005). Application of chemical nematicides is the most widely used strategy, especially in intensive production systems involving high-value crops (Haydock et al., 2006). However, concerns about environmental health have raised the motivation to search for alternative methods that are less harmful to non-target species and to the environment (Pinkerton et al., 2000). An area that is fast gaining interest is the application of natural enemies coupled with application of organic amendments (Akhtar & Malik, 2000; El-Sherif et al., 2007). This approach optimizes the ecological synergies between biological components of the ecosystem, enhancing efficiency of soil processes in order to maintain soil fertility, productivity and crop protection. This strategy aims at minimizing the adverse effects on non-target organisms with the ultimate goal of enhancing biodiversity and ecosystem health (Pinkerton et al., 2000; Steinbeger et al., 2001). Information is required on

options that can be adopted to reduce chemical use without compromising quality and crop productivity. The objective of this study was to determine the efficacy of locally available organic amendments, a commercial neem-based product and a nematophagous fungus in suppression of plant parasitic nematodes associated with carnations.

2. Materials and Methods

2.1 Experimental Site

The experiments were conducted under greenhouse conditions between January 2008 and March 2009 at James Finlays Company Farm, Kericho. Plots measuring 1 m x 4 m were laid out in randomized complete block design with six replications. Soil samples were collected prior to application of the treatments. Five soil sub-samples, each consisting of about 500 cm^3, were randomly collected to a depth of 30 cm from the three middle rows of each plot. The soil sub-samples were thoroughly mixed to form a composite sample which was placed in plastic bags and transported to the laboratory and stored at 10^oC. Soil sampling was repeated at 90 and 180 days after transplanting carnations into the plots.

The modified Baermann funnel technique (Hooper et al., 2005) was used to extract nematodes from 200 cm^3 of soil. The soil was spread on a double layer of milk filters supported by a sieve then placed in a shallow dish before adding water to a level where it just touched the soil. After 24 hours, the sieve was carefully removed and the nematodes suspension concentrated by passing through a series of four 45µm-aperture sieves and the nematodes collected from each of the sieves. Aliquots of 1 ml of a well-agitated nematode suspension was pipetted into a counting slide and observed under a light microscope. Counting was repeated for three aliquots and the mean nematode count recorded. Nematodes were then placed in vials and stored at 4^oC awaiting fixation. Nematodes were killed by subjecting them to a temperature of 55 – 60^oC for two minutes and then fixing them in 3% of formalin (Hooper, 2005). Twenty five nematodes from each suspension were selected for identification to the genus level based on morphological features (Hunt et al., 2005).

2.2 Organic Amendment for Plant Parasitic Nematodes Control

The materials tested were sugarcane bagasse which is a fibrous residue of cane stalks left over after the crushing and extraction of the juice. Tea and flower composts are decomposed green materials derived from leaves and shoot trimmings from grading sheds. Molasses (a by-product of sugarcane processing) is thick syrup derived from the processing of the sugarcane into sugar. Neem (Achook®) is a commercial botanical pesticide extracted from the neem tree and contains azadirachtin (0.15%). *Paecilomyces lilacinus* (PL plus®) is a nematophagous fungi known to parasitizes nematode eggs and is formulated in powder form containing 4×10^9 spores/gram of strain 251. Fenamiphos (Nemacur 5Gr®) is a non-volatile systemic nematicide that has been registered for use on a wide range of crops in Kenya. Plots that were not subjected to any treatment were considered as control.

Sugarcane bagasse, tea and flower composts were sun-dried until a constant mass was achieved in about 7 days. The composts and bagasse were incorporated into the soil, just before planting, using a hand hoe at the rate of 300 tons/ha as recommended for greenhouse usage (McSorley & Gallagher, 1995). Molasses was applied at the rate of 667 ml/m^2 (Schenck, 2001) while neem was applied following the manufacturer's recommendation at 1.5 ml/m^2, dissolved in one litre of water. *Paecilomyces lilacinus* was applied at the rate of 2 kg/ha in planting holes while fenamiphos was broadcasted and mixed into the soil, one week before planting, at the rate of 30 g/m^2. All the materials were applied once. One month old rooted spray carnation cultivar White Natila cuttings were transplanted into the plots at a spacing of 12.5 x 25 cm to a achieve a plant density of 32 plants/m^2. Treatments were arranged in a randomized complete block design replicated six times.

Root galling and eggmass indices were quantified using a scale of 1-9 where: 1 = 0 galls/eggs masses, 2 = 1-5, 3 = 6-10, 4 = 11-20, 5 = 21-30, 6 = 31-50, 7 = 51-70, 8 = 71-100, and 9 = 100 galls/egg masses (Sharma et al., 1994). All the data collected was subjected to analysis of variance and where applicable means were compared using LSD at $P\leq0.05$.

3. Results

Plant parasitic nematodes belonging to sixteen genera were identified in plots where carnation had been grown as a monocrop for over ten years. The genera identified were *Scutellonema, Helicotylenchus, Meloidogyne, Pratylenchus, Tylenchus, Hemicyclophora, Tylenchorhynchus, Rotylenchus, Tylenchulus, Criconema, Trichodorus, Hoplolaimus, Hemicriconemoides, Longidorus, Xiphinema* and *Paratylenchus* spp. (Table 1). The most predominant nematodes were in the genera *Scutellonema, Helicotylenchus* and *Meloidogyne* spp., with 100, 81 and 100 % frequencies of occurrence, respectively. The mean numbers of nematodes belonging to the genus *Scutellonema* was highest followed by those in the genus *Meloidogyne*.

Table 1. Plant parasitic nematodes associated with intensive carnation production under greenhouse conditions

Nematode genera	% Density (in 200 cm^3 soil)	Mean numbers	Frequency of occurrence (%)
Scutellonema	14.7	22	100
Helicotylenchus	13.6	13	81
Meloidogyne	11.6	15	100
Pratylenchus	10.5	13	64
Tylenchus	7.8	11	72
Hemicyclophora	7.4	6	50
Tylenchorynchus	6.1	11	61
Rotylenchus	5.5	10	48
Tylenchulus	5.3	10	42
Criconema	3.4	6	39
Trichodorus	3.0	7	41
Hoplolaimus	2.9	6	30
Hemicriconemoides	2.3	6	24
Longidorus	2.2	7	22
Xiphinema	2.1	7	20
Paratylenchus	1.6	7	18

Differences in numbers of all the plant parasitic nematodes were significant among the treatments applied (Figure 1). 90 days after treatment, fenamiphos and neem led to the highest reductions of 72% and 71% in nematode populations, respectively. Sugarcane bagasse was the least effective among the materials tested, causing a 31% reduction in nematode numbers compared to the control. Fenamiphos was the superior treatment, but it was not significantly different from neem. Among the organic materials, molasses was more effective than bagasse.

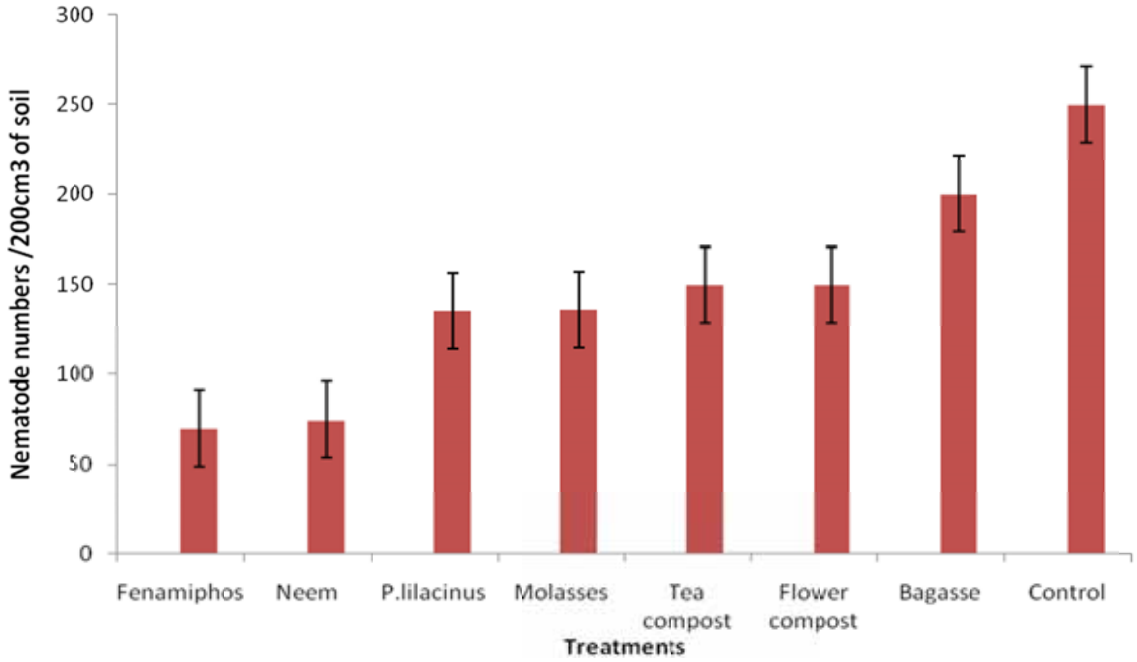

Figure 1. Effects of seven control agents on plant parasitic nematodes in carnations

A decline in nematode numbers was recorded in plots treated with fenamiphos and neem from planting up to 180 days after planting. The nematode numbers declined 90 days after treatment with *P. lilacinus* but an increase was recorded at 180 days. Numbers of plant parasitic nematodes was about two times more in the control compared to sugarcane bagasse, which was the least effective among the treatments at the end of the experiment.

Application of the different treatments had variable effects on plant parasitic nematodes from different genera (Table 2). The effects of sugarcane bagasse, tea compost and molasses were not significantly different on the number of nematodes in the genera *Hemicriconemoides, Pratylenchus, Paratylenchus, Trichodorus* and *Tylenchus.* Nematodes in the genera *Meloidogyne*, *Tylenchorhynchus*, *Rotylenchus*, *Tylenchus* and *Hoplolaimus* were suppressed by all the materials tested as shown in table 2. With the exception of fenamiphos and neem, the rest of the treatments did not cause significant effects on nematodes in the genus *Trichodorus*. The effect of *P. lilacinus* was not significant to nematodes in the genera *Hemicyclophora, Paratylenchus, Trichodorus* and *Tylenchus*.

Treating carnation plots with the various materials resulted in significant reduction in eggmass production and galling induced by root-knot nematodes (Table 3). The lowest eggmass indices (1.5) were observed in plots treated with neem or *P. lilacinus* while the highest (3.9) was recorded in plots treated with bagasse and molasses, excluding the control. Among the plots under treatment, the highest galling index (2.1) was observed in carnation treated with bagasse while plots treated with neem recorded the least (1.4). The highest reduction in galling index (69%) was recorded in plots treated with neem ,followed by the one treated with fenamiphos.

Table 2. Effect of different treatments on plant parasitic nematodes from different genera associated with carnations

Nematode genera	Numbers of nematodes in 200 cm^3 soil							
	Bagasse	Tea compost	Flower compost	Fenamiphos	Neem	Molasses	*P lilacinus*	Control
Scutellonema	18.1b	14.7b	18.9b	9.7b	14.2b	12.2b	61.1a	68.3a
Meloidogyne	20.8b	15bc	21.4b	4.4d	8.9cd	21.1b	20..6b	34.4a
Pratylenchus	20.8ab	12.5cd	11.9cd	6.7d	8.9d	16.4bc	18.3abc	23.6a
Tylenchus	13.9ab	13.3ab	6.4c	5.6c	6.1c	7.8bc	18.3a	17.1a
Tylenchorynchus	9.7b	8.6bc	8.9bc	1.9d	3.3cd	7.2bcd	3.1cd	26.9a
Rotylenchus	7.8bc	8.1b	8.3b	2.9c	6.9bc	6.9bc	8.5b	18.3a
Tylenchulus	5.8bc	7.8bc	6.4bc	3.9c	4.2bc	3.9c	9.2b	18.7a
Trichodorus	4.7ab	5.8a	5.0ab	1.1c	3.1bc	5.3ab	3.6abc	5.6ab
Hoplolaimus	4.2b	4.4b	3.9b	1.7b	1.4b	2.8b	4.4b	10.0a
Hemicriconemoides	3.9a	3.3ab	5.0a	0.8b	1.1b	4.7a	3.1ab	4.4a
Paratylenchus	2.2abc	2.5abc	2.8abc	0.8c	0.8c	1.7bc	3.3ab	4.2a

Data are means of 18 samples. Means followed by the same letter(s) along rows are not significantly different ($P \leq 0.05$).

Table 3. Effect of different treatments on eggmass (EMI) and galling (GI) caused by root-knot nematodes in carnations

Treatments	EMI	Galling index (GI)	% Reduction in GI
Bagasse	3.9b	2.1b	53%
Tea compost	3.1c	1.7c	62%
Flower compost	2.9c	1.8c	60%
Fenamiphos	1.7d	1.5d	67%
Neem	1.5d	1.4d	69%
Molasses	3.9b	1.9c	58%
P. lilacinus	1.5d	1.9c	58%
Control	6.2a	4.5a	-

Data are means of 18 samples. Means followed by the same letter(s) within columns are not significantly different ($P \leq 0.05$).

4. Discussion

Out of the sixteen plant parasitic nematodes associated with carnations, members of the genera *Scutellonema*, *Meloidogyne* and *Helicotylenchus* were the most frequently encountered. This finding is consistent with previous reports on distribution of plant parasitic associated with different cropping systems in Kenya (Kimenju et al., 1998; Kandji et al., 2003). Among the nematodes that were widespread in carnation plots, *Meloidogyne* spp. present the greatest threat, given their damage potential as endo-parasites, compared to the rest which are ectoparasites. Apart from the direct effects that root-knot nematodes have on plants, they form synergistic complexes with other disease causing organisms leading to severe destruction and reduction in yield (Back et al., 2002; Masse et al., 2002).

This study has demonstrated that amending soils with organic substrates as well as incorporating a biological control agent (*P. lilacinus*) and neem resulted in significant reduction of numbers of plant parasitic nematodes associated with carnation. The organic substrates tested namely bagasse, tea and flower composts and molasses were suppressive to all genera of plant parasitic nematodes in carnation plots. In addition, the amendments reduced the reproductive potential of root knot nematodes as measured using eggmass densities. These findings are in agreement with previous reports by Akhtar and Malik (2000), Agyarko and Asante (2005) who found that incorporating organic amendments into the soil, have a an effect on soil organisms.

Organic substrates offer an exciting alternative or supplement to other strategies, with the ultimate goal of reducing chemical usage in general and nematicides in particular (Akhtar & Malik, 2000). The mode of action stems from the decomposition process that leads to changes in the physical and chemical properties of the soil. According to Sanchez-Moreno and Navas (2007), the nematode community is strongly influenced by changes in soil systems since nematodes are highly dependent on soil properties. When incorporated into the soil, organic substrates undergo a series of processes that release NH_4^+, formaldehyde, phenols and volatile fatty acids, among other compounds (Walker, 2004; Wang et al., 2004). The compounds may act individually or collectively to stimulate build-up of beneficial microbes that are antagonistic to plant parasitic nematodes (Kerry, 2000; Akhtar & Malik, 2000; Viaene et al., 2006). In addition to the direct effects on nematodes, organic amendments also increase the water holding capacity of the soil, improve the soil structure and release nutrients into the soil (Walker, 2004). These attributes are known to increase plant's ability to overcome the negative effects of nematode infestation (Mcsorley & Gallagher, 1995).

Application of organic substrates led to an initial increase in numbers of some plant parasitic nematodes but there was an ultimate reduction in the numbers. The delay in response of nematodes to application of organic substrates may be explained by the fact that a substantial amount of time is required for the breakdown of the substrates and subsequent release of the active ingredients into the soil (Widmer & Abawi, 2002; Kimenju et al., 2004).

In this study, the efficacy of *P. lilacinus* was clearly demonstrated as a promising strategy in carnation production for the control of root-knot nematodes. *Paecilomyces lilacinus* is an ubiquitious soil hyphomycete which parasitizes eggs of root-knot nematodes thus regulating populations of the nematodes in field soil (Schenck, 2004). While, Khalil et al. (2012) found that *P. lilacinus* was the most effective bioagent between others against *M. incognita* on tomato under greenhouse conditions for galls, egg masses and nematode population in soil by 66.67, 75.97 and 85.22%, respectively. Also, Kiewnick and Sikora (2006) recorded that the fungal biocontrol agent, *P. lilacinus* strain 251 (PL251) was potential to control the root-knot nematode *Meloidogyne incognita* on tomato. The action of *P. lilacinus* against plant parasitic nematodes was interpreted in multitude investigations. Khan et al. (2006) and Khan et al. (2004) recorded the directed penetration of fungal hypha to the female cuticle of *M. javanica* by transmission electron microscopy. While, Park et al. (2004) reported that *P. lilacinus* could be produce leucino toxin and other nematicidal compounds. In the laboratory test this fungus infested eggs of *M. incognita* and destroys the embryos within 5 days because of simple penetration of the egg cuticle by individual hypha aided by mechanical and/or enzymatic activities, in addition to killing juveniles and females of *M. incognita* and *Globodera pallida* (Jatala, 1986). The pesticidal properties of azadirachtin, the active ingredient in neem, have been clearly documented (Akhtar & Malik, 2000; Agyarko & Asante, 2005). According to Agyarko and Asante (2005), neem based products reduced egg hatching and the mobility of nematode juveniles. Several other compounds namely salannin, nimbidin, thionemone ammonia, phenols, formaldehyde and fatty acids are released during decomposition of neem-based products (Javed et al., 2007) which antagonises the nematodes reproduction. in the same trend both Khalil et al. (2012) and Saad et al. (2012) found that Azadirachtin (Achook 0.15%) effective against *Meloidogyne* sp. on tomato plants. It was suggested that neem may be effect as repellent compound on plant parasitic nematodes (Khalil, 2013).

Carnation growing is mainly done under monoculture in intensive production systems. According to Giller et al. (1997) monoculture is one of the practices that deny the soil the long-term benefits of functional and taxonomic

diversity. The practice results in biophysical, chemical and hydrological changes that interfere with the biological equilibria in the soil (Freckman & Ettema, 1993). Application of non-chemical strategies and especially organic substrates may help to maintain the soil's productive potential.

5. Conclusion and Recommendations

From the study, it is evident that organic amendments can play a role in managing plant parasitic nematodes in high value crops. Further work is required to assess to the potential of combining different methods for plant parasitic nematodes.

References

Agyarko, K., & Asante, J. S. (2005). Nematode dynamics in soil amended with neem leaves and poultry manure. *Asian Journal of Plant Science, 4*, 426-428. http://dx.doi.org/10.3923/ajps.2005.426.428

Akhtar, A., & Malik, A. (2000). Roles of organic soil amendments and soil organisms in the biological control of plant parasitic nematodes: a review. *Bio resource Technology, 74*, 35-47. http://dx.doi.org/10.1016/S0960-8524(99)00154-6

Back, M. A., Haydock, P. P., & Jenkinson, P. (2002). Disease complexes involving plant parasitic nematodes and soilborne pathogens. *Plant Pathology, 51*, 683-697. http://dx.doi.org/10.1046/j.1365-3059.2002.00785.x

El-Sherif, A. G., Refaei, A. R., El-Nagar, M. E., & Salem, M. (2007). Integrated management of *Meloidogyne incognita* infecting eggplant by certain organic amendments, *Bacillus thuringiensis* and oxamyl with reference to N P K and total chlorophyll status. *Plant Pathology Journal, 6*, 147-152. http://dx.doi.org/10.3923/ppj.2007.147.152

Freckman, D. W., & Ettema, C. H. (1993). Assessing nematode communities in agroecosystems of varying human intervention. *Agriculture Ecosystems and Environment, 45*, 239-261. http://dx.doi.org/10.1016/0167-8809(93)90074-Y

Giller, K. E., Beare, M., Lavelle, P., Izac, A. M., & Swift, M. J. (1997). Agricultural intensification, soil biodiversity and agroecosystem function. *Applied Soil Ecology, 6*, 3-16. http://dx.doi.org/10.1016/S0929-1393(96)00149-7

Haydock, P. J., Woods, S. R., Grove, G., & Hare, M. C. (2006). Chemical control of nematodes. In R. N. Perry & M. Moens (Eds.), *Plant Nematology* (pp. 392-408). Wallingford, Cambridge: CAB International. http://dx.doi.org/10.1079/9781845930561.0392

Hooper, D. J., Hallmann, J., & Subbotin, S. A. (2005). Methods for extraction, processing and detection of plant and soil nematodes. In M. Luc, R. A. Sikora, & J. Bridge (Eds.), *Plant Parasitic Nematodes in Subtropical and Tropical Agriculture* (2nd ed., pp. 53-86). CAB International. http://dx.doi.org/10.1079/9780851997278.0053

Horticultural Crops Development Authority (HCDA). (2000). Statistics of horticultural crops. *HCDA Annual report*.

Horticultural Crops Develpment Authority (HCDA). (2005). Horticultural sector policy objectives. *HCDA Annual report*.

Hunt, D. J., Luc, M., & Manzanilla-Lopez, R. H. (2005). Identification, morphology and biology of plant parasitic nematodes. *Plant parasitic nematodes in subtropical and tropical agriculture, 2*, 11-52. http://dx.doi.org/10.1079/9780851997278.0011

Jatala, P. (1986). Biological control of plant parasitic nematodes. *Ann. Rev. Phytopathol., 24*, 453-489. http://dx.doi.org/10.1146/annurev.py.24.090186.002321

Javed, N., Gowen, S. R., El-Hassan, S. A., Inam-ul-Haq, M., Shahina, F., & Pembroke, B. (2007). Efficacy of neem (*Azadirachta indica*) formulations on biology of root-knot nematodes (*Meloidogyne javanica*) on tomato. *Crop Protection, 27*, 36-43. http://dx.doi.org/10.1016/j.cropro.2007.04.006

Kandji, S. T., Ogol, C. K., & Albrecht, A. (2003). Crop damage by nematodes in improved-fallow fields in Western Kenya. *Agro forestry Systems, 57*, 51-57. http://dx.doi.org/10.1023/A:1022949431814

Kerry, B. (2000). Rhizosphere interactions and the exploitation of microbial agents for the biological control of plant-parasitic nematodes. *Annual Review of Phytopathology, 38*, 423-441. http://dx.doi.org/10.1146/annurev.phyto.38.1.423

Khalil, M. S. (2013). Abamectin and Azadirachtin as Eco-friendly Promising Biorational Tools in Integrated Nematodes Management Programs. *J Plant Pathol Microb, 4*, 174. http://dx.doi.org/10.4172/2157-7471.1000174

Khalil, M. S., Kenawy, A., Ghorab, M. A., & Mohammed, E. E. (2012). Impact of microbial agents on *Meloidogyne incognita* management and morphogenesis of tomato. *J. Biopest., 5*(1), 28-35.

Khan, A., Williams, K. L., & Nevalainen, H. K. M. (2004). Effects of Paecilomyces lilacinus protease and chitinase on the egg shell structures and hatching of Meloidogyne javanica juveniles. *Biol. Cont., 31*, 346-352. http://dx.doi.org/10.1016/j.biocontrol.2004.07.011

Khan, A., Williams, K. L., & Nevalainen, H. K. M. (2006). Infection of plant parasitic nematodes by Paecilomyces lilacinus and Monacrosporium lysipagum. *Biol. Cont., 51*, 659-678.

Kiewnick, S., & Sikora, R. A. (2006). Biological control of the root-knot nematode Meloidogyne incognita by Paecilomyces lilacinus strain 251. *Biol. Cont., 38*, 179-187. http://dx.doi.org/10.1016/j.biocontrol.2005.12.006

Kimenju, J. W., Muiru, D. M., Karanja, N. K., Nyongesa, M. W., Miano, D. W., & Mutua, G. K. (2004). Assessing the role of organic amendments in management of root-knot nematodes on common bean, *Phaseolus vulgaris* L. *Tropical Microbiology and Biotechnology, 3,* 14-23.

Kimenju, J. W., Waudo, S. W., Mwang'ombe, A. W., Sikora, R. A., & Schurter, R. P. (1998). Distribution of lesion nematodes associated with maize in Kenya and susceptibility of maize cultivars to *Pratylenchus zeae*. *African Crop Science Journal, 6*, 367-375.

Masse, D., Pate, E., Ndiaye-Faye, N., & Cadet, P. (2002). Effect of fallow improvement on the nematode community in the Sudanian region of Senegal. *European Journal Soil Biology, 38*, 205-211. http://dx.doi.org/10.1016/S1164-5563(02)01149-4

McSorley, R., & Gallagher, R. N. (1995). Effect of yard waste compost on plant-parasitic nematode Densities in Vegetable Crops. *Journal of Nematology, 27*, 545-549.

Park, J. O., Hargreaves, J. R., McConville, E. J., Stirling, G. R., Ghisal-berti, E. L., & Sivasithamparam, K. (2004). Produc tion of leucinostatins and nematicidal activity of Australian isolates of Paecilomyces lilacinus (Thom) Samson. *Lett. Appl. Microbiol., 38*, 271-276. http://dx.doi.org/10.1111/j.1472-765X.2004.01488.x

Phyllis, J. (1997). Outlook on plant nematodes and their control in Cyprus. In M. A. Maqbool & B. Kerry (Eds.), *Plant Nematode Problems and their Control in the Near East Region*. Food and Agricultural organization of the United Nations, Rome

Pinkerton, J. N., Ivors, K. L., Miller, M. L., & Moore, L. W. (2000). Effect of solarization and cover crops on populations of selected soil-borne plant pathogens in western Oregon. *Plant Diseases, 84,* 952-960. http://dx.doi.org/10.1094/PDIS.2000.84.9.952

Saad, A. S. A., Massoud, M. A., Ibrahim, H. S., & Khalil, M. S. (2012). Activity of nemathorin, natural product and bioproducts against root-knot nematodes on tomatoes. *Archives of phytopathol. and plant prot., 45*(8), 955-962. http://dx.doi.org/10.1080/03235408.2012.655145

Sánchez-Moreno, S., & Navas, A. (2007). Nematode diversity and food web condition in heavy metals polluted soils in a river basin in southern Spain. *European Journal of Soil Biology, 43*, 166-179. http://dx.doi.org/10.1016/j.ejsobi.2007.01.002

Schenck, S. (2001). Molasses soil amendment for crop improvement and nematode management. *Vegetable Report 3*. Hawaii Agriculture Research Center.

Sharma, S. B., Sikora, R. A., Greco, N., Di Vito, M., & Caubel, G. (1994). Screening techniques and sources of resistance to nematodes in cool season food legumes. *Euphytica, 73*, 59-66. http://dx.doi.org/10.1007/BF00027182

Sikora, R. A., & Fernandez, E. (2005). Nematode parasites of vegetables. In M. Luc, R. A. Sikora, & J. Bridge (Eds.), *plant parasitic nematodes in subtropical and tropical agriculture* (2nd ed., pp. 319-392). CAB International. http://dx.doi.org/10.1079/9780851997278.0319

Steinberger, Y., Liang, W. E., Savkina, T., & Barness, G. (2001). Nematode community composition and diversity associated with a topoclimatic transect in a rain shadow desert. *European Journal of Soil Biology, 37*, 315-320. http://dx.doi.org/10.1016/S1164-5563(01)01107-4

Viaene, N., Coyne, D. L., Kerry, B. R. (2006). Biological and cultural management. In R. N. Perry & M. Moens (Eds.), *Plant Nematology* (pp. 346-369). Wallingford, Cambridge: CAB International. http://dx.doi.org/10.1079/9781845930561.0346

Walker, G. E. (2004). Effects of *Meloidogyne javanica* and organic amendments, inorganic fertilizers and nematicides on carrot growth and nematode abundance. *Nematol. Medit., 32*, 181-188.

Wang, K. H., McSorley, R., & Gallagher, R. N. (2004). Effect of *Crotalaria juncea* amendment on squash infected with *Meloidogyne incognita*. *Journal of Nematology, 36*, 290-296.

Widmer, T. L., & Abawi, G. S. (2002). Mechanism of suppression of *Meloidogyne hapla* and its damage by a green manure of Sudan grass. *Plant Diseases, 84*, 562-568. http://dx.doi.org/10.1094/PDIS.2000.84.5.562

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Susceptibility of *Gossypium mustelinum* Populations to the Main Cotton Diseases in Brazil

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Abstract

The present study was conducted in five *Gossypium mustelinum* populations that were phenotyped and genotyped to quantify their resistance to the major diseases that affect cultivated cotton (*G. hirsutum*) in Brazil. Four *G. mustelinum* populations, in addition to genotypes of *G. hirsutum* and *G. barbadense* (resistant or susceptible controls), were phenotyped for resistance to cotton blue disease, angular leaf spot, common mosaic and ramulose. Artificial inoculation of cotton plants with causative agents of cotton blue disease, angular leaf spot and ramulose, as well as those naturally infested with common mosaic virus, showed that all *G. mustelinum* accessions were susceptible to every disease studied. Four microsatellite markers linked to disease resistance genes for cotton blue disease, angular leaf spot and root-knot nematodes (*Meloidogyne* spp.) in *G. hirsutum* were used for genotyping of the five populations. The markers amplified different alleles from those associated with resistance genes in cultivated cotton, revealing polymorphisms different from reported cases of *G. hirsutum* resistance. The susceptibility to all diseases studied may represent a phytosanitary risk for the *in situ* conservation of natural *G. mustelinum* populations.

Keywords: *in situ* conservation, SSR marker, natural populations, wild cotton

1. Introduction

In situ conservation of landraces or wild relatives of cultivated plants can be hindered if crops are cultivated near these populations. One possible interference is the genetic mischaracterization of wild populations due to gene flow (Groot et al., 2003). This possibility has been discussed with greater emphasis after the commercial release of genetically modified cultivars (Ellstrand et al., 1999; Darmency, 2008). Despite the importance of gene flow, other factors may render *in situ* conservation more complex independently of whether the cultivated genotypes are genetically modified or not.

Commercial crops or other exotic plants introduced by man can trigger outbreaks of pests and diseases that, depending on the distance, can affect related wild species or local varieties (landraces). The impact on genotypes previously present in the region depends on several factors, including the level of genetic resistance to diseases in cultivated and wild genotypes (Alexander, 2010). The understanding of epidemics in commercial crops has been widely discussed in recent years (Anderson et al., 2004; Fisher et al., 2012); however, little is known about the levels of resistance in wild populations. In some pathosystems, the causative agent of the disease has a wide range of hosts, including related species. In such cases, co-cultivation of susceptible species may have a negative impact on natural populations.

There are well-reported cases where populations of native species have been negatively affected by the introduction of cultivated genotypes, such as *Ulnus* spp. (North America, Europe and Southeast Asia), *Castanea dentata* (Western U.S.), Eucalyptus (Australia) and *Nassela pulchra* (California) (Gilbert, 2002; Anderson et al., 2004). Among native grasses in California, for example, an increased incidence of pathogens due to coexistence with cultivated exotic grasses caused a reduction in their reproductive performance, reducing the number of individuals descended from wild populations (Malmstrom et al., 2005).

Wild populations and landraces comprise a dynamic germplasm from the point of view of variability because they are subjected to all evolutionary factors. If a new, very aggressive evolutionary agent is introduced into the environment, the selection resulting from the adaptation of populations to this new agent can reduce their genetic basis, jeopardizing the conservation of the variability not associated with the new agent and preventing proper *in situ* conservation. Brazil is an important center of origin and diversity of wild and cultivated populations, including several species of *Manihot* (Fukuda et al., 2005), *Passiflora* (Cervi, 2006), *Zea mays* L. (Carvalho et al., 2002), *Phaseolus vulgaris* (Burle et al., 2010), *G. hirsutum* var. *marie galante* (Menezes et al., 2010), *Oryza glumaepatula* (Brondani et al., 2005) and *Gossypium mustelinum* (Barroso et al., 2010), the species of focus in the present study.

Gossypium mustelinum is one of five allotetraploids of this genus, which includes the cultivated cotton plants (*G. hirsutum* and *G. barbadense*). The species is a native of Brazil and endemic to the northeast region, primarily found in the biome of Caatinga (Pickersgill et al., 1975; Barroso et al., 2010). There are no crops found within its area of incidence; however, there is a potential for expansion of the cultivated cotton areas in surrounding areas (Brazilian Native and Naturalized Cotton [ALBRANA], 2013). There are small foci of coexistence with cultivated cotton. They result from maintenance of plants in backyards and from propagule dispersal between producing and consuming regions, with no observed impact on wild populations (Alves et al., 2013). This is an indication that impacts may depend on broader sympatry, where a greater number of cultivated individuals are within the vicinity.

Pathogens that infect commercial crops can also cause diseases in other species of allotetraploid cotton plants, due to their phylogenetic proximity (Gilbert & Webb, 2007). Thus, the introduction and spread of pathogens from commercial cotton farms may be a threat to the conservation of *G. mustelinum* populations. The main diseases affecting commercial cotton crops in Brazil are cotton blue disease, caused by the *Polerovirus* Cotton leafroll dwarf virus - CLRDV; angular leaf spot or blight, caused by the bacterium *Xanthomonas citri* subsp. *malvacearum*; ramulose, caused by the fungus *Colletotrichum gossypii* var. *cephalosporioides*; root-knot disease, caused by the nematode *Meloidogyne incognita*; and gray mildew disease, caused by the fungus *Ramularia areola* (Suassuna & Coutinho, 2011). Historically, phytosanitary problems with these diseases increased with the expansion and intensification of cotton production in the region. Although one cannot deny the economic and social benefits of cotton production in Brazil, which is the fifth largest cotton producer in the world, its cultivation should not occur at the expense of *in situ* conservation and maintenance of important native genetic resources.

Although collection and sampling expeditions for *ex situ* conservation of populations of interest have been conducted, *in situ* conservation should not be excluded. *In situ* conservation allows continuity of the evolutionary process of populations, deriving new variations, which may be introduced to germplasm banks (Negri & Tiranti, 2010). Both methods are complementary and are more efficient for the conservation of total genetic diversity when used together (Sun et al., 2012). In regards to *G. mustelinum*, efforts have been made to employ both methods (Barroso et al., 2010). Therefore, knowledge of the genetic resistance of *G. mustelinum* to the major cotton crop diseases may help us to understand the scale of threats to its conservation. In the present study, the resistance of *G. mustelinum* populations to major cotton diseases in Brazil was measured.

2. Materials and Methods

2.1 Plant Material

Gossypium mustelinum cotton plants were collected in the tributaries of the Contas river basin in the State of Bahia, Brazil. These plants were located in four tributaries: Jacaré Creek, Quixaba Creek, Serra Azul Creek and Riachão Creek. A total of 205 plants were used in this study.

In the resistance evaluation tests (phenotyping), seeds collected *in situ* from populations of Jacaré Creek (24 plants), Serra Azul Creek (23 plants) and three tributaries of the Riachão Creek I (22 plants), II (22 plants) and III (20 plants) were separated into different treatment groups. Controls for cotton blue disease included the genotypes Delta Opal (*G. hirsutum* - resistant) and FM 966 *(G. hirsutum* - susceptible); for angular leaf spot, Delta Opal (resistant) and PA 0435 (*G. barbadense* - susceptible) and for ramulose, PA 0435 (moderately resistant) and BRS Cedro (*G. hirsutum* - susceptible).

2.2 Preparation of Inoculum, Inoculation and Evaluation of Incidence/Severity

Assessment for angular leaf spot and common mosaic was conducted under field conditions in a randomized block design with five replications, and each plot was comprised of two rows of three meters with one plant per meter. For angular leaf spot, artificial inoculations were made. The inoculum was obtained from *Xam* isolates previously identified as race 18 cultured in *523 KADO* medium for 48 hours and diluted with saline solution (0.85% NaCl), and adjusted to 0.3 optical density at 600 nm absorbance, which corresponds to 10^8 CFU (Colony Forming Units).

Inoculation was performed using the syringe method, in which approximately 1.0 mL of bacterial suspension was injected under pressure at three different points on the abaxial surface of three leaves of each plant. Evaluation was performed 48 hours after inoculation by observing for the presence or absence of anasarca symptoms at the inoculation points. The same test was used to evaluate, via natural infection, the resistance to common mosaic disease caused by whitefly-transmitted geminivirus.

Assessment of cotton blue disease was carried out in a protected screenhouse. The trial was conducted in a randomized block design with five replications, with an experimental unit consisting of one plant grown in soil from the base of the screenhouse. All plants were inoculated using viruliferous aphids (*Aphis gossypii*) collected from previously cultivated cotton plants with typical cotton blue disease symptoms (inoculum source plant). The inoculations were performed in the different treatments at 20, 27 and 34 days after plant emergence (DAE). At each inoculation time, at least five viruliferous aphids, maintained in the inoculum source plants, were transferred to different treatment plants. Disease assessment was qualitatively performed (with or without symptom development) 30 and 60 days after the first inoculation.

Assessment of ramulose resistance was measured in an experiment conducted in a greenhouse. The experiment was conducted in a randomized block design with five replications, and the experimental unit consisted of one plant grown in a pot (20 liters). Three monosporic isolates of the pathogen were previously multiplied in potato dextrose agar (PDA) medium and maintained in a biochemical oxygen demand (BOD) incubator (25°C and 12 hours light) for 30 days. A spore suspension at a concentration of 5 x 10^5 spores/mL was prepared. At 30 and 37 DAE, plants were inoculated with the aid of a hand sprayer. Each pot was covered for 24 h with a plastic bag (100 liter volume) forming a humid chamber. After removal from the humid chamber, the environment was maintained with relative air humidity above 80% with the aid of microsprinklers that sprayed a mist for 1 minute every 3 hours. Thirty days after the second inoculation, a severity assessment was conducted using a scale from 1 to 5, with which the disease index was calculated (Oliveira et al., 2010). Due to the inadequate normal distribution of the data, the pair-to-pair difference between treatments for ramulose severity obtained from the average scores was tested using the nonparametric *Mann-Whitney test.*

2.3 Genotyping With Microsatellite Markers

All collected *G. mustelinum* plants (n = 205) were genotyped using microsatellite markers linked to resistance genes (Table 1). Three *G. hirsutum* cultivars were used as controls for the presence of resistance alleles: Delta Opal, resistant to cotton blue disease and angular leaf spot; M315, resistant to root-knot nematode; and FM 966, resistant to angular leaf spot and susceptible to cotton blue disease and root-knot nematode.

Five simple sequence repeat (SSR) primer pairs labeled with fluorochromes were used for genotyping. These SSR loci are physically linked to genes that confer resistance to major cotton diseases: DC20027, linked to the *Rghv1* gene that confers resistance to cotton blue disease (Fang et al., 2010); CIR246, linked to the *B12* gene, which confers resistance to angular leaf spot (Xiao et al., 2010); and CIR316M (Shen et al., 2006) and BNL3661 (Guitiérrez et al., 2010), which are associated with two different loci that control root-knot nematode tolerance. Polymerase chain reactions were performed in a tetraplex system using the Multiplex PCR Kit (Qiagen). Electrophoresis of amplified fragments was performed on an ABI3100 DNA analyzer (Applied Biosystems). Fragment size (bp) was estimated using GeneScan 2.1 with ROX500 and edited with the aid of GeneMapper 3.5 software (Applied Biosystems). SSR marker-assisted analysis in *G. mustelinum* was carried out by comparing the amplified alleles to the loci of the SSRs that were associated with resistance genes in *G. hirsutum*.

Table 1. Statistical significance matrix between populations of *G. mustelinum* (Jacaré Creek, Serra Azul Creek, Riachão Creek I, II, III) and controls (BRS Cedro - susceptible and PA 0435 - moderately resistant)

	Jac	SA	RRI	RRII	RRIII	PA 0435
SA	0.47					
RRI	0.38	0.45				
RRII	0.50	0.60	0.73			
RRIII	0.79	0.76	0.31	0.38		
PA 0435	0.94	0.94	0.50	0.60	0.80	
BRS Cedro	0.03*	0.04*	0.02*	0.01*	0.05*	0.04*

Mann-Whitney test (0.05%).

3. Results

Figure 1. *G. mustelinum* plants with symptoms of foliar diseases. (a) cotton blue disease, (b) angular leaf spot, (c) common mosaic and (d) ramulose

In the assessment of cotton blue disease, it was observed that only the Delta Opal cultivar did not develop symptoms of viral disease. All *G. mustelinum* plants and the susceptible control (FM 966) showed typical symptoms of the disease (stunting, leaf rolling and yellowing of veins) (Figure 1a). The severity of symptoms observed in *G. mustelinum* was similar to that observed in plants of the susceptible *G. hirsutum* cultivar, with reduced height of plants and inhibited formation of the reproductive structures.

All populations of *G. mustelinum* developed symptoms of angular leaf spot at the inoculation points. Lesions that initially had a soaked appearance later became brown (Figure 1b). Symptoms were identical to those observed in susceptible controls and different from resistant plants, in which dry and well-defined lesions that were typical of hypersensitivity were formed. Although not artificially inoculated, due to the high pressure of whitefly (*Bemisia tabaci*), it was possible to quantify the incidence of plants with common mosaic symptoms, a virosis transmitted by this insect and caused by the *Abutilon mosaic* geminivirus (Figure 1c). All populations of *G. mustelinum* were susceptible to common mosaic of cotton. Because the inoculation occurred in an uncontrolled manner, plants that had no contact with the virulent insects did not develop symptoms of the virosis. Common mosaic occurrence ranged between 10 and 25% of plants in all treatments. The same incidence levels were also observed in commercial crops of susceptible cultivars, and the asymptomatic plants represent escaped plants.

All populations of *G. mustelinum* developed initial symptoms of ramulose (rounded and necrotic leaf spots), especially in younger leaves (Figure 1d). However, disease progression was not as dramatic as in *G. hirsutum.* After death of the apical meristems, which led to loss of dominance and favored the formation of lateral shoots, there was no development of disease symptoms in secondary branches which had normal vegetative growth. The moderate resistance control, *G. barbadense*, and all treated *G. mustelinum* plants had scores ranging from 2 to 4. On average, the score index attributed to the disease for *G. mustelinum* populations ranged from 2.6 to 3.0. Significant differences in severity were observed only in the comparison of *G. mustelinum* populations with the susceptible control (BRS Cedro), with statistical significance of P=0.03 (Jacaré), P=0.04 (Serra Azul), P=0.02 (Riachão I), P=0.01 (Riachão II) and P=0.05 (Riachão III). There was no significant difference between the populations of *G. mustelinum* and the moderately resistant control (Table 1).

Primers for five SSR loci linked to disease resistance genes in *G. hirsutum* amplified SSR fragments in *G. mustelinum* (Table 2). The allelic composition of each locus analyzed was determined in populations of *G. mustelinum* and the control cultivars. The primer DC20027 amplified two loci, a monomorphic (DC20027-1) 182 bp fragment present in all accessions and cultivars, as described by Fang et al. (2010). The locus linked to the resistance gene (*Rghv1*) for cotton blue disease was polymorphic, in which four SSR alleles were detected: 202, 200, 196 and 198 bp. The 200 bp fragment was present in only one individual of the Jacaré population and was the same allele found in *G. barbadense* accessions and in disease-susceptible cultivars. Two alleles present in *G. mustelinum* have not yet been observed in *G. hirsutum*, with sizes of 196 and 198 bp. The 196 bp allele appeared more frequently in all populations, being constant in the Jacaré population and with the lowest frequency in the Riachão Creek population.

Table 2. Composition and allele frequency of SSR loci linked to disease resistance genes in populations of *G. mustelinum* (Jacaré Creek, Quixaba Creek, Serra Azul Creek, Riachão Creek I, II, III) collected in the basin of the Contas River, Bahia and of controls (*G. hirsutum*)

Primer	Allele	Population of *G. mustelinum*						*G. hirsutum*		
		Jac	Qx	S. A.	RRI	RRII	RRIII	Delta	FM 966	M315
DC20027	182	1.000	1.000	1.000	1.000	1.000	1.000	-	-	-
	196	0.880	1.000	0.984	0.588	0.588	0.588	-	-	-
	198	0.080	-	0.016	0.412	0.412	0.412	-	-	-
	200	0.040	-	-	-	-	-	-	1.000	1.000
	202	-	-	-	-	-	-	1.000	-	-
CIR246	146	-	-	-	-	-	-	1.000	1.000	1.000
	154	0.020	0.976	1.000	0.440	0.440	0.440	-	-	-
	156	0.980	0.024	-	0.560	0.560	0.560	-	-	-
CIR316M	193	0.021	-	-	-	-	-	-	-	-
	198	-	-	-	-	-	-	1.000	1.000	-
	201	-	-	-	-	-	-	1.000	1.000	1.000
	206	0.979	1.000	1.000	1.000	1.000	1.000	-	-	-
	210	-	-	-	-	-	-	-	-	1.000
BNL3661	183	0.091	1.000	1.000	1.000	1.000	1.000	-	-	-
	185	-	-	-	-	-	-	-	-	1.000
	189	0.909	-	-	-	-	-	-	-	1.000
	195	1.000	-	-	0.709	0.709	0.709	1.000	1.000	-
	199	-	1.000	0.170	0.013	0.013	0.013	-	-	-
	201	-	-	-	0.278	0.278	0.278	-	-	-
	203	-	-	0.542	-	-	-	-	-	-
	205	-	-	0.288	-	-	-	-	-	-

A 156 bp allele amplified with the CIR246 primer was common in *G. hirsutum* and in *G. mustelinum*. This allele is associated with susceptibility to race 18 of *X. citri* subsp. *malvacearum*. In addition to this allele, another allele of 154 bp was also observed in only *G. mustelinum*. The resistance-associated allele of 146 bp in *G. hirsutum* was not observed in any of the wild individuals.

Primers CIR316M and BNL 3661, used for marker-assisted selection in *G. hirsutum*, amplified two loci each. All of these loci were polymorphic in *G. mustelinum*, except for the CIR316M_201 bp locus present in all accessions used in the present study. The CIR316M_206 bp allele belongs to the same locus described by Wang, Ulloa, and Roberts (2006); however, it differs from the CIR316M_210 bp allele, which is linked to QTL that confer increased tolerance to the root-knot nematode. This allele was observed only in *G. mustelinum* in homozygous condition in all individuals of all populations except for an individual of the Jacaré population, which showed a 193 bp allele. This allele was also amplified in accessions of *G. barbadense*.

The highest number of alleles was observed for the primer BNL3661. The QTL-associated with root-knot nematode tolerance in wild species showed two SSR alleles of 183 and 189 bp that were not observed in *G. hirsutum*. The first was observed in all subjects of the Quixaba, Serra Azul and Riachão Creek populations, and the second was observed at high frequency in the Jacaré population (Table 2). This primer also amplified a second locus with four SSR alleles, of which only the BNL3661_195 allele was present in cultivars of *G. hirsutum* classified as susceptible. Therefore, based on the absence in *G. mustelinum* of SSR alleles of the CIR316M and BNL3661 loci and their presence in resistant cultivars of *G. hirsutum* (201/210 bp and 185/189 bp, respectively), the root-knot nematode resistance conferred by these alleles was not expected to be present in the wild populations of this study.

4. Discussion

In environments with no or few signs of degradation, *G. mustelinum* has good multiplication and dissemination capacity (Barroso et al., 2010). The species mainly inhabits areas with longer water availability within the semiarid region in which it occurs, such as the banks of rivers and ponds. However, anthropogenic factors cause damage to the population, particularly the clearing of riparian vegetation which is usually associated with use as a pasture for cattle and goats (Alves et al., 2013). In addition, the commercial cultivation of cotton in close proximity to natural populations can introduce new problems. A likely problem would be related to phytosanitary issues, such as diseases. As reviewed by Gilbert et al. (2002), the introduction of new pathogens or increased inoculum pressure from those that already exist, arising from multiplication in commercial areas, can compromise the local survival of native plant species.

There was no detectable genetic resistance in *G. mustelinum* for the major diseases that usually affect cotton cultivars in Brazil. The plants of this species showed symptoms similar to those observed in susceptible cultivars for all diseases tested. Therefore, it is likely that the damage caused will also be similar. With angular leaf spot, the disease causes reduced plant vigor and productivity, with loss rates ranging from 20-70% in susceptible cultivars (Hillocks, 2010). Moreover, the bacteria are able to lodge inside the seed and cause death of the seedlings. Plants from infected seeds that do not suffer tipping serve as a source of bacterial inoculum to healthy plants. Therefore, the ability to produce offspring is reduced.

Plants of the tested populations of *G. mustelinum*, when inoculated with CLRDV, developed cotton blue disease symptoms similar to susceptible plants of *G. hirsutum*. Infected plants had reduced development, and when the infection occurred in the early stages of the vegetative phase, it did not produce flowers. When infected after the onset of flowering, the reproductive structures were smaller than those of healthy plants. This reduction in seed production under controlled conditions is indicative of reduced adaptability under field conditions because the multiplication of individuals would be affected dramatically once the disease is introduced in natural populations of *G. mustelinum*.

The average ramulose severity in *G. mustelinum* was lower than the average of the susceptible control. This reduced severity might be due to the less effective genes present in this species. However, although noticeable, the level of resistance is very low and the plants still develop symptoms of the disease despite later vegetative recovery. Similar to angular leaf spot, ramulose reduces plant productivity, and the pathogen houses in the seed, causing seedling damping-off and serving as a source of inoculum.

There may be damage to natural populations in the event of introduction or increased incidence of diseases that are common in cotton crops in Brazil. The damage will be proportional to the amount of disease in the populations. If there is increased frequency of a pathogen, natural plant populations may develop the disease and have reduced reproductive capacity and competitive performance with other species present in the habitat. Therefore, diseases pose a real threat to *in situ* maintenance of existing variability in *G. mustelinum*.

One of the most likely ways of introducing diseases within populations is the cultivation of cotton in regions where wild species occur. By 2011, cotton crops in the two microregions where the populations under study were present (Vitória da Conquista and Jequié) occupied only 255 ha (Brazilian Institute of Geography and Statistics [IBGE], 2011). This low value, when associated with the lack of cotton crops in nearby populations, suggested that it is unlikely to have negative interferences with materials cultivated in natural populations. In fact, except for common mosaic found in only one plant, none of the other diseases were observed in the field during collection expeditions or on subsequent visits, indicating that there was little or no *in situ* inoculum pressure. Also during the field visits, the presence of the vector for cotton blue disease virus (*Aphis gossypii*) was observed; however, the *G. mustelinum* plants showed no disease symptoms. It is possible that the commercial cultivation of cotton contributes to increase the population of viral vector insects, such as whiteflies and aphids, which may introduce or increase virus transmission capacity, as observed in other plant species (Alexander, 2010; Malmstrom et al., 2005).

The introduction of commercial cultivation of cotton plants in places where there is high native variability must be carefully considered. Due to the low diversity and high inbreeding within populations of the species (Barroso et al., 2010; Alves et al., 2013), the introduction of new pathogens for which there is no genetic resistance would jeopardize the survival of wild genotypes and increase the genetic bottleneck effect found in the species. From the point of view of conservation, the importance of wild cotton should not be neglected, hitherto little known as a genetic resource. Furthermore, it is possible that resistance gene are present at low frequency in the populations and that they have not been verified due to the sampling process.

The SSR loci linked to disease resistance genes in commercial cultivars amplified an allelic composition distinct in wild relatives, making them useful tools for rapid identification of introgression of resistance alleles in natural

populations of *G. mustelinum*.

Preventive strategies for the conservation of natural populations are increasingly necessary because small populations present in the basin of the Contas River do not have sources of resistance to any of the pathogens studied. The response to susceptibility may lead to a decrease in seed production. Thus, the path of the persistence of natural populations of *G. mustelinum* is uncertain, considering the low genetic diversity within the populations. Therefore, collection measures for *ex situ* conservation, periodic monitoring and continuous review of exclusion zones in view of the discovery of new wild populations are necessary to reduce the threat of loss of diversity as well as the uncertainty of its continuity.

5. Conclusion

- *G. mustelinum* populations collected in the basin of the Contas River were susceptible to cotton blue disease, angular leaf spot, common mosaic and ramulose.
- Primers for SSR loci linked to disease resistance genes in commercial cotton cultivars amplified distinct allelic compositions in wild relatives, making them as useful tools for rapid identification of introgression of resistance alleles in natural populations of *G. mustelinum*.
- There is a phytosanitary risk arising from the introduction of cotton crops (*G. hirsutum*) near natural populations of *G. mustelinum*, which may result in the reduction of these populations due to a likely increase in the incidence and severity of the diseases studied.

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References

Albrana. (2013). *Brazilian Native and Naturalized Cotton*. Retrieved June 13, 2013, from http://www.cnpa.embrapa.br/albrana

Alexandre, H. M. (2010). Disease in natural plant populations, communities, and ecosystems: insights into ecological and evolutionary processes. *Plant Disease, 5*, 492-502. http://dx.doi.org/10.1094/PDIS-94-5-0492

Alves, M. F., Barroso, P. A. V., Ciampi, A. Y., Hoffmann, L. V., Azevedo, V. C. R., & Cavalcante, U. (2013). Diversity and genetic structure among subpopulations of Gossypium mustelinum (Malvaceae). *Genetic Molecular Research, 12*, 597-609. http://dx.doi.org/10.4238/2013.February.27.9

Anderson, P. K., Cunningham, A. A., Patel, N. G., Morales, F. J., Epstein, P. R., & Daszak, P. (2004). Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends in Ecology and Evolution, 10*, 535-544. http://dx.doi.org/10.1016/j.tree.2004.07.021

Barroso, P. A. V., Hoffmann, L. V., Batista, C. E., Freitas, R. B., Alves, M. F., Silva, U. C., & Andrade, F. P. (2010). In situ conservation and genetic diversity of three populations of Gossypium mustelinum Miers (ex Watt). *Genetics Resource and Crop Evolution, 57*, 343-349. http://dx.doi.org/10.1007/s10722-009-9472-9

Brondani, R. P. V., Zucchi, M. I., Brondani, C., Rangel, P. H. N., Borba, T. C. O., Rangel, P. N., … Vencovsky, R. (2005). Genetic structure of wild rice Oryza glumaepatula populations in three Brazilian biomes using microsatellite markers. *Genetica, 125*, 115-123. http://dx.doi.org/10.1007/s10709-005-4916-4

Burle, M. L., Fonseca, R. F., Kami, J. A., & Gepts, P. (2010). Microsatellite and genetic structure among common bean (Phaseolus vulgaris L.) landraces in Brazil, a secondary center of diversity. *Theoretical and Applied Genetics, 121*, 801-813. http://dx.doi.org/10.1007/s00122-010-1350-5

Carvalho, V. P., Ruas, P. M., Ruas, C. F., Ferreira, J. M., & Moreira, R. M. P. (2002). Assessment of genetic diversity in maize (Zea mays L.) landraces using inter simple sequence repeat (ISSR) markers. *Crop Breeding and Applied Biotechnology, 4*, 557-568. Retrieved from http://www.sbmp.org.br/cbab/siscbab/uploads/c8128f42-566c-e4be.pdf

Cervi, A. C. (2006). *O gênero Passiflora L. (Passifloraceae) no Brasil, espécies descritas após o ano de 1950*. Adumbrationes ad Summae Editionem, 16, 1-5. Retrieved June 9, 2013, from http://bibdigital.rjb.csic.es/PDF/Adumb_16.pdf

Darmency, H. (1994). The impact of hybrids between genetically modified crop plants and their related species: introgression and weediness. *Molecular Ecology, 3*, 37-40. http://dx.doi.org/10.1111/j.1365-294X.1994.tb00040.x

Ellstrand, N. C., Prentice, H. C., & Hancock, J. F. (1999). Gene flow and introgression from domesticated plants into their wild relatives. *Annual Review of Ecology and Systematics, 30*, 539-63. http://dx.doi.org/10.1146/annurev.ecolsys.30.1.539

Fang, D. D., Xiao, J., Canci, P. C., & Cantrell, R. G. (2010). A new SNP haplotype associated with blue disease resistance gene in cotton (Gossypium hirsutum L.). *Theoretical and Applied Genetics, 120*, 943-953. http://dx.doi.org/10.1007/s00122-009-1223-y

Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., & McCraw, S. L. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature, 484*, 186-194. http://dx.doi.org/10.1038/nature10947

Fukuda, W. M. G., Cavalcanti, J., Fukuda, C., & Costa, I. R. S. (2005). Variabilidade genética e melhoramento da mandioca (Manihot esculenta Crantz). *Recursos Genéticos e Melhoramento de Plantas para o Nordeste Brasileiro*. Retrieved from http://www.cpatsa.embrapa.br/catalogo/livrorg/mandioca.pdf

Gilbert, G. S. (2002). Evolutionary ecology of plant diseases in natural ecosystems. *Annual Review of Phytopathology, 40*, 13-43. http://dx.doi.org/10.1146/annurev.phyto.40.021202.110417

Gilbert, G. S., & Webb, C. O. (2007). Phylogenetic signal in plant pathogen-host range. *Proceedings of the National Academy of Sciences, 104*, 4079-4983. http://dx.doi.org/10.1073/pnas.0607968104

Groot, M. H. M., Wiel, C. C. M., Tienderen, P. H., & Nijs, H. C. M. (2003). *Hybridization and introgression between crops and wild relatives*. University of Amstersdam & Plant Research International. 55p. Retrieved from http://www.postbus5100.compleet-en-actueel.nl/pdf/27428k47b3.pdf

Guitiérrez, O. A., Jenkins, J. N., McCarty, J. C., Wubben, M. J., Hayesn, R. W., & Callahan, F. E. (2010). SSR markers closely associated with genes for resistance to root-knot nematode on chromosomes 11 and 14 of Upland cotton. *Theoretical and Applied Genetics, 121*, 1323-37. http://dx.doi.org/10.1007/s00122-010-1391-9

Hillocks, R. J. (2010). Cotton diseases and their control in the 21º century. In P. J. Wakelyn & M. R. Chaudhry, (Eds), *Cotton: Technology for the 21st century*. *International Cotton Advisory Committee* (pp. 155-180).

Ibge. (2011). *Brazilian Institute of Geography and Statistics* (Instituto Brasileiro de Geografia e Estatística). Retrieved October 26, 2013, from http://www.sidra.ibge.gov.br/bda/tabela/listabl.asp?c=1612&z=&o=

Malmstrom, C. M., McCullough, A. J., Johnson, H. A., Newton, L. A., & Borer, E. T. (2005). Invasive annual grasses indirectly increase virus incidence in California native perennial bunchgrasses. *Oecologia, 145*, 153-164. http://dx.doi.org/10.1007/s00442-005-0099-z

Menezes, I. P. P., Barroso, P. A. V., Hoffmann, L. V., Lucena, V. S., & Giband, M. (2010). Genetic diversity of mocó cotton (Gossypium hirsutum race marie-galante) from the northeast of Brazil: implications for conservation. *Botany, 88*, 765-773. http://dx.doi.org/ 10.1139/B10-045

Negri, V., & Tiranti, B. (2010). Effectiveness of in situ and ex situ conservation of crop diversity. What a Phaseolus vulgaris L. landrace case study can tell us? *Genetica, 138*, 985-998. http://dx.doi.org/10.1007/s10709-010-9485-5

Oliveira, M. A. C., Duarte, J. B., Morello, C. L., Suassuna, N. D., & Oliveira, A. B. (2010). Inheritance of resistance to Colletotrichum gossypii var. cephalosporioides in cotton. *Crop Breeding and Applied Biotechnology, 10*, 65-73. Retrieved from http://www.sbmp.org.br/cbab/siscbab/uploads/c8eb9793-fabe-8438.pdf

Pickersgill, B., Barrett, S. C. H., & Lima, A. D. (1975). Wild cotton in northeast Brazil. *Biotropica, 7*, 42-54. http://dx.doi.org/10.2307/2989799

Shen, X., Becelaere, G. V., Kumar, P., Davis, R. F., May, O. L., & Chee, P. (2006). QTL mapping for resistance to root-knot nematodes in the M-120 RNR Upland cotton line (Gossypium hirsutum L.) of the Auburn 623

RNR source. *Theoretical and Applied Genetics, 113*, 1539-1549. http://dx.doi.org/10.1007/s00122-006-0401-4

Suassuna, N. D., & Coutinho, W. M. (2011). Manejo das principais doenças do algodoeiro no cerrado Brasileiro. In E.C. Freire (Ed.), *Algodão no cerrado do Brasil* (2th ed., pp. 567-612). ABRAPA, Brasília.

Sun, J. C., Cao, G. L., Ma, J., Chen, Y. F., & Han, L. Z. (2012). Comparative genetic structure with single-origin pairs of rice (Oryza sativa L.) landraces from in situ and ex situ conservation programs in Yunnan of China using microsatellite markers. *Genetics Resource and Crop Evolution, 59*, 1611-1623. http://dx.doi.org/10.1007/s10722-011-9786-2

Wang, C., Ulloa, M., & Roberts, P. A. (2006). Identification and mapping of microsatellite markers linked to a root-knot nematode resistance gene (rkn1) in Acala NemX cotton (*Gossypium hirsutum* L.). *Theoretical and Applied Genetics, 112*, 770-777. http://dx.doi.org/10.1007/s00122-005-0183-0

Xiao, J., Fang, D. D., Bhatti, M., Hendrix, B., & Cantrell, R. (2010). A SNP haplotype associated with a gene resistant to Xanthomonas axonopodis pv. malvacearum in upland cotton (*Gossypium hirsutum* L.). *Molecular Breeding, 25*, 593-602. http://dx.doi.org/10.1007/s11032-009-9355-y

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Intercropping of Corn With Some Selected Legumes for Improved Forage Production: A Review

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Abstract

Low forage quality and low corn yield experienced due to continuous monoculture resulting from persistent soil depletion in the developing world have generated the need for a sustainable practice to improve quality and yield of aforementioned. This review examines the salient issues that relates to the effect of intercropping some selected legume in different cropping patterns with corn in order to improve the yield and forage quality of corn, and that of quality feed/forage production. Two legumes species namely: Bambara groundnut and Peanut were the key crops focused with the main corn crop in this review work.

Keywords: intercropping, legumes, forage, quality, production, cereals

1. Introduction

Improving forage yield and quality has been in the Centre stage in the last few years in the livestock sub sector of agriculture. Achieving this noble target is viewed in many ways among which is intercropping of cereals with legumes. Intercropping, which is the simultaneous cultivation of crops is a predominant cropping system in developing countries; it is currently accomplished in many portions of the world (Francis, 1986). It is an advanced agro technic (Thayamini & Brintha, 2010) of growing two or more crops at the same time during the same season in the same piece of land (Geiler et al., 1991). The system has been shown not only to be more efficient than sole cropping (Remison, 1978) but also to improve the overall ecology (Adelana, 1984). It is eminent to point out that to produce additional food from less expanse of land through more efficient use of natural means with minimal impact on the environment in order to meet the increasing population request (Amos et al., 2012). The main idea of intercropping is to get improved productivity per unit land area and time, and also impartial and judicious exploitation of land resources and farming inputs including labour. Most studies on intercropping focused on productive and sustainable system, ie on the legume-cereal intercropping (Fusuo & Li, 2003). Legumes in maize based cropping systems are considered to be better alternatives for securing nitrogen economy and increasing yield of maize besides bonus yield, greater productivity per unit time and space and higher net returns of intercropping system over monoculture (Thayamini & Brintha, 2010). Its effect on N input from symbiotic nitrogen fixation into the cropping system and reduction of negative impact on the environment are eminent (Jensen, 1996) Intercropping delivers a fast and good ground shield and also allows the roots to adventure soil nutrients at several depths (Steiner, 1991). The traditional cultivators seem to have unconsciously planned their cropping system with a view of maintaining the soil richness because intercropping produces a constant and workable agro ecosystem. Ijoyah and Fanen (2012) further reports that the choice of crop combination is key to successful intercropping. Incompatibility factors such as planting density, root system and nutrient competition need to be considered (Ijoyah & Jimba, 2012). Farmers practice intercropping with a wide array of crops, consisting ordinarily of a major crop and other insignificant crops, however, it is pertinent that the selection of compatible crops be given priority as this depends on their growth habit, land, light, water and fertilizer utilization (Thayamini & Brintha, 2010). Intercropping plays a vital role in subsistence food production in both advanced and emerging countries (Adeoye et al., 2005). Legumes can relocate fixed N to intercropped cereals through their joint

growing period and this N is an imperative resource for the cereals (Bhagad et al., 2006). In a general note, Shafik and Soliman (1999) put it that intercropping may lead to overall yield advantage. In intercropping systems, all the environment resources utilized to maximize crop production per unit area per unit time. Several researches have been reported on intercropping (Mandal et al., 1990; Brintha & Seran, 2009; Ijoyah, 2012). And mostly focusing on cereal-legume based (Ofori & Stern, 1987; Hugar & Palled, 2008) and proved successful.

1.1 Corn

Maize (*Zea mays* L) is an annual crop of great importance, it was domesticated from America. It is a cereal crop belonging to the Family Poaceae that is used as a source of carbohydrate to both human (in the developing countries) and animal feed worldwide due to its high feeding value (Undie et al., 2012) it is recently used in production of biofuel. It is equally well accepted for feed ingredient and can contribute up to 30% protein, 60% energy, and 90% starch in animal diet (Dado, 1999). Maize is one of the important crops occupying third position next to wheat and rice in cereal production in the world. Maize has been recognized as a common component in most intercropping system. It seems to lead as the cereal constituent of intercrop and is regularly combined with dissimilar legumes (Maluleke et al., 2005). Maize yield is generally higher in high solar intensities, lower night temperatures and lower incidence of pest and diseases (Adesoji et al., 2013).

1.2 Bambara Nut

Bambara Groundnut (*Vigna subterranean* L verdc) is a crop belonging to the legume specie of the family fabaceae (Bamshaiye et al., 2012), and is regarded as the third most important crop after groundnuts and cowpeas in Africa (Alhassan et al., 2012). Bambara nut is an herbaceous, intermediate (0.30 – 0.35 m in height), annual plant with creeping stems at ground level. The entire plant is similar to peanut, flat with compound leaves of three leaflets. The leaves with erect petiole are alternate and trifoliate. The peduncles are auxiliary, elongating from the stem nodes, each peduncle bearing one to three flowers. The flowers which are pale yellow are borne on the freely branching stem, which after fertilization grows down towards the soil, taking the developing seed in it (Jane et al., 2012). It forms pods and seeds on or just below the ground. It is seen as a snack or food supplement but not a lucrative cash crop (Bamshaiye et al., 2012). It is used for both human and animal consumption. (Bamshaiye et al., 2012). It is conventionally classified as a bean, but its seeds are actually dug from the ground like peanut. The crop is indigenous to Africa (Jane et al., 2012). Although occasionally grown in Asia, South America, Oceania and elsewhere, its cultivation is rare outside the African continent (Hillocks et al., 2012). The distribution of wild bambara groundnut is known to extend from Jos Plateau and Yola in Nigeria, to Garoua in Cameroon (Goli, 1997). The crop can produce high yield levels with estimated world annual production at 330,000 tonnes. It has several natural agronomic advantages including high nutritional value, drought resistance (Bamshaiye et al., 2012). It is resilient to high temperature and is fit for marginal soils where other leguminous crops cannot be developed (Alhassan et al., 2012), and does well on a deeply ploughed field with a fine seedbed or ridges (on water logged soils), allowing the plant to bury its developing fruit (Alhassan et al., 2012). It grows best in average annual rainfall of between 900 – 1000 mm. it is also cultivated at altitude's ranging from 0-1550 meters (Wamba et al., 2012). The plant has latent to improve malnutrition and increase food accessibility. The seed which vary in shapes , sizes, and colour with some being round ,or elliptical in shape and a cream, brown red mottled or black colours and weight ranging between 280 and 320 g. the seed makes a complete food, as it contains sufficient amount of protein (19%), carbohydrate (63%), and fat (6.5%) (Bamshaiye et al., 2012). It is richer than groundnut in essential amino acid such as isoleucine, leucine, methionine, phenylalanine, threonine, and valine (Alhassan et al., 2012). The harvest of this crop is achieved when the plant attain maturity by the entire foliage turning yellow and dries up. The duration of the crop life cycle is between 100-180 days Bambara groundnuts have for long been used as animal feed (Jane et al., 2012) and seeds have been successfully used to feed chicks (Alhassan et al., 2012). The leaves are suitable for animal grazing, because they are rich in nitrogen and phosphorus (Jakusko & Belel, 2009; Bamshaiye et al., 2012). Livestock, especially goats are very fond of the stem or stalk, which they are allowed to graze on at the end of the season (Bamshaiye et al., 2012). Also the leaves can be pounded with those of lantana trifolia L., then water is added to create a solution used to rinse livestock as a defensive against ticks. This solution is used as a pesticide on vegetables (Bamshaiye et al., 2012). Also the flour could also be used as composite flour used for cereal based confectionaries e.g. Biscuit\ Cakes, bread (Alhassan et al., 2012).The excerpt from the nut particularly the protein extracts can be used directly in cosmetic Inventions and offers specific and prominent effects. The nut can also be used rather freely to replace the high-prized lumps of meat without foregoing adequate nourishment (Bamshaiye et al., 2012).

1.3 Peanut

Ground nut (*Arachis hypogaea* L) is an important annual legume worldwide. Iit is the 13th worldwide most important food crop, and fourth important oilseed crop (Smith, 2002) known for its oilseed, food and animal feeds (Mangasini et al., 2012) the crop is mostly involved in crop rotation in the sub Saharan Africa. Its world production stood at 28.5 million tons/annum. (ICRISAT, 2009). About 90 % of the world's groundnut production happens in the tropical and semi-arid tropical areas (Hamidou et al., 2013), in countries including: China, India, Nigeria, Indonesia, Senegal, USA, Argentina, and South America (Mangasini et al., 2012). The crop requires about 500 mm to 1600 mm of annual rainfall on a well-drained light sandy loamy soil (Taru et al., 2008). Abundant of the world's groundnut production areas are characterized by high temperature and low or unpredictable rainfall even though it was reported that Groundnut is delicate to temperature (Vara Prasad et al., 1999). Plant reactions to high temperature differ with plant type and phenological periods (Wahid et al., 2007). Reproductive developments are evidently affected by high temperatures in utmost plants, which lead to concentrated crop yield (Hamidou et al., 2013). The crop bears so many local names including Peanut, Earthnuts, Monkey-nuts, Goober. High quality easily digestible protein (25%), edible oil (50%), and carbohydrates (20%) are contained in the seed (Sorrensen et al., 2004; Musa et al., 2012)

1.4 Intercropping System

The practice of cultivating two or more crops in the same space and at the same time is common among smallholder farmers (Seran & Brintha, 2010), this common combination in intercropping systems mostly involves cereal legumes (Ijoyah, 2012), particularly Maize – soybean, maize – cowpea, maize – groundnuts, millet-groundnuts, and rice – pulses (Matusso et al., 2012). Series of research work have been reported by scientist on cereal- legume intercropping (Waddington et al., 2007; Egbe, 2010; Osman et al., 2011; Ijoyah, 2012), with intercropping successes as compared to monocrop. With this, farmers can produce to exploit location specific agro-climatic circumstances for improved production (Bhagat et al., 2006). Intercropping systems is known to make a more efficient use of growth factors as they capture and make a better use of radiant energy (Matusso et al., 2012), available water and nutrients (Sullivan, 2003). Prevent pest and diseases, suppress weeds and maintain and improve soil fertility (Sanginga & Woomer, 2009, Seran & brintha, 2010). Cropping system refers to the spatial and temporal arrangement of different crops to exploit natural resources and enhance productivity per unit area and time (Gurigbal, 2010). The spatial arrangement of crops helps in the effective utilization of land, soil moisture, nutrients and solar radiation. This is brought about by choosing appropriate crops of varying morpho-physiological nature and planning their planting geometry to reduce mutual competition for resources and enhance complementarities to increase overall productivity. In general, this is achieved by intercropping systems (Gurigbal, 2010).

1.5 Benefits of Intercropping

The low input and high risk environment of the smallholder farmer benefits enormously from intercropping (Rana & Pal, 1999). Cereal and legumes which has become a popular combination among farmers was probably due to legumes ability to combat erosion and raise soil fertility levels (Matusso et al., 2012). Flexibility, maximization of profit , minimization of risk, soil conservation and soil fertility improvement are some of the principal reasons for smallholder farmers to intercrop their farms/crops(Matusso et al., 2012). Further to that, they have the potentials to give higher yield than sole crops, greater yield stability and efficient use of nutrients (Seran & Brintha, 2010). Similarly, better weeds control, improvement of quality by variety while cereal crops require larger area to produce same yield as cereals in an intercrop system (Ijoyah, 2012).

1.6 Problems of Intercropping

Reduction in yield of component crop may occur due to intense competition (Thole, 2007). The situation in which two or more plants share the same growth factors each far below their combined demands and in the same environment is known as competition (Thole, 2007). The basic morpho-physiological changes and agronomic features such as fertilizer application, sowing time, and proportion of crop mixture are basic determinants of competition between component crops. Where constituent crops are arranged in certain rows, the degree of competition is determined by the comparative growth rates, growth duration and proximity of roots of the diverse crops. The cereal component in a cereal-legume intercrop has advanced growth rate, height advantage, and a more widespread rooting system which gives it upper hand in competition with associated legumes. Ofori and Stern (1987) reported that the yield of the legume component decline on normal by about 52% of the sole crop yield whereas the cereal yield was condensed by only 11%. Significantly, it was noted that the cereal constituent depresses the legume in an intercrop. This was attributed to abridged photosynthetic active radiation of the legume by the screening from cereal crop.

1.7 Effect of Intercropping on Growth and Yield of Maize

Maize has been recognized as a common component in most intercropping system in the tropics (Ijoyah, 2012). Fawusi and wanki (1982) reported a high leaf area index and light interception for maize in mixture over sole crops. While Prasad and brooks (2005) found an increase in maize plant density to significantly affect the LAI in maize soybean intercropping. Thus, increase in the growth of maize was reported by Adesoji et al. (2013) to be as result nitrogen effects that lead to increase cell division, cell expansion and increase in size of all its morphological parts. Also Reddy and Reddi (2007) observed separately the grain yield of maize to have increased after intercropping with groundnut and green gram. The purpose of maximum maize –legume association is to reach a full yield of the maize plus selected legume yield (Chui & Richards, 1984). But however, reported decline in yield of maize as a result of varying spacing in intercrop with cowpea. This further agrees with the report of Gangwar and Sharma, (1994) that there was decreased yield of maize due to intercropping of legumes namely cowpea, clusterbean, sunhemp and dhiancha. Also experiment conducted at the Indian Agric. Research Institute revealed a significant dry matter accumulation of maize and groundnut intercropped in the 1:1 row ratio arrangement (Aravind kumar, 2004). Similarly, Maluleke et al. (2005) found maize dry matter was reduced with increasing Lablab population. Mangasini et al. (2012) found the vegetative growth of component crop in a mixture is affected by intercropping. Thayamini and Brintha (2010) noted that the planting pattern of the maize and legume did not affect the yield of maize. Chui and Richards (1984) reports that intercropping hindered maize tasseling and silking by up to 2 days, particularly at the full population concentration of soybeans. Intercropping maize with cowpea was seen to significantly decrease ear length, cob length, dry cob weight, dry grain yield and dry total plant biomass (Egbe et al., 2010). Ali and Mohammad (2012) observed that the highest dry leaf/dry stem yield and total protein of plant was related to forage corn intercropping with Karaj and Multicut respectively. Yield increased that was noticed in a maize/soybean strip intercropping arrangement were primarily due to the upsurge in the boarder rows of maize together to soybeans (Li et al., 2001) Plant density affects both intra- and interspecific competition and has particularly a strong effect on grain yield of maize (Flores-sanchez et al., 2013). Maize - legume intercrop could substantially increase the quality and quantity of forage (Ali & Mohammad, 2012). Farmers' field was however noticed to have had the highest amount of vegetative biomass when legume crops are intercropped with maize (Amos et al., 2012).

1.8 Effect of Intercropping on Growth and Yield of Legumes

Several research works have been reported on the response of legumes to intercropping predominantly with annual cereal crops. Bhagad et al. (2006) reported that Intercropping arrangement did not influence 100 - kernel mass of groundnut, however, number of pods per hill, weight of pods apiece hill and per cent shelling were significantly subjective due to different treatments. Research work also revealed that space for higher cereals can be altered to a certain degree without reducing its yield while providing a more promising environment for the intercropped legume (Chui & Richards, 1984). Hongchun et al. (2013) reported that intercropping with maize did not disturb fresh weight of peanut associated with monocropping. The use of twin rather than single irregular rows of each species improved intercrop soybean yield without materially varying maize performance comparative to mono cropping (Maluleke et al., 2005). Intercropping significantly condensed the number of soybeans leaves per plant by 58%, leaf area index (LAI) by 75% and phytomass at start seed – filling by 78% (Maluleke et al., 2005), however, Chui and Richards (1984) maintained that grouping maize plants at three to a hill enlarged intercrop soybean leaves per plant, LAI and phytomass relative to the conservative maize planting of one plant per hill. Soybeans yield was concentrated by up to 90% in intercropping with maize in the equal row (Dalal, 1977). Legumes are viewed as serious component in conservation Agriculture (Meyer, 2010).

1.9 Effect of Intercropping on Nutrients Uptake

When peanut and maize raise together, phytosiderphore released from maize roots may mobilize Fe (III) and profit the iron nutrition of peanut plant (Fusuo & Li, 2003). Peanut/maize intercropping is known to progress Fe nutrition in all peanut tissues (Hongchun et al., 2013). Enhancement in the Fe nutrition of peanut intercropped with maize was mainly caused by rhizosphere collaboration between peanut and maize (Zuo et al., 2000). Thus, Hongchun et al. (2013) further states that in peanut/maize intercropping, the secretion of phytosiderophores from maize in the intercrop arrangement may contribute to the improvement of Fe nutrition of the peanut. Even when Geiler (2001) reported soil pH to have extensively influence nodulation and can make deficiency of some essential nutrients such as P and Mo, it was further reported that intercropping greatly augments Fe and Zn concentration in seeds of peanut (Hongchun et al., 2013). Li et al. (2001) reported that nitrogen acceptance by maize in an intercrop is greater as relate to sole cropping. The greater N acquisition by a non - legume crop intercropped with a legume is often reported in literature (Francis, 1986; Vandermeer, 1989; Stern, 1993). This may probably be due to the effect of competition. However, nitrogen attainment by soybeans was not significantly affected by intercropping.

Similarly, phosphorus achievement by soybean was significantly amplified by P application under intercropping and by intercropping below P application (Li et al., 2001). Legumes as a catch crop can reduce nitrate and K leaching (Askegaard & Eriksen, 2008), and act not only as a N2 fixing crop but also as a catch crop by taking up additional soil minerals N,P, and K . (Flores-sanchez et al., 2013). These findings make legumes an important tool in the cropping systems where N and K are the major yield limiting factors (Flores-Sanchez et al., 2011, 2012a). Rusinamhodzi et al. (2012) reports that deficiencies of micro nutrients such as Zinc, molybdenum and boron in the field may bound legume growth as well as limit nitrogen fixation. At Main Agricultural Research Station, Dharwad, Karnataka, uptake of nitrogen, phosphorus, potassium by maize was found to reduced significantly due to intercropping (263, 13 and 138 NPK kg ha^{-1}) as against sole cropping (305, 16 and 188 NPK kg ha^{-1}). Uptake of nitrogen with greengram (284 kg ha^{-1} was significantly developed than with cowpea (239 kg ha^{-1}) and soybean (247 kg ha $^{-1}$-1) as intercrops, the nitrogen uptake was maximum in 1:1 row ratio (274 kg ha^{-1}) compared to 1:2 row ratio (251 kg ha^{-1}) (Kanakeri, 1991). Further, among different intercrops, cowpea noted maximum uptake of nitrogen (68 kg ha^{-1}), phosphorus (2 kg ha^{-1}) and potassium (18 kg ha^{-1}) followed by soybean with 60, 2 and 18 kg ha^{-1} NPK, separately.

1.10 Intercrop Productivity

Intercrop productivity, otherwise called yield advantage is core in any intercrop studies. Production systems involving inter planted food crops are widespread in tropical latitudes (Thayamini & Brintha, 2010). Intercrops are greatest productive when the component crop varies greatly in growth duration so that their maximum condition for growth resources occurs at different periods (Ijoyah, 2012). The interaction of several factors will optimize the most effective use of restrictive resources in intercrop (Fukai & Trenbath, 1993). These factors range from the genetic constitution of the component crops to environmental and agronomic manipulation of the microenvironment (Fukai & Trenbath, 1993). High intercrop productivity is attained if early maturing constituent is grown with little interference from the late growing crop. Thus, the choice of accurate cultivars and agronomic manipulations to certify the most effective use of limiting resources is key part for high crop yield (Thayamini & Brintha, 2010). The biggest yield advantage and complementary effect occur when component crops have different growing periods to make their demand on resources at different times (Ijoyah, 2012). Fukai (1993) maintained that legumes are a shared component of an intercrop, and their skill to fix nitrogen often supports the productivity of the intercrop, or subsequent crops. In comparison to sole cropping yield benefit have been recorded in many intercropping system, including: maize/bean, sorghum/soybean, maize/cowpea, wheat/mungbeans, wheat/chickpea, maize/fababeans etc (Li et al., 2001). Most published intercropping mixture with significant yield advantage were from legume/non legume combination (Li et al., 2001).

1.10.1 Land Equivalent Ratio (LER)

This is the relative area of land under monocrop which is needed to obtain the yield produced in intercropping (Wiley, 1979). Rao and Willey (1980) showed a clear variation in duration of maturity of component crop was due to largely the advantage in yield, which clearly allowed in this combination for a good resource use with time. Khan et al. (1992) in an experiment involving maize and soybean recorded a high LER of 1.40 as a result of sowing them in same rows, while a low LER of 0.95 involving the same crops was noted but on alternate rows. In Brazil, Raposa et al. (1995) recorded high LER in intercrop involving 2:2 row arrangements than with monocrop. Yield advantages in maize based intercropping were also reported in Ethiopia (Fininsa, 1997) that LER for intercrop was far above that of monocrop with maximal relative yield advantage of 28%. Similarly, altered maize (75 cm), rice bean (30 cm) row proportions recorded yield advantage in terms of land use, and for moisture. LER values in 1:2 row ratio at 100 per cent + zero per cent fertilizer (maize 60/90 cm-rice bean 30 cm), in 2:3 at 100 per cent fertilizer (maize 150/15-rice bean 30 cm) and in 2:5 at 100 per cent + 100 per cent fertilizer were 1.84, 1.87 and 1.97 respectively (Lakra et al., 2000).

1.10.2 Area Time Equivalent Ratio (ATER)

The LER method was modified by Hiebsch and Macollam (1980) to include the duration of the crop present on the land from planting to harvest. This method is known as the area time equivalent ratio (ATER). In maize + cowpea/soybean intercropping system, the yield advantages ranged from 22 to 32 per cent based on LER method 19 to 25 per cent based on ATER method over sole crops and thus LER productivity estimates were greater than that of ATER (Allen & Obura, 1983). The higher ATER (1.38) was recorded in the maize (3 plants m^{-2} *Phaseolus vulgaris* in 1:2 row ratio than *Phaseolus vulgaris* and maize grown as sole crops (Gardner & Kisakye, 1990). At Pantnagar, in maize based intercropping systems, Halikatti and Banarasilal (1998) recorded higher ATER value (1.18) with one row of blackgram followed by two rows of blackgram between maize pairs compared to other cropping systems. Similarly, Pandita et al. (2000), also reported that maize and *Phaseolus*

vulgaris at 1:2 row ratio gave the maximum ATER (1.48) with highest maize equivalent yield (78.8 q ha^{-1}). At Dharwad, in maize based intercropping systems, Mohan (2003) recorded higher ATER value (1.65) with two rows of rice bean between maize spaced at 90 cm followed by two rows of soybean (1.63) and frenchbean (1.51) compared to other cropping systems.

1.11 Effects of Bambara Nut Intercrop on Growth and Yield of Maize

Bambara's ground nut potential of contributing to food security has fuelled an increasing research interest (Jane et al., 2012). Karikari (2001) reported that intercropping maize with Bambara nut did not affect the number of cobs and weight of seed in maize. Similarly it was also reported that yield did not reduce nor increase in the intercrop of sorghum and Bambara nuts (Gabatshele et al., 2012). On the contrary, Ogah and Ogbodo (2012) reported a significant increase in total grain yield of maize when intercropped with Bambara nut than in the sole crop maize. The total percentage yield loss due to stem borer infestation in maize was also reported to be significantly lower in intercrop with Bambara nut. He further maintain that the 2:2 maize/Bambara nut intercrop produced meaningfully higher number of cobs, quantity of seeds, and seed weight with less infestation than 1:1 maize/Bambara intercrop (Ogah and Ogbodo 2012). Maize plants that were grown in intercrop with Bambara nut were also found to have a significant reduction in the density of larvae, number of borers and percentage dead heart (Ogah & Ogbodo, 2012).

1.12 Effects of Groundnuts on Growth and Yield of Corn

Groundnut is very commonly intercropped with maize in Southeast Asia and Africa. It was reported from west Cameroons that groundnuts a major crop are grown with maize at a fairly low density with impressing high yield per pure stand (Reddy & Reddi, 2007). Another study, revealed a very rapid growth rate of millet in intercrop, with ground nuts achieving 8134 kg/ha of dry matter in 85 days. Sole groundnut growth rate however was slower, and achieved 4938 kg/ha of dry matter in 105 days. Also observed in another study of Groundnuts/Maize intercropping was the mean yield of groundnut was significantly higher when sown four (4) weeks earlier than maize. Generally, several reports revealed that on the maize/groundnut combination is that g/nut yield is readily depressed by competition from Maize (Thayamini & Brintha, 2010). Conversely, ICRISAT reported a poor maize growth in Maize/Groundnut intercrop that was without N-fertilizer application, and there was no visual evidence of growth being any better if the groundnuts intercrop were present. However, where nitrogen was applied to the maize, the growth was suppressed (Thayamini & Brintha, 2010), and the residual benefits rapidly diminished (Rao & Willey, 1980). Bhagad et al. (2006) further emphasized that the yield Mechanisms of maize like length of cob and regular weight of cob were meaningfully higher once groundnut + sweet corn were intercropped in 3:1 ratio and provided with 125% RDF. Also Koli (1975) reported a little productivity of maize-groundnut mixture which he say was possibly due to relatively high maize population such that the nearness of maize to groundnut did not make for considerable spatial complementary among the two crops.

1.13 Effect of Legumes Intercrop and Cropping System on Soil Fertility

Soil fertility problems are not only an agronomic issue, but also strongly related to economic and social issues. Intercropping tend to ameliorate some of the fertility constraint of poor farmlands. Adeleke and Haruna (2012) mentioned that pulses are usually intercropped with cereals and advance land productivity over soil amelioration. In a study, Vesterager et al. (2008) found maize and cowpea intercropping as beneficial on nitrogen poor soil. Maize /cowpea intercropping increases the amount of nitrogen, phosphorus, and potassium contents associated to monocrop of maize (Dahmardeh et al., 2010). Degraded and infertile soils are realized as a result of continuous monocropping and insufficient organic matter reprocessing coupled with occurrence of rainfall variability marked by common dry spells account for low crop yield (Amos et al., 2012). It was further noted that the understanding of the fact that maintenance and improvement of soil fertility cannot be exclusively through the use of predictable fertilizers (Amos et al., 2012). As a trait in legumes as cover crops, conservation involves minimum soil disturbance, permanent sol cover with living or dead plant resources, and diversified crop rotation and associated by legumes crops (Amos et al., 2012). Adeleke and Haruna (2012) also in the result of their findings revealed increase in total nitrogen after cropping any of the four legumes (soybean, cowpea, lablab and groundnut) and when the land was left fallow. This monumental increase in the total nitrogen was probably due to the ability of the legumes to fix atmospheric nitrogen in the soil through symbiotic N fixation. This symbiosis alone accounts for more than 20% of global biological nitrogen fixation and has been calculated to contribute 45-50 million tons of fixed N to agriculture each year (Geiler, 2001). Also the higher Cat ion Exchange Capacity (CEC) which plots that were previously cropped to legumes and had compared with the previous maize plot and fallow plots could be attributed to the leaf litter droppings which more or less serve as mulch and later decomposed to add nutrients to the soil (Adeleke & Haruna, 2012).

1.14 Resource Use

Intercropping systems can allow for spatial and temporal increase in nutrients uptake (Flores-sanchez et al., 2013). Spatial nutrients uptake can be increased through the increasing root mass (Undie et al., 2012), while temporal advantage in nutrients uptake occur when crops in an intercropping system have their peak nutrients demands at different times (Anders et al., 1996). Similarly, plants species with differing root and uptake patterns, like the case of legumes/cereals in intercrop, more efficient use of available nutrients may occur (Matusso et al., 2012), and higher uptake of nitrogen in the intercrop have been reported (Seran & Brintha, 2010; Undie et al., 2012; Flores-sanchez et al., 2013) whereas in intercrop their similar root orientation tends to compete together at the same surface level (Hamidou et al., 2013). Intercropping amid high and low canopy crops is a mutual practice in tropical agriculture. Total system light Interception is resolute by crop geometry and foliage architecture (Trenbath, 1986). In intercropping between high and low cover crops is to improve light interception and hence yields of the smaller crops requires that they be planted among sufficiently wider rows of the taller ones (Seran & Brintha, 2010). A favorable microclimate is created by intercropping for the lower plants growth (Azam-Ali et al., 1990). Keating and Carberry (1993) have reported a better use of solar radiation by intercropping soybeans and Maize. Further to that, intercropping enhanced the efficient use of strong light by maize and weak light by groundnuts which subsequently lead to yield advantage (Jiao et al., 2008). A combined leaf canopy might make better special use of light (Waddington and Edward, 1989). Growth of plants in any cropping system is vital and is determined by the availability of water and it efficient use lead to increase use of other resources (Dahmardeh et al., 2010). Water capture by intercrops is 7% higher than as compared to mono crop (Morris & Garrity, 1993). Chui and Richards (1984) further maintained that during competition light obviously increase internode elongation on soybeans. Further to that, a delay in sowing of four weeks was long enough to avoid interspecific competition for light and nutrients and allow a good establishment of both maize and roselle (Flores-sanchez et al., 2013). Despite the beneficial effects of the intercropping to the cereal crops, it may also quicken soil nutrient depletion, particularly for phosphorous, due to added efficient use of soil nutrients and higher exclusion through the harvested crops (Mucheru-Muna et al., 2010). However, Chalka and Nepalia (2006) found that maize intercropped with soybean produced significantly lower NPK depletion and higher N uptake. And, recent efforts on replenishment of soil fertility in Africa have been through the introduction of legumes as intercrop and/or in rotation to minimize external inputs (Sanginga & Woomer, 2009).

1.15 Above and Below Ground Interaction in Intercrop

Light is a vital factor that determines yield (Jeyakumaran & Seran, 2007) especially when two morphologically dissimilar crops with different periods of maturity are intercropped (Ijoyah, 2012). Most of the advantages gotten from growing crops in intercrops come largely from the ways in which the crop mixtures balance each other in their exploitation of the environment (Oyewole, 2010). Indeed corn canopy architecture plays a significant role in the amount of sunlight radiation intercepted by other crops sown in an intercropping pattern (Metwally et al., 2012). The reduction of light intensity caused by the corn plant reduces the photosynthetic capacity of a second crop in an intercrop pattern (Metwally et al., 2012). Crop biomass buildup depends on light interception by leaves and on the effectiveness, with which the intercepted light is used to produce dry matter (Oyewole, 2010). Yield is determined principally by crop biomass, which in turn is determined by the quantity of radiation intercepted by the crop canopy (Oyewole, 2010). Any influence on the plant canopy either as a result of plant shading, which may result from intercropping, or other resources will affect yield. Crops - weeds competition is well - known by growth habit of crops (Dimitrios et al., 2010). Increased leaf cover in intercropping system helps to reduce weeds population once the crops are established (Beets, 1990). Flores-Sanchez et al. (2013) reported the contribution of above ground and below ground interaction of maize/wheat to be 50 and 59% respectively due to increase in nitrogen uptake. In a report by Hongchun et al. (2013), that through inter-specific root connections, peanut/maize intercropping contribute to the peanut nourishment of some nutrients elements including improvement in shoot zinc (Zn), Phosphorus (P), and Potassium (K) concentration. The nitrogen (N) productivity in both peanut and maize are improved. Mixed grown cereal and legumes have many advantages in terms of growth and some other agronomical properties (Singh et al., 1986; Putnam et al., 1986). There are also significant handicaps of mixed grown component crops such as root competition for water and nutrients and competition for light (Ofori & Stern, 1987; Portes, 1984). Innis (1997) explained that water loss in the soil is reduced by various root systems, these increases transpiration and tend to produce a microclimate cooler than the surrounding. Flores-sanchez et al. (2013) further reported that the aboveground biomass of maize was not affected by legume intercrop neither in the maize monoculture nor in the maize-roselle mixture. It is clear that intercropping patterns caused a significant reduction in light interception through adjacent corn plants and produced taller component crop (Metwally et al., 2012). Legume residues generally create a mulching layer that increases the physical barrier for

early germination; such effects do require sufficient residual organic material on the soil surface (Flores-Sanchez et al., 2013). In the soil, facilitative root interaction are most likely to be of great importance in nutrient-poor soil and low input agro ecosystem due to the crisis in inter specific competition or facilitation for plants growth factors (Dahmardeh, 2013). Maize benefit from intercropping with peanut due to extensive root system of maize for absorption of water and nutrients , and possibly that peanut via N fixation could secret H^+ in soil (Flores-Sanchez et al., 2013); this acidification of the rhizosphere could improve the dissolution of phosphorus in the high pH soil (Dahmardeh, 2013). Previous works reported that multiplicative processes in groundnut are sensitive to temperature. Increasing air and soil temperatures condensed fruit-set, number of pods and yield in groundnut (Hamidou et al., 2013). In addition, Oyewole (2010) showed that pod yield of groundnut genotypes declined by more than 50% when flowering and pod formation happened when maximum temperatures averaged 40 °C. Nitrogen and phosphorus connections at the root zone of Bambara groundnut in the soil was reported to be the most probable reason for increases experiential in its growth and yield characters (Nweke & Emeh, 2013).

1.16 Forage Yield and Quality

Maize – legume intercrop could considerably increase forage quantity and quality and lessening condition for protein supplement (Ali & Mohammad, 2012). An important measure in grass land resource is the yield of forage; this defines the volume of dry matter obtainable to livestock (Shi et al., 2013). Thus, Legume – cereal configuration is considered as a management approach in producing both quality and quantity forage (Hamdollah, 2012). Intercropping cereals and legumes is important due to some potential benefits including the enhancement of forage quality through the complimentary outcome of two or more crops grown instantaneously on the same part of land (Hamdollah, 2012). As forage quality increases crude fiber also increase, while crude protein and ether extract of grasses declines, hence a negative association exist between forage quality and above ground biomass (Shi et al., 2013). Legume cultivars were reported to give a significant effect on plants heights, leaf fresh yield, stem fresh yield, total fresh yield, leaf dry yield, and entire proteins in plants (Ali & Mohammad, 2012). It was also found that intercropping of maize with pulses meaningfully affected dry matter yield (tha^{-1}). Crude protein (CP) yield also was pointedly affected by intercropping systems as the highest CP yield was for the intercropping maize with Mungbeans (Hamdollah, 2012). Different ecological and social factors effects maize forage yield and quality (Emine et al., 2010). Shi et al. (2013) also found that environmental features such as temperature and soil fertility to disturb physiological developments, and so impact forage quality. High forage harvest means more crude fiber but fewer ether extract and crude proteins. It is the measure of dry matter made available to be consumed by Livestock (Shi et al., 2013). Many scholars reported that plant mass affects completely forage yield and maximum of its quality mechanisms (Jiwang et al., 2004; Emine et al., 2010). Corn is a high yield, high-energy forage crop (Jorge & Joseph, 1999). It was maintained that the characteristic of good fodder corn is high forage yield, high digestibility (Emine et al., 2010). The lost in the food value of the forage (Leaves and stalk) in corn is rewarded by the grain making high quality forage need remained informed as an important facet of forage crop production (Hamdollah, 2012). Corn forage has low attentiveness of protein as related to legume forage (Jorge & Joseph, 1999). Although cereals are extensive used in livestock nutrition, for their high dry material production and low price (Ghanbari-Bonjar, 2000), they have low sustenance value due to their low forage worth (Hamdollah, 2012). The total vitality value of corn forage does not vary much, it offers about 72% total digestible nutrients (TDN) whether it is browsed while green and emergent, or at prime of life (Troy, 2011). Forage quality of legume is high but has low dry matter making (Ross et al., 2005). The NDF and ADF contents are important in rations formulation because they reflect the amount of forage that can be used up by animals (Lithourgidis et al., 2006).

Protein content in forage may likely to decrease as dry matter yield increased (Foster & Malhi, 2013), the protein content in forage tended to be superior for the cereals with the early planting date and lowest existences to harvest. Acid Detergent Fiber (ADF) content of forage retorted inversely to protein content of forage, but has a tendency to increase as dry matter yield increased with increased days to harvest (Foster & Malhi, 2013). Widdicombe and Thelen (2002) reported that crude protein contents of forage maize were negatively related with plant density. Also, it was described that maize ear fraction and plant density had an undesirable relationship (Emine et al., 2010). Yilmaz et al. (2007) also reported that ear percentage decreased with cumulative plant density. Further to that, crude protein satisfied of forage corn decreased with increased plants density (Emine et al., 2010), while on the contrary, Jiwang et al. (2004) maintained that Acid detergent fiber (ADF) and CP contents increased with increased plants bulk. Hamdollah (2012) further reports that the CP yield and dry matter of formed forages increased by intercropping as associated with the maize monoculture. Also a significant reduction in NDF and ADF content was noticed during the legume /maize intercrop. This resulted in increased feed digestibility (Hamdollah, 2012).

2. Conclusion

The relevance of improved forage production for our growing livestock industry is a key factor in the modern day crop production system. Intercropping which demonstrated a high technical potentials of crop – to – crop interaction for the better and higher quality food production, is in the lead in improving and ensuring the quality and quantity of food, for man, feed for animals, quality raw materials for the growing industries as well as a good environmental sustainability (such as improved fertility of soils, reduced prevalence of crop diseases in the field). In this review, critical areas of intercropping as they affect production especially in some legumes intercropped with maize were highlighted, and the advantages or otherwise were discussed. It is therefore imperative to adventure further into more studies to discover or rediscover the treasures of intercropping of different crops for the betterment of our life.

References

Adelana, B. O. (1984). Evaluation of maize-tomato mixed-cropping in south-western Nigeria. *Indian J. Agric. Sci., 24*(7), 564-569.

Adeleke, M. A., & Haruna, I. M. (2012). *Residual nitrogen contribution from grain legume to the growth and development of succeeding Maize crop*.

Adeoye, G. O., Sridhar, M. K. C., Adeoluwa, O. O., & Akinsoji, N. A. (2005). Evaluation of naturally decomposed solid waste from municipal dump sites for their manorial value in southwest Nigeria. *Journal of Sustainable Agriculture, 26*(4) 143-152. http://dx.doi.org/10.1300/J064v26n04_09

Adesoji, A. G., Abubakar, I. U., Tanimu, B., & Labe, D. A. (2013). Influence of Incorporated short duration legume fallow and nitrogen on maize (*Zea mays L*.) growth and development in northern guinea savannah of Nigeria.*American-EuroasianJ. Agric. And Env. Sci., 13*(1), 58-67.

Alhassan, G. A., Kalu, B. A., & Egbe, O. M. (2012). Influence of planting densities on the performance of intercropped bambara groundnut with cowpea in Makurdi, Benue state, Nigeria. *International Journal of Development and Sustainability, 1*(3).

Ali, S., & Mohammad, H. S. (2012). Forage yield and quality in intercropping of forage corn with different cultivars of berseem clover in different levels of nitrogen fertilizer. *Journal of Food, Agriculture and Environment, 10*(1), 602-604.

Allen, J. R., & Obura, R. K. (1983). Yield of corn, cowpea and soybean under different intercropping systems. *Agronomy Journal, 75*, 1005-1009. http://dx.doi.org/10.2134/agronj1983.00021962007500060032x

Amos, R. N., Jens, B. A., & Symon, M. (2012). On farm evaluation of yield and economic benefits of short term maize legume intercropping systems under conservation Agriculture in Malawi. *Field crop research, 132*, 149-157.

Anders, M. M., Potdar, M. V., & Francis, C. A. (1996). The significance of Intercropping in cropping systems. In O. Ito, C. Johansen, J. J. Adu-Gyamfi, K. Katayama, J. V. D. Kumar, K. Rao, & T. J. Rego (Eds.), *Dynamics of roots and nitrogen in cropping systems of the semi-arid tropics*. Japan International Research Center for Agricultural Sciences. *International Agricultural Series No. 3 Ohwashi, Tsukuba, Ibavaki 305, Japan.* http://dx.doi.org/10.1016/j.agee.2007.05.008

Arvindkumar, Singh, S. N., & Gajendra, G. (2004). Influence of planting ratio and Fertilizer application on dry matter production LAI and nutrient content and uptake by maize and groundnut in intercropping. *Annals of Agricultural Research, 25*(2), 283-288.

Askegaard, M., & Eriksen, J. (2008). Residual effect and leaching of N and K in cropping systems with clover and ryegrass catch crops on a coarse sand. *Agriculture, Ecosystems and Environment, 123*(1-3), 99-108.

Bamshaiye, O. M., Adegbola, J. A., & Bamshaiye, E. I. (2012). Bambara groundnut: An under- utilized nut in Africa. *Advances in Agricultural Biotechnology, 1*(2011), 60-72.

Beets, W. C. (1990). Raising and sustaining productivity of small holder systems in the tropics: *A handbook of sustainable Agricultural development.* Alkamaar, Netherlands: Agbe Publishing.

Bhagad, S. B., Chavan, S. A., Zagade, M. V., & Dahiphale, A. V. (2006). Intercropping groundnut and sweet corn at different fertility levels and row proportions. *Indian Journal of Crop Science, 1*(1-2), 151-153.

Brintha, I., & Seran, T. H. (2009). Effect of paired row planting of raddish (*Raphanus SativusL*.) intercropped with vegetable amaranths (*Amaranths tricolor* L.) on yield components in sandy regosol. *J. Agric. Sci., 4*, 19-28.

Chalka, M. K., & Nepalia, V. (2006). Nutrient uptake appraisal of maize intercropped with legumes and associated weeds under the influence of weed control. *Indian Journal of Agricultural Research, 40*, 86-91.

Chui, J. A. N., & Richard, S. (1984). Influence of spatial arrangement of maize on performance of an associated soybeans intercrop. *Field crop Res.,* 8, 187-198. http://dx.doi.org/10.1016/0378-4290(84)90062-5

Dado, R. G. (1999). Nutritional benefit of specialty Maize grain Hybrid in Dairy diets. *Journal of Animal Science,* 197-207.

Dahmardeh, M. (2013). Intercropping two varieties of Maize (*Zea mays* L) and Peanut (*Arachishypogaea* L): Biomass yield and intercropping advantage. *Int. J. Agric. And Forestry, 3*(1), 7-11.

Dahmardeh, M., Ghanbari, A., Syahsar, B. A., & Ramrodi, M. (2010). The role of intercropping maize (*Zea mays* L) and cowpea (*Vignaunguiculata* L) on yield and soil chemical properties. *Afr. J. Agric. Res., 5*, 631-636.

Dalal, R. C. (1977). Effects of intercropping of maize with soybeans on grain yields. *Tropical Agric. (Trinidad), 54*, 189-191.

Dimitrios, B., Panyiota, P., Aristidis, K., & Aspasia, E. (2010). Weed suppression effects of maize- vegetable in organic farming. *Int. J. Pest Mang, 56*, 173-181. http://dx.doi.org/10.1080/09670870903304471

Egbe, O. M., Alibo, S. E., & Nwueze, I. (2010). Evaluation of some extra-early-and early- maturing cowpea varieties for intercropping with maize in southern guinea savanahof Nigeria. *Agriculture and Biology Journal of North America.* http://dx.doi.org/10.5251/abjna.2010.1.5.845.858

Emine, B. C., Celik, N., & Bayram, G. (2010). Yield and quality of forage Maize as influence by plant density and nitrogen rates . *Turkish Journal of Field Crops, 15*(2), 128-132.

Fawusi, M. O. A., & Wanki, S. B. C. (1982). Plant density effects on growth, yield, leaf area index and light transmission on intercropped maize (*Zea mays* L.) and cowpea (*Vignaunguiculata* L. Walp). *Nigeria Journal of Agricultural Sciences, 99*, 19-23.

Fininsa, C. (1997). Effect of planting pattern, relative planting data and intra-row spacing on aharicot bean/ maize intercrop. *African Crop Science Journal, 5*, 15-22. http://dx.doi.org/10.4314/acsj.v5i1.27866

Flores-Sanchez, D., KleineKoerkamp-Rabelista, J., Navarro-Garza, H., Lantinga E. A., Rossing, W. A. H., & Kropff, M. J. (2011). Diagnosis of agro-ecological engineering of maize-based smallholder farming systems in Costa Chica, Guerrero state, Mexico. *Nutrient Cycling in Agro- Ecosystems*.

Flores-Sanchez, D., Pastor, A., Janssen, B. H., Lantinga, E. A., Rossing, W. A. H., & Kropff, M. J. (2012). Comparison of organic and inorganic nutrient inputs for productivity enhancement in smallholder maize-based systems in South West Mexico. http://dx.doi.org/10.1007/s10705-011-9455-z

Flores-Sanchez, D., Pastor, A., Janssen, B. H., Lantinga, E. A., Rossing, W. A. H., & Kropff, M. J. (2013). Exploring Maize-Legume intercropping systems in South West MexicoAgroecology and Sustainable Food Systems.

Foster, A., & Malhi, S. S. (2013). Influence of Seeding date on growing seasons conditions on forage yield and quality of four annual crops in Northeastern Saskatchewan. *Communication in Soil Science and Plants Analysis,* 44, 884 -891. http://dx.doi.org/10.1080/00103624.2012.747610

Francis, C. A. (1986). *Multiple cropping systems*. Macmillan, Newyork.

Fukai, S. (1993). Intercropping- bases of productivity. *Field crops Research, 34, 239-245.* http://dx.doi.org/10.1016/0378-4290(93)90116-5

Fukai, S., & Trenbath, B. R. (1993). Processes determining intercrop productivity and yield of component crops. *Field crops Research, 34*, 247 -271. http://dx.doi.org/10.1016/0378-4290(93)90117-6

Fusuo, Z., & Li, L. (2003). Using competitive and facultative interaction in intercropping systems enhances crop productivity and nutrients use efficiency. *Plant and soil, 248*, 305-312. productivity and nutrients use efficiency. *Plant and soil, 248*, 305-312. http://dx.doi.org/10.1023/A:1022352229863

Gabatshele. M. L., Marokane, T. K., & Mojeremane, W. (2012). Effects of intercropping on the performance of maize and cowpeas in Botswana. *Journal of Agriculture and Forestry, 2*(6), 307-310. http://dx.doi.org/10.5923/j.ijaf.20120206.07

Gangwar, K. S., & Sharma, S. K. (1994). Fodder legume intercropping in maize (*Zea mays*) and its effect on succeeding wheat (*Triticumaestivum*). *Indian Journal of Agricultural Sciences, 64*(1), 38-40.

Gardner, F. P., & Kisakye, J. (1990). Productivity of bean/maize intercrops as influenced by bean type and planting date and maize density. *Proceedings Soil and Crop Science Society of Florida, 49*, 139-146.

Geiler, K. E. (2001). *Nitrogen fixation in tropical cropping system* (2nd ed.). CABI publishing, Wallingford, UK. http://dx.doi.org/10.1079/9780851994178.0000

Geiler, K. E., Ormesher, J., & Awa, F. M. (1991). Nitrogen transfer from Phaseolus bean to intercropping maize measured using 15-N enrichment and 15-N isotope dilution methods. *Soil Biol. Biochem, 23*, 239-246.

Ghanbari-Bonjar, A. (2000). *Intercropping field bean (Viciafaba) and wheat (Triticum aestivumL.) as a low-input forage*. PhD thesis. Wye College, University of London, UK.

Goli, A. E. (1997). Conservation and improvement of bambaragroundnut (*Vigna subterranean* (L.) Verdc.). In Helle et al. (Eds), *Bibliographical Review*, Proceedings of an International Workshop held at Harare, Zimbabwe (pp. 4-10).

Gurigbal, S. (2010). *The soybean: Botany, Production and Uses*. Ludhiana, India: Cabi Publishers. http://dx.doi.org/10.1079/9781845936440.0000

Halikatti, S. I., & Banarasilal, B. (2011). Production Potentiality of Maize as Influenced by Planting Geometry, Mulching and Grain Legume Intercropping. *Karnataka Journal of Agricultural Sciences*, *11*(4).

Hamdollah, E. (2012). Yield and Quality of forage produced in intercropping of maize (*Zea mays* L.) with cowpea (*Vignasinensis* L.) and Mungbean (*Vigna radiate* L.) as double cropped. *J. Basic Appli. Sci. Res., 2*(1), 93-97.

Hamidou, F., Halilou, O., & Vadez, V. (2013). Assessment of Groundnut under combined heat and Drought stress. *J. Agronomy and Crop science, 199*, 1-11. http://dx.doi.org/10.1111/j.1439-037X.2012.00518.x

Hiebsch, C. K., & Macollam, R. E. (1980). Area time equivalency ratio. A method for evaluating the productivity of intercrops. *Agronomy Journal, 75*, 15-22.

Hillocks, R. J., Bennett, C., & Mponda, O. M. (2012). Bambara nut: A Review of utilisation, market potential and crop improvement,*African Crop Science Journal, 20*(1), 1-16.

Hongchun, X., Shen, H., Zhang, L., Zhang, Y., Guo, X., Wang, P., … Zuo, Y. (2013). Comparative proteomic analysis for assessement of the ecological significance of maize and peanut intercropping. *Journal of Proteomics, 78*, 447-460. http://dx.doi.org/10.1016/j.jprot.2012.10.013

Hugar, H. Y., & Palled, Y. B. (2008). Studies on maize-vegetable intercropping systems. *Karnataka J. Agric. Sci., 21*, 162-164.

ICRISAT. (2009). International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Groundnut. Retrieved 17 May 2013, from http://www.icrisat.org/groundnut/GroundNut.htm

Ijoyah, M. O. (2012). Review of intercropping research on cereal- vegetable based cropping system, *Scientific Journal of crop Science, 1*(3), 55-62.

Ijoyah, M. O., & Fanen, F. T. (2012). Effects of different cropping pattern on performance of maize-soybean mixture in Makurdi, Nigeria. *J. Crop Sci., 1*(2), 39-47.

Ijoyah, M. O., & Jimba, J. (2012). Evaluation of yield and yield components of maize (*Zea mays* L.)and okra (*Abelmoschus esculentus*) intercropping system at Makurdi, Nigeria. *J. Bio. Env. Sci., 2*(2), 38-44.

Innis, W. H. (1997). *Intercropping and the scientific basis of traditional Agriculture* (1st ed.). London: Intermediate Technology Publications Ltd.

Jakusko, B. B., & Belel, M. D. (2009). The effects of different level of phosphorous on quantitative characters/yield and productivity of bambaran groundnuts(Vigna subterranean) in Yola. *Techno Science African Journal, 311*, 13-15.

Jane, G. A., Godfrey, W. N., Gideon, N. M., & David, M. M. (2012). NaCl salinity affects germination,growth,physiology and biochemistry of Bambara groundnut. *Brazilian Journal of Plant Physiology, 24*(3), 151-160. http://dx.doi.org/10.1590/S1677-04202012000300002

Jensen, E. S. (1996). Grain yield, symbiotic N2 fixation and interspecific competition for inorganic N in pea-barley intercrops. *Plant Soil, 182*, 25-38. http://dx.doi.org/10.1007/BF00010992

Jeyakumaran, J., & Seran, T. H. (2007). Studies on intercropping capsicum (*Capsicum annum* L.) withBushitao (Vigna unguiculata L.) *Proceedings of the 6th Annual Research Session* (pp. 431-440), Oct. 18-19, Trinconalee campus, EUSL.

Jiao, N. Y., Zhao, C., Ning, T. Y., Hou, L. T., Fu, G. Z., Li, Z. J., & Chen, M. C. (2008). Effect of maize peanut intercropping on economic yield and light response of photosynthesis. *Chinese J. Applied Ecol., 19*, 981- 985

Jorge, A. C., & Joseph, G. L. (1999). Plant density and hybrid Influence in corn forage yield and quality. *Agron. J, 91*, 911 -915. http://dx.doi.org/10.2134/agronj1999.916911x

Kanakeri, V. V. (1991). *Studies on intercropping of legumes in kharifmaize and their Residual effect on succeeding wheat*. M.Sc.(Agri.) Thesis, University of Agricultural Sciences, Dharwad.

Karikari, S. K. (2003). A decade of Bambara groundnut agronomic research at the Botswana College of Agriculture. *UNISWA J. of Agric., 12*, 24-28.

Keating, B. A., & Carberry, P. S. (1993). Resource capture and use in intercropping: solar radiation. *Field Crop Res., 34*, 273-301. http://dx.doi.org/10.1016/0378-4290(93)90118-7

Khan, Z., Saeed, A., Zada, K., & Ahmad, S., (1992). Biologic and intercrop studies on yield and nitrogen fixation of soybean and maize. *Sarhad Journal of Agriculture, 8*, 613-622.

Koli, E. S. (1975). Pure cropping and mixed cropping of maize and groundnuts. *Neth. J . Agric. Sci., 26*, 344-353.

Li, L., Sun, J., Zhang, F., Li, X., Yang, S., & Rengel, Z. (2001). Wheat/maize or wheat/soybean strip intercropping I yield advantage and interspecific interaction on nutrients. *Field Crop Res., 71*, 123-137. http://dx.doi.org/10.1016/S0378-4290(01)00156-3

Lithourgidis, A. S., Vasilakoglou, I. B.,Dhima, K. V., Dordas, C. A., & Yiakoulaki, M. D. (2006). Forage yield and quality of common vetch mixtures with oat and triticale in two seeding ratios. *Field.Crops. Res., 99*, 106- 113. http://dx.doi.org/10.1016/j.fcr.2006.03.008

Maluleke, M. H., Bediako, A. A., & Ayisi, K. K. (2005). Influence of maize-lablab intercropping on Lepidopterous stem borer infestation in maize. *J. Entom., 98*, 384-388. http://dx.doi.org/10.1603/0022-0493-98.2.384

Mandal, B. K., Dhara, M. C., Mandal, B. B., Das, S. K., & Nandy, R. (1990). Rice, Mungbeans, Soybeans, Peanut, Ricebean, and Blackgram yields under different intercrop systems. *Agrons. J., 82*, 1063-1066. http://dx.doi.org/10.2134/agronj1990.00021962008200060006x

Mangasini, A. K., Mwanahawa, L. M., Arbogast, G. M., & Neema, P. K. (2012). Agronomic factors limiting groundnut production: A case of smallholder farming in Taboraregion. *17th Annual Research Workshop* Dar es Salaam, Tanzania; March 28-29, 2012

Matusso, J. M. M., Mugwe, J. N., & Mucheru-Muna, M. (2012). Potential role of cereal-legume intercropping systems in integrated soil fertility management in smallholder farming systems of sub-Saharan Africa Research Application Summary. *Third RUFORUM Biennial Meeting 24-28 September 2012, Entebbe, Uganda*

Metwally, A. E. A., Shafik, M. M., & Tamer, A. W. (2012). Effect of intercropping corn on Egyptian cotton characters.*The J. cotton Sci., 16*, 210-219.

Meyer, R. (2010). Low-input intensification in agriculture chances for small-scale farmers in developing countries. *GAIA-Ecol. Perspect. Sci. Soc., 19*, 263-268.

Mohan, H. M. (2003). *Maize based intercropping studies with grain legumes in vertisols*. M.Sc. (Agri.) Thesis, University of Agricultural Sciences, Dharwad.

Morris, R. A., & Garrity, D. P. (1993). Resource capture and utilization in intercropping: water. *Field Crop Res., 34*, 303-317. http://dx.doi.org/10.1016/0378-4290(93)90119-8

Mucheru-Muna, M., Pypers, P., Mugendi, D., Kungu J., Mugwe, J., Merckx, R., & Vanlauwe, B. (2010). Staggered maize-legume intercrop arrangement robustly increases crop yields and economic returns in the highlands of Central Kenya. *Field Crops Research, 115*, 132-139. http://dx.doi.org/10.1016/j.fcr.2009.10.013

Musa, M., Singh, A., Abubakar, L., Noma, S. S., Alhassan, J., & Haliru, B. S. (2012). Influence of cultivar and sokoto phosphate rock levels on the yield and yield components of groundnuts (*Arachis hypogea* L) in dry sub-humid sokoto area, Nigeria. *Nigerian Journal of Basic and Applied Sci., 20*(1), 49-54.

Nweke, I. A., & Emeh, H. O. (2013). The response of Bambara ground nut (*Vigna subterranean* (L) verdc) to phosphate fertilizers levels in IgbarianSouthEast Nigeria. *IOSR J. of Agric & Vet. Sci., 2*(1), 28-34. http://dx.doi.org/10.9790/2380-0212834

Ofori, F., & Stern, W. R. (1987). Cereal-legume intercropping systems. *Adv. Agron., 41*, 41-90. http://dx.doi.org/10.1016/S0065-2113(08)60802-0

Ogah, E. O., & Ogbodo, E. N. (2012). Assessing the impact of biodiversity conservation in the management of Maize Stalk Borer (Busseolafusca F.) in Nigeria. *Current Trends in Technology and Science Vol. II Issue IIPg 234 -238.*

Osman, A. N., Ræbild, A., Christiansen, J. L., & Bayala, J. (2011). Performance of cowpea (*Vignaunguiculata*) and Pearl Millet (*Pennisetumglaucum*) Intercropped under *Parkiabiglobosa*in an Agroforestry System in Burkina Faso. *African Journal of Agricultural Research, 6*(4), 882-891.

Oyewole, C. I. (2010). Maize (*Zea mays* L.)-Okra (Abelmoschus esculentus(L) Moench) intercropping as affected by cropping pattern in kogi state, Nigeria. *ContinentalJ. Agronomy, 4*, 1-9.

Pandita, A. K., Shah, M. H., & Bali, A. S. (2000). Effect of row ratio in cereal-legume intercropping systems on productivity and competition functions under Kashmir conditions. *Indian Journal of Agronomy, 45*, 48-53.

Portes, T. D. A. (1984). Profile of light interception and yields of six bean (*Phaseolus vulgaris* L.) cultivars of different growth habits intercropped with maize (*Zea mays* L.), *Field Crop Abst., 37*(6), 491.

Prasad, R. B., & Brooks, R. M. (2005). Effects of varying maize density on intercropped maize and soybeans in Nepal.*Exp.Agric., 41*, 365-382. http://dx.doi.org/10.1017/S0014479705002693

Putnam, D. H., Herbert, S. J., & Vargas, A. (1986). Intercropped corn-soybean density studies, II.Yield composition and protein. *Exp. Agric., 22*, 373-381. http://dx.doi.org/10.1017/S0014479700014629

Rana, K. S., & Pal, M. (1999). Effect of intercropping systems and weed control on crop-weed competition and grain yield of pigeonpea. *Crop Res., 17*, 179-182.

Rao, M. R., & Willey, R. W. (1980). Evaluation of yield stability in intercropping studies On sorghum/pigeonpea. *Experimental Agriculture, 16*, 105-106. http://dx.doi.org/10.1017/S0014479700010796

Raposo, J. A. D. E. A., Schuch, L., Assis, F. N. D. E., Machado, A. A., & De-Assis, F. N. (1995). Intercropping of maize and beans in different plant arrangements anddensities in pelotas, pesquisa. *Agropecuaria Brasilleira, 30*, 639-647.

Reddy, T. Y., & Reddi, G. H. S. (2007). Principles of Agronomy (pp. 468-489). Kalyan Publishers India.

Remison S. U. (1978). Neighbour effects between maize and cowpea at various levels of N and P. *Expl. Agric., 14,* 205-212. http://dx.doi.org/10.1017/S001447970000870X

Ross, S. M., King, J. R., Donovan, J. T., & Spaner, D. (2005). The productivity of oats and berseem clover intercrops. I. Primary growth characteristics and forage quality at fourdensities of oats. grass. *Forage. Sci., 60*, 74-86. http://dx.doi.org/10.1111/j.1365-2494.2005.00455.x

Rusinamhodzi, L., Corbeels, M., Justice, N., & Ken, E. G. (2012). Maize- Grain legume intercropping is an attractive option for ecological intensification that reduces climatic risk for smallholder farmers in central Mozambique. *Field Crop Res., 136*, 12-22. http://dx.doi.org/10.1016/j.fcr.2012.07.014

Sanginga, N., & Woomer, P. L. (2009). Integrated soil fertility management in Africa: Principles, Practices and Development Process. (Eds.). *Tropical Soil Biology and Fertility*. Institute of the International Centre for Tropical Agriculture. Nairobi.

Seran, T. H., & Brintha, I. (2010). Review on maize based intercropping. *Journal of Agronomy, 9*(3), 135-145. http://dx.doi.org/10.3923/ja.2010.135.145

Shafik, M. M., & Soliman, A. M. (1999). Effect of intercropping grain sorghum and soybean on yield and yield components. *Proc.1st conf. Recent Technologiesin Agric. Cairo Univ. 27-29, Nov. Vol. 11, 277-283*

Shi, Y., Ma, Y., Ma, W., Liang, C., Zhao, X., Fang, J., & He, J. (2013). Large scale patterns of forage yield and quality across Chinese grasslands. *Chinese Science Bulletin, 58*(10), 1187-1199. http://dx.doi.org/10.1007/s11434-012-5493-4

Singh, N. B., Singh, P. P., & Nair, K. P. P. (1986). Effect of legume intercropping on Enrichment of soil nitrogen, bacterial activity and productivity of associated maize crops. *Exp. Agric., 22*, 339-344. http://dx.doi.org/10.1017/S0014479700014587

Smith, S. (2002). Growing corn with companion crop legumes for high protein silage, *Green book Energy and Sustainable Agriculture Program* (pp. 68-70). Minnesota Dep. Agric. USA.

Sorrensen, R., Butts, C., Lamb, M., & Rowland, D. (2004). *Five years of subsurface drip irrigation on Peanut*. UGA/CPES Research and Extension Bulletin No.2004.

Steiner, K. G. (1991). Overcoming soil fertility constraints to crop production in West Africa: Impact of traditional and improved cropping systems on soil fertilizer. In A. U. Mokwunye (Ed.), *Alleviating Soil Fertility constraints to increased crop production in West Africa* (pp. 69-91). http://dx.doi.org/10.1007/978-94-011-3224-4_7

Stern, W. R. (1993). Nitrogen fixation and transfer in intercrop systems. *Field Crops Res., 34*, 335-356. http://dx.doi.org/10.1016/0378-4290(93)90121-3

Sullivan, P. (2003). *Intercropping principles and production practices*. Appropriate Technology Transfer for Rural Areas Publication. Retrieved from http://www.attra.ncat.org

Taru, V. B., Kyagya, I. Z., Mshelia, S. I., & Adebayo, E. F. (2008). Economic efficiency of resource use in Groundnut production in Adamawa State of Nigeria. *World Journal of Agricultural Sciences, 4*(S), 896-900.

Thayamini, H. S., & Brintha, I. (2010). Review on Maize based intercropping. *Journal of Agronomy, 9*(3), 135-145. http://dx.doi.org/10.3923/ja.2010.135.145

Thole, A. (2007). *Adaptability of soybeans [Glycine max(L)merr] varieties to intercropping under leaf stripped and detasselled Maize (Zea mays L.)*. M. Sc thesis, Department of crop science, University of Zimbabwe.

Trenbath, B. R. (1986). Resource use by intercrops. In C. A. Francis (Ed.), *Multiple cropping systems* (pp. 57-86). New York: Macmillan publishing co.

Troy, S. (2011). Graze Standing Corn for Forage Flexibility. *Angus Journal, 3*(6), 118.

Undie, U. L., Uwah, D. F., & Attoe, E. E. (2012). Effect of intercropping and crop arrangement on yield and productivity of late season Maize/soybean mixtures in the humid environment of South Southern Nigeria. *Journal of Agricultural Science, 4*(4), 37-50. http:dx.doi.org/10.5539/jas.v4n4p37

Vandermeer, J. (1989). *The ecology of intercropping*. New York: Cambridge University press. http://dx.doi.org/10.1017/CBO9780511623523

Vara Prasad, P. V., Craufurd, P. Q., & Summerfield, R. J. (1999). Fruit number in relation to pollen production andviability in groundnut exposed to short episodes of heat stress. *Annals of Bot., 84*, 381-386. http://dx.doi.org/10.1006/anbo.1999.0926

Vesterager, J. M., Nielsen, N. E., & Hogh-Jensen, H. (2008). Effects of cropping history and phosphorus source on yield and nitrogen fixation in sole and intercropped cowpea- maize systems. *Nutrients Cycling Agrosystems, 80*, 61-73. http://dx.doi.org/10.1007/s10705-007-9121-7

Waddington, S. R., & Edward, A. F. (1989). Research methods for cereal- legume intercropping. *Proceedings of the Workshop on research Methods for cereals /legumes intercropping in Eastern and Southern Africa* (pp. 69-69). Jan. 23-27, Malawi. http://dx.doi.org/10.1017/S0014479707005303

Waddington, S. R., Mekuria, M., Siziba, S., & Karigwindi, J. (2007). Long-term yield sustainability and financial returns from grain legume-maize intercrops on a sandy soil in subhumidNorth Central Zimbabwe. *Experimental Agriculture, 43*, 489-503.

Wahid, A., Gelani, S., Ashraf, M., & Foolad, M. R. (2007). Heat tolerance in plants: An overview. *Env. Exp. Bot., 61*, 199-223. http://dx.doi.org/10.1016/j.envexpbot.2007.05.011

Wamba, O. F., Taffouo, V. D., Youmbi, E., Ngwene, B., & Amougou, A. (2012). Effects of organic nutrient sources on the growth, total chlorophyll and yield of three Bambara groundnut landraces in the coastal region of Cameroon. *J. Agron., 11*(2), 31-42. http://dx.doi.org/10.3923/ja.2012.31.42

Widdicombe, D., & Thelen, K. D. (2002). Row width and plant density effect on corn forage hybrids. *Agron. J., 94*, 326 -330. http://dx.doi.org/10.2134/agronj2002.0326

Willey, R. W. (1979). Intercropping its importance and research needs, Part-I, Competitionand yield advantages. *Field Crop Abstracts, 32*, 1-10.

Yilmaz, S., Gozubenli, H., Kruskan, O., & Atis, I. (2007). Genotype and plant density effect on corn (*Zea mays* L.) forage yield. *Asian J. Plant Sci,* 6(3), 538-541. http://dx.doi.org/10.3923/ajps.2007.538.541

Zam-Ali, S. N., Matthews, R. B., Williams, J. H., & Peacock, J. M. (1990). Light use, water uptake and performance of individual components of sorghum groundnut intercrop. *Exp. Agric., 26,413-427.* http://dx.doi.org/10.1017/S0014479700001344

ZHANG, J. W., Chang-hao, H. U., Kong-jun, W. A. N. G., Shu-ting, D. O. N. G., & Peng, L. I. U. (2004). Effects of plant density on forage nutritive value of whole plant corn. *Agricultural Sci. in China, 3*(11), 842-848.

Zuo, Y. M., Zhang, F. S., Li, X. L., & Cao, Y. P. (2000). Studies on the improvement in iron nutrition of peanut by intercropping with maize on a calcareous soil. *Plant Soil, 220*, 13-25. http://dx.doi.org/10.1023/A:1004724219988

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Measuring Agricultural Support for Tajikistan

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Abstract

This paper endeavours to estimate the agricultural support for Tajikistan based on OECD Producer Support Estimate (PSE) methodology. In accordance to this methodology, the PSE and related other support measure indicators as well as the Nominal Rate of Protection (NRP) are calculated.

The results of the PSE calculation show that agricultural producers in Tajikistan are supported. The support originates mainly from transfers from consumers to producers and other transfer from consumers, while the budgetary payments to producers on national and regional level within specific commodity programmes are insignificant. The transfer from consumers to producers can be explained by higher prices consumers pay for imported commodities in comparison to world market prices due to both, higher insurance and freight costs. However, in comparison to the importance of the agricultural sector in Tajikistan's economy, the extent of agricultural support is estimated as rather low.

Keywords: Tajikistan, agricultural, support, nominal, protection, coefficient, producer, consumer

1. Introduction

Agriculture plays a crucial role in the economic growth of Tajikistan. The agricultural sector has undergone substantial changes after independence, caused, inter alia, by developments in agricultural policy. Already in 1991 international trade barriers were removed and agricultural prices were liberalized. After years of deep decline, the sector has recovered and became a backbone of the economy. In 2009, the share of agricultural production in GDP, transfers from the sector to the state budget in form of tax revenues, and the share of the sector in export (via cotton lint, vegetables and fruits) were 20, 33, and 30 percent respectively (Agency on Statistics [AS], Tajikistan in Figures, 2010).

The growth of the agricultural sector in recent years is related to land reform. Although, land in Tajikistan remains exclusively under state ownership" (Article 13 of the Constitution of the Republic of Tajikistan) and cannot be privatized, land use-rights can be transferred to individuals. Peasant (*dehkan*) farms hold 98.5 percent of the total agricultural land. As of January 2013 the numbers of peasant (*dehkan)* farms reached 71,857 and the land held by *dehkan* farms was 98.5 percent of total agricultural land (January 2013, Committee on Land, Geodesy and Cartography of the Government of the Republic of Tajikistan). Nearly 90 percent of agricultural products are produced by the private sector: 63 percent by households' subsidiary plots (except cotton), and 29 percent by peasant (*dehkan*) farms (AS, Agriculture Sector in Tajikistan, 2010).

Apart from the land reforms, there is no state incentive to further sectoral growth in Tajikistan. Agricultural workers receive the lowest wage in the economy and agricultural producers do not receive any explicit/implicit support, e.g. through concession on taxes or interest rates. The agricultural sector of Tajikistan is rather liberal - except the strategic commodity of cotton. The value chain of cotton production, processing and marketing is still highly distorted and inefficient: Tajikistan applies an export tax for cotton (10 percent). The input market for cotton producers was distorted by the monopsony position of cotton investors until 2008. The situation is changing slightly after a governmental decision to give producers a chance selling their harvest to any cotton ginners in 2009. Furthermore, in 2010, the Government of Tajikistan started introducing concessions on interest rates for agricultural producers and reduced land taxes for land allocated under cotton by 50 percent.

Assessing and quantifying policy measures and agricultural (dis)incentives allows their evaluation and comparison across time and between countries. For Tajikistan such calculations do not exist yet. This paper is going to fill this

information gap by estimating indicators of agricultural support for Tajikistan. There are three approaches internationally used to estimate agricultural support: the agricultural price distortion estimate of the World Bank, the Aggregate Measure of Support (AMS) of the World Trade Organization (WTO), and the Producer Support Estimate (PSE) of the OECD.

The World Bank approach uses indicators such as Nominal Rate of Assistance (NRA), Relative Rate of Assistance (RRA), Consumer Tax Equivalent (CTE) and Trade Bias Index (TBI), which focus mainly on government-imposed distortions that create a gap between domestic prices and border prices under free market conditions (Anderson, 2010; Anderson, 2009; Anderson & Swinnen, 2008; Anderson & Valdes, 2008; Anderson & Valenzuela, 2008; Anderson et al., 2008). One advantage of the Relative Rate of Assistance (RRA) is that this indicator considers both, agricultural and non-agricultural sectors assistance, and thus defines which country's sectoral policy regime has an anti- or pro-agricultural bias (Anderson, 2010).

The Producer Support Estimate (PSE) methodology of the OECD, developed in the early 1980s, was the conceptual basis for the Aggregate Measure of Support (AMS) in the General Agreement on Tariffs and Trade (GATT) Uruguay Round (Huang, 2010). WTO as the successor of GATT applies the AMS for monitoring the countries' commitment within WTO agreements on agriculture. Both measures, AMS and PSE, focus on the agricultural sector solely. They are related to each other but differ in purpose and measurement (Note 1). The PSE is an indicator for the annual monetary transfer of consumers and tax payers to agricultural producers measured at farm gate level. It quantifies agricultural support through (policy) interventions compared to a situation without such interventions, irrespective of form, target, and impact on agricultural production and income. Producers' support measured by OECD may originate through market transfers, transfers to producers from taxpayers, transfer to producers from consumers, other transfers from consumers, budgetary transfers (transfers to producers from taxpayers, transfers to consumers from taxpayers and price levies), market price support, payment to producer based on output and payment per ton. The AMS quantifies state monetary support in form of direct state expenditures as well as income transfers of consumers to agricultural producers due to market price distortions caused by state interventions. It includes all state interventions that are requested to be reduced in the frame of WTO agreements (amber box), like direct payments, input subsidies, market price support, and interest subsidies. Non-product specific elements (green box) are not included in the AMS thus making it less general than the PSE.

The application of the various approaches depends on the availability of data, specific advantages/constraints of the approaches and further research requirements. The main advantage of the PSE approach is its monitoring of time series and the cross-country comparability due to its consistent methodology. Thus, this paper applies the PSE methodology of the OECD to estimate support in Tajikistan's agricultural sector for the period 2000-2007.

2. Methodology and Data Sources

The OECD database on producer and consumer support (OECD, 2013) incorporates most OECD economies (Note 2) and some other economies (Note 3). PSE indicators, that are actually a set of indicators arising from each other, are used for the analysis of policy impacts, serve as input data and basis for international trade negotiations, and can serve as a tool for monitoring and evaluation of agricultural policy developments. For example, the PSE database is applied in the Policy Evaluation Partial Equilibrium Model (Tongeren, Kimura, & Le Thi, 2012) and the GTAPEM - General Equilibrium Model. The latter is a general equilibrium model that is based on the Global Trade Analysis Project (GTAP) incorporating key features of former models (OECD, 2009b).

In accordance to the PSE methodology, this study calculates the producer support and other support measure indicators as well as the Nominal Rate of Protection (NRP).

The OECD PSE methodology suggests that the list of commodities necessary for the calculation of the Market Price Support (MPS) and other support indicators should at least represent 70% of the total value of agricultural production. This analysis covers almost 90% of commodities (Note 4).

In accordance with OECD methodology, some commodity calculations do not consider the producer price at the farmgate level, but rather their passing through some processing stages (Note 5) in order to ensure the comparability of the same commodity at farmgate and border level. This is the case e.g. with sugar beet and cane, rice paddy, meat in live weight, and raw cotton. Therefore, in this study, the producer price of respective commodities is not always the one at farmgate level for unprocessed commodities (Note 6).

In this analysis the calculation of support indicators is done for the period 2000-2007. The periods 1990-1999 (Note 7) and 2008-2012 (Note 8) could not be calculated due to unavailability of certain data. The PSE and other support indicators have been calculated for each commodity as well as for the agricultural sector as a whole.

For the calculation of support indicators it is necessary to consider marketing margins (Note 9) and quality and weight adjustments. Quality adjustments were done for most of the commodities either on farmgate or border level in order to ensure comparability and thus reliable calculation.The border price (Note 10) was calculated according to the net trade situation and depending on the reliability and availability of official data.

Following data were applied in this analysis of Tajikistan's agricultural sector support: commodity balances (Note 11), producer and border prices at farmgate level, value of production, data on marketing margin (cost of processing, transportation and handling costs), data on quality and weights, exchange rates, insurance and freight costs, applied tariffs and taxes, and budgetary and other transfers on national and regional levels.

The sources of data include national statistics of the Ministry of Agriculture of the Republic of Tajikistan, the Tax Committee under the Government of the Republic of Tajikistan, the National Bank of Tajikistan and the Customs Service under the Government of the Republic of Tajikistan as well as international statistics from FAOSTAT, the United States Department of Agriculture (Foreign Agricultural Services USDA-FAS), and the Agency of Statistics of the Republic of Kazakhstan.

3. Results

This section presents the results and steps of the calculated agricultural support indicators. Detailed tables of results (total, producer and consumer supports, other support indicators as well as Nominal Rate of Protection) are presented in the appendix (A1-A18).

3.1 Producer Support Estimate and Other Support Indicators

The PSE provides information on agricultural support through various (policy) interventions while the issue of trade distortions is not addressed. Policy-related transfers, for example decoupled payments to farmers (i.e. the payment to producers that are not related to production of commodities), are likely to have much less effect on supply and demand of agricultural commodities even if such payments are not in place (OECD, PSE Manual, 2010b). Sometimes the policy regarding the sector is changed (Note 12) while the value of PSE is not. In this case, in order to detect the change in policy, each component of PSE should be examined.

The PSE component in accordance to OECD's 2007 revision are: (a) Support based on commodity output that includes MPS and payments based on output; (b) payments based on input use; (c) Payments based on current Area (A), Animal Numbers (AN), Receipts (R) or Income (I); (d) Payments based on non-current A/AN/R/I, production required; (e) Payments based on non-current A/AN/R/I, production not required; (f) Payments based on non-commodity criteria; (g) miscellaneous payments.

From the above listed PSE components, only MPS, which is one component of support based on commodity output, indicates the transfers from consumers and taxpayers to producers, while the rest of components indicate the transfers from the state budget to producers of agricultural commodities. On the other hand, it might be misleading to say that a change in the value of PSE is necessarily related to a change in policy. For instance, the value of support might change under changing world market conditions and exchange rate changes which might lead to a change in the value of PSE regardless of agricultural policy changes over time.

The PSE alongside budgetary payments also include transfers from taxpayers to producers and other transfers which do not require actual monetary disbursement from state budgets at the national and regional levels. The transfer from taxpayers to producers and other transfers are financed by domestic consumers who purchase the agricultural commodities at a price above the international level (OECD, PSE Manual, 2010b).

3.2 Nominal Protection Coefficient and Nominal Rate of Protection

The OECD's producer Nominal Protection Coefficient (pNPC) is the ratio between the Producer Price (PP) and the Border Price (BP), including to the former the ratio between the payments per ton on current output (PO_i) and quantity of produced commodity (QP_i), both measured at farmgate level (OECD, 2000).

$$pNPC_i = \frac{\left(PP_i + \frac{PO_i}{QP_i}\right)}{BP_i} = \frac{PP_i}{BP_i} + \frac{\frac{PO_i}{QP_i}}{BP_i} \quad (1)$$

The Nominal Rate of Protection (NRP) is the most frequently used measure of protection because of its relatively limited data requirements and its ability to capture most of the market distortion effects (Thomson & Metz, 1998). The producer NRP is defined as the ratio between the Market Price Differential (MPD) and the Border Price (Equation 2).

$$pNRP_i = \frac{MPD_i}{BP_i} = \frac{(PP_i - RP_i)}{BP_i} = \frac{PP_i}{BP_i} - 1 \quad (2)$$

The producer NRP can be derived from the producer NPC:

$$pNRP_i = \left(pNPC_i - \left(\frac{\frac{PO_i}{QP_i}}{BP_i} \right) - 1 \right) * 100 \quad (3)$$

The consumer NRP (cNRP) simply equals cNPC minus unity because the latter is simply the ratio between the Producer Price and Border Price, both measured at farmgate level.

$$cNRP_i = (cNPC_i - 1) * 100 \quad (4)$$

It should be noted that the producer and consumer NPC are coefficients, while the producer and consumer NRPs are ratios.

3.3 Calculation of Agricultural Support Indicators for Various Commodities

The respective sub-sections provide information on the steps of calculation of the agricultural support indicators of various commodities.

3.3.1 Wheat, Maize, and Other Grains

"Wheat". The calculation of PSE for wheat requires certain adjustments beforehand: first, with regard to the quality of production and second, referring to marketing and transport margins.

Quality adjustments are necessary to compare domestically produced and imported wheat. Wheat produced in Kazakhstan is of significantly higher quality than that produced in Tajikistan. Kazakhstan produces both hard and soft wheat (Agency of Statistics of Republic of Kazakhstan, 2010). Tajikistan imports mainly hard wheat from Kazakhstan which is not comparable to the soft wheat that is produced in Tajikistan. In order to ensure a comparison of "like with like" it is assumed that the quality of soft wheat is the same in both countries. The quality adjustment was done as follows: the difference between the producer price for hard and soft wheat in Kazakhstan is calculated (almost in average 20 percent for the observed period). Therefore, the border price for hard wheat (BP_h) is equal to unity minus the price differential between the border prices of hard and soft wheat (ΔP) multiplied by the border price for soft wheat (BP_s).

$$BP_h = (1 - \Delta P) * BP_s \quad (5)$$

Such quality adjustment ensures that a homogenous product is compared at farmgate and border levels. As a second option, it is theoretically possible to adjust the quality of soft wheat, i.e. the price of domestically-produced soft wheat to the border price of hard wheat. In this case, the value of domestic (soft) wheat production will increase later in the calculation. An increase of the value of production will further lead to an increased gross value of agricultural commodities in total and thus to an increased total value of production of MPS commodities. Due to this calculation dilemma of the second approach, the first option was chosen, i.e. imported hard wheat was adjusted to domestic soft wheat and not vice versa.

Both cases (increased and decreased producer and border prices due to quality adjustments) lead to adjustments in the value of: MPD; MPS; market transfer (transfer to producers from consumers, other transfers from consumers); budgetary transfer (transfer to producers from taxpayers); pNPC and cNPC; percentage of pNPC and cNPC; PSE and consumer support estimates (CSE); producer and consumer Nominal Assistance Coefficient (NAC); producer Single Commodity Transfer (SCT); and percentage SCT.

Besides the quality adjustment, marketing and transport margins also have to be considered in order to ensure the comparability of border and producer prices. Transportation and handling costs between the border and the domestic wholesale market (T_1) should be added to the CIF price in the case of an import situation (in the case of an export situation these costs have to be subtracted from the FOB price). On the next level, the handling and transportation costs between the wholesale market and the farmgate (T_2) are subtracted (in both cases import-CIF and export-FOB). And finally, the costs of possible processing (S) of the raw farm product into the final imported/exported products are subtracted.

In the case of wheat, of which Tajikistan is a net importer, and as far as the data on handling and transportation costs (T_1 and T_2) are not available, it is logically assumed that they cancel each other out. But in the case of an export situation, all transportation costs have to be subtracted (OECD, PSE Manual, 2010b). The costs of processing a farm product (S) into the final imported (exported) good must always be taken into account and deducted from the CIF price.

"Maize and other grains". There is an insignificant production level of maize and other grains (barley, oats and rye) in Tajikistan and most of it is used as feed. In order to calculate the PSE for these crops, the Ukrainian border

price was taken as a border (reference) price because Ukraine is the main exporter of these crops to Tajikistan. In order to obtain the border (reference) price, the FOB price in Ukraine (FOB_{other}) for these products are taken as a Tajik border price with insurance and freight costs (IF) added.

In order to ensure the reliability of the calculation of the PSE for other grains, which include barley, oats and rye, the weighted average producer and border prices for the single grain crops were taken, instead of the simple arithmetical mean. Also, the costs for insurance and freight (IF) were weighted. This is due to significant differences in transit costs through the territory of Uzbekistan depending on the final destination in Tajikistan – the north part (Sughd region) or the southern part (Khatlon region and the capital city of Dushanbe). For example, transportation costs are half when delivered to the North as to the South (Chabot & Tondel, 2011). Furthermore, depending on the use of maize and other grains (for human consumption or feed), the IF costs are weighted by the population and number of livestock in each region. Altogether, the weighted average insurance and freight costs for delivery to the North and to the rest of Tajikistan (South and capital city Dushanbe) are calculated as shown in Equation 6:

$$IF_c = (\mathrm{Sh}^{\mathrm{n}}_{\mathrm{pl}} * IF_n) + (\mathrm{Sh}^{\mathrm{r}}_{\mathrm{pl}} * IF_r) \quad (6)$$

where, $\mathrm{Sh}^{\mathrm{n}}_{\mathrm{pl}}$= share of population and livestock in the northern part of the country, $\mathrm{Sh}^{\mathrm{r}}_{\mathrm{pl}}$ = share of population and livestock in the rest of the country, IF_c = country-weighted average insurance and freight cost, IF_n = insurance and freight cost for delivery to the northern part of the country, IF_r = insurance and freight cost for delivery to the rest of the country.

Finally, the producer and border prices for other grains are derived from the weighted producer and border prices of barley, oats and rye respectively (Equations 7 and 8).

$$\mathrm{PP}_{\mathrm{og}} = \frac{(\mathrm{B}^{\%}_{\mathrm{og}} * \mathrm{PP}_{\mathrm{b}}) + (\mathrm{O}^{\%}_{\mathrm{og}} * \mathrm{PP}_{\mathrm{o}}) + (\mathrm{R}^{\%}_{\mathrm{og}} * \mathrm{PP}_{\mathrm{r}})}{100\%} \quad (7)$$

$$BP_{\mathrm{og}} = \frac{(B^{\%}_{\mathrm{og}} * BP_b) + (O^{\%}_{\mathrm{og}} * BP_o) + (R^{\%}_{\mathrm{og}} * BP_r)}{100\%} \quad (8)$$

where, $\mathrm{PP}_{\mathrm{og}}$ = producer price for other grains, $\mathrm{BP}_{\mathrm{og}}$ = border price for other grains, $\mathrm{B}^{\%}_{\mathrm{og}}$ = share of Barley in Other grains, $\mathrm{O}^{\%}_{\mathrm{og}}$ = share of Oats in Other grains, $\mathrm{R}^{\%}_{\mathrm{og}}$ = share of Rye in Other grains, PP_b, PP_o, PP_{r}, BP_{b}, BP_{o}, BP_{r} = producer and border prices for barley, oats and rye respectively.

The average weight of barley, rye and oats in other grains for the period for 2000-2007 were 97.2, 1.0 and 1.8 percent respectively. It should be noted that the weight of each of these crops in other grains fluctuates significantly each year, but their own calculation in each year was taken as a weight and used for share of each crop.

3.3.2 Cotton

Tajikistan is a net exporter of cotton. Raw cotton passes through stages of processing before final delivery to consumers; consequently, producer prices should be compared at the refined level. The refined level for cotton is reached after processing of raw cotton in a gin and separating cottonseed from cotton lint. Therefore, the estimate of MPS at this analysis refers to cotton lint at the gin and the border.

The indirect producer price (PP_{cl}) for cotton lint at the farmgate level is derived from the border price. PP was calculated based on the deduction of all taxes from the border FOB price (FOB_{cl}) and handling and transportation costs (T_1 & T_2), as shown in Equation 9. Processing costs (S) were not deducted because the producer price and the border price are shown at the same processing stage, in the form of cotton lint.

$$PP_{cl} = FOB_{cl} - T_1 - T_2 - TR_{TUGE} - VAT - C_t \quad (9)$$

where, TR_{TUGE} = tariff rate of Tajik Universal Goods Exchange (15 percent), VAT = Value added tax (until 2009 the rate was 20 percent, later 18 percent), C_t = Customs Tax (10 percent applied to exports of cotton lint).

3.3.3 Rice

The commodity rice was considered in the analysis as milled rice. Some weight adjustment has been done in order to compare milled rice at farmgate and border levels. The total production of milled rice (QP_{rme}) is derived by multiplying the production of paddy rice (QP_{rpe}) with the extraction rate (0.66) of paddy to milled rice.

The weighted average producer price at farm gate level for milled rice (PP_{rme}) is obtained by dividing the weighted average producer price of paddy rice at farm gate level (PP_{rpe}) by extraction rate. The same approach is used for deriving the border price for milled rice equivalent.

The value of production of milled rice equivalent (VP_{rme}) can be expressed as:

$$VP_{mre} = \left(QP_{rpe} * ER\right) * \left(\frac{PP_{rpe}}{ER}\right) = QP_{mre} * PP_{rme} = VP_{rpe} * ER \quad (10)$$

where, QP_{rpe}= quantity of produced paddy rice; ER= extraction rate; PP_{rpe}= producer price of paddy rice at farm gate level; QP_{rme}= quantity of produced of milled rice; VP_{rpe}= value of production of paddy rice equivalent.

The processing costs of transforming paddy rice into milled rice are not taken into account at farmgate and border levels, assuming that they cancel each other out.

Due to the unreliability of border prices for imported rice from China (the main rice exporter to Tajikistan), the Chinese producer price for rice paddy is taken as a reference border price and added to IF costs for delivery to the Tajik border.

3.3.4 Fruits and Vegetables

Potatoes, onions, tomatoes, lemons, grapes and apples produced in Tajikistan are internationally traded commodities. Grapes and lemons are net export commodities of Tajikistan. Depending on the season, onions, tomatoes and apples are either export or import commodities, but for this analysis, Tajikistan is considered as a net exporter because the exports prevail over imports most of the year. The import of potatoes exceeds its exports, but it should be noted that potatoes are also used as feed. Tajikistan is self-sufficient with its potato production and on average during the period 2000-2007 almost 15 percent of domestically produced potatoes were used as feed, while for the same period the quantity of imports was less than the quantity of feed used. The import of potatoes takes place during the winter season with Pakistan being the main country of origin.

One of the challenges of calculation is the unreliability and consistent underestimation of the border price for potatoes as there is significant illegal trade and available data are erratic. Recorded CIF prices are often less than the producer prices in the country of origin (Pakistan, Russia) which is not realistic (Note 13). In order to calculate the border price for potatoes despite these problems, the farmgate price in Pakistan was taken plus insurance and freight costs to the Tajik border.

Similar data problems exist with onions, lemons, tomatoes, grapes and apples where the Tajik producer price (at farmgate level) is higher than the border price for these exported commodities, although no export subsidy policy is in place in Tajikistan.

In order to define the border price for the exported commodities (onions, tomatoes, lemons, grapes and apples) the MPD was calculated. The MPD for these commodities is equal to the producer price (PP_i) multiplied by the ratio of average *ad valorem* tariffs (tr_i) for commodities and unity plus *ad valorem* tariffs.

$$MPD_i = PP_i * \frac{tr_i}{1+tr_i} \quad (11)$$

Then the border reference price is calculated as the producer price plus market price differential.

It should be noted that there is no input support, neither governmental payments based on output to producers nor export subsidies at the country or regional level.

3.3.5 Livestock Commodities

"Milk". Based on the former OECD methodology, until 2004 the border reference price for milk for all countries was derived from the farmgate milk price in New Zealand, adjusted for milkfat content and transportation costs. The calculation of protection indicators for milk is complex because of two reasons: first, raw milk is not traded internationally, and second, the valuable component of raw milk - the milkfat - varies. Therefore, the border reference price for raw milk in 2007, in accordance with the new OECD methodology, was derived implicitly based on trade prices of skimmed milk powder (SMP) and butter (OECD, 2010b). The quality adjustment for milk was done for imported and domestically produced milk components, taking into account such components as milkfat content in butter, SMP, and raw milk, as well as the non-fat solids content of butter, SMP, and raw milk. As the reference price for butter (82%) and SMP, the CIF price in Northern Europe was taken (OECD, 2010a). IF costs were added for delivery to the Tajik border. The milk fat content in butter (CIF) refers to data from the University of Guelph, Institute of Dairy Science and Technology. For the calculation of the implicit border price of

raw milk, Western data on non-fat solids content of raw milk (USAID, 2003; Salathe & Price, 1992), non-fat solids content of butter (USAID, 2003), milkfat content of SMP, and milkfat content of raw milk (FAO, 2004) are used due to the unavailability of official Tajik statistics. Based on these data, the implicit reference border price for raw milk is calculated stepwise, derived from the price of the two milk components milkfat and non-fat solids.

The implicit border price of milkfat (X) is defined by the border price of butter and SMP and calculated as follows:

$$X = \frac{(dBP_s - cBP_b)}{(ad - bc)} \tag{12}$$

where a = milkfat content of butter, b = non-fat solids content of butter, c = milkfat content in SMP, d = non-fat solids content of SMP, BP_b = butter border price and BP_s = SMP border price.

The implicit border price of non-fat solids (Y) is also defined by the border price of butter and SMP:

$$Y = \frac{(aBPs - bBPb)}{(ad - bc)} \tag{13}$$

Finally, the implicit reference border price of raw milk (BPim) is obtained from the calculated reference border prices of butter and SMP (Equation 14):

$$BP_{im} = \alpha BPb + \beta BPs \tag{14}$$

where α = Share of butter price in milk price, ? 2 = Share of SMP price in milk price.

As a next step, the processing margin is calculated by subtracting the producer price for manufacturing quality milk from the implicit wholesale price of raw milk in the domestic market. The marketing margin (MM) of butter and SMP can be expressed and calculated in two ways (Equations 15 and 16):

$$MM = (\alpha WP_b + \beta WP_s) \tag{15}$$

$$MM = (\alpha WPb + \beta WP_s) - C \tag{16}$$

where, WP_b = wholesale price of butter in the domestic market, WP_s = wholesale price of SMP in the domestic market, PP_m = producer price of milk, C = average processing margin of butter and SMP in four major exporters (New Zealand, the European Union, Australia and the United States of America).

The share of butter price in milk price (α) and the share of SMP price in milk price (β) is defined and calculated as follows:

$$\alpha = \frac{de - bf}{ad - bc} \tag{17}$$

$$\beta = \frac{(af - ce)}{(ad - bc)} \tag{18}$$

In the case of unavailability of data on the wholesale price of butter and SMP in the domestic market (like Tajikistan) prices can be derived alternatively in three ways. The first way is by using technical coefficients: the production of 1 ton of (82% fat) butter requires 8780 liters of milk (4%); the production of 1 ton of (0.5% fat) Skimmed Milk Powder (SMP) requires 20,850 liters of 4% fat milk (FAO, 2009). The producer price for raw milk is multiplied by the required liters of milk for producing butter and SMP, and then added by the domestic processing costs, interest rates of processors, and transportation costs to deliver to the wholesale market. Such an approach can be used if there is no implicit or explicit price support of milk producers and processors. The second way is to use the wholesale price for butter and SMP of the main importing and exporting countries (OECD, Agricultural Outlook, 2010a) and to complement it with IF costs for delivery to the country border, taxes, and costs of delivery from the border to domestic wholesale markets. The third way, if consumer prices (AS, Prices in Tajikistan, 2010) are available, is to subtract the percentage of the retail price (converted to an absolute value) from the consumer price.

After the implicit reference border price of raw milk has been calculated, the further steps of calculating the PSE for milk are the same as for all MPS commodities.

"Beef and veal". The producer price of boneless beef (PP_{bb}) is defined as a ratio of the producer price for beef in live animal weight equivalent (PP_{bl}) and a weight-adjustment coefficient (WA_{bb}) (tons of boneless beef obtained from one ton of live animal).

The reference border price of boneless beef (RP_{bl}) is equal to the difference between the border price (BP_{bb}) and marketing margin (MM_{bb}) multiplied by a quality adjustment (QA_{bb}) for boneless beef.

Due to unreliable official data of the Customs Committee of Tajikistan, the border price (CIF) for the period 2003-2007 was estimated by comparing the domestic consumer and producer prices with the producer price in India (Note 14). So far, as in the calculation of the producer price for boneless beef, the marketing margin (MM) is not taken into account, it is logically assumed that MM for the reference border price is zero, while the quality adjustment coefficient is equal to unity. In this case, the reference price is simply equal to the border price for boneless beef.

"Sheep and Goats". Sheep and goat meat produced in Tajikistan is not officially traded in international markets and the country is self-sufficient. In order to calculate the support indicators, the New Zealand producer price (FG_{other}) was taken as a border price, added to insurance and freight costs (IF) for delivery to the Tajik border.

"Poultry and Eggs". During the period 2000-2007, on average, only 12 percent of all consumed poultry meat was produced in Tajikistan, the rest was imported. New Zealand's producer price was taken as a border price and IF costs added.

The majority of egg imports comes from Iran (90% of total imported eggs) and to a lesser degree from India, Pakistan and China. As with beef, an official CIF price can be found; however it does not reflect the real price and is underestimated. For this reason the real border price was calculated based on the comparison of producer prices (at farmgate) in the aforementioned countries. For 2000-2002 the producer price in India and for 2003-2007 the Iranian producer price was taken as a farmgate price respectively.

3.4 Excess Feed Cost

Wheat, oats, maize, barley, millet, rye, sorghum, cotton and sunflower seedcakes, rape and mustard seed, potatoes, beans, apples, bran and other vegetables are mainly used as feed in Tajikistan.

Excess feed cost (EFC) is a supplementary cost resulting from MPS on quantities of crops domestically produced and consumed as feed by livestock producers (OECD, Glossary of Agricultural terms, 2000). Its estimation is necessary in order to eliminate double counting of price transfers in the aggregation of MPS across commodities when deriving a national level of MPS (OECD, PSE Manual, 2010b). In order to avoid double-counting, the EFC should be deducted from PSE for livestock and CSE for commodities that are used as feed. EFC calculation, therefore, allows calculating the quantity of domestic feed crops that are used for beef and veal, pork, sheep and goat, poultry and egg production. If livestock producers pay lower prices for feed crops (in this case EFC is negative), it leads to a decrease of producer prices for livestock commodities and *vice versa*. In this case, consumers as well pay a lower price for livestock commodities, and conversely. All these would be true in the case of an open and perfectly competitive economy. On the basis of all the foregoing, it can be stated that the EFC is an important component of agricultural support estimates.

Most feed crops are produced domestically and the share of imported feed during the observed periods is, on average, 0.1 percent. EFC is not calculated for imported feed crops such as molasses, soybean cake and other cereals.

In order to estimate the EFC, the quantity of consumed feed crops was defined and the share of each feed commodity in total feed was calculated. The share of all livestock (sheep and goats, cattle beef, dairy cows, horses, yaks and poultry) in total feed demand is calculated. Half of the cattle in Tajikistan are calves, so that the equivalent used for them (0.6) is less than that for full-grown bulls or dairy cows (1.0). Required (oat) feed is based on 2.12 tons of oats required for feeding one animal per year (Sedik, 2009). Based on such assumption, the required oat feed is 1.27 tons per year per calf. The share of feed consumed by sheep and goats is equal to 26 percent of total feed consumption, the share by beef cattle and dairy cows are 24 and 42 percent respectively, horses and yaks consume 5 percent, while poultry consume the remaining 3 percent of domestically produced feed. The shares of each feed within total feed are defined. In the next step, total feed is proportionally distributed among the different livestock for the years under question.

Excess Feed Cost (EFC_i) is equal to market price differential (MPD_j) multiplied by the quantity of domestically produced and consumed feed (QC_j^i).

For most crops (feed and non-feed) the producer price in Tajikistan is almost two times higher than the border price (FOB) in most neighboring exporter countries and the producer prices in those countries. When feed from potential third exporter countries is delivered to the Tajik border the actual border price exceeds the producer price due to high insurance and freight costs and the relatively high transportation costs for transit through Uzbek territory. Thus, the import of feed is not profitable, despite the relatively high producer prices in Tajikistan.

4. Discussion

Based on the study results presented, this part will pay particular attention to three key findings on measuring agricultural support in Tajikistan: first, the factors influencing the value of PSE and other support indicators; second, favoured form of land use (mainly cereals and cotton) and livestock production; third, the reliability of official data and its handling.

First, there are several factors which influence the value of agricultural support. A change in producer or border prices leads to a price gap between them, hence to fluctuation of MPS, and consequently PSE, CSE and other support indicators. Also, such fluctuation can be due to the appreciation (or depreciation) of the national currency (in the case of a trading situation). Those factors occur for most of the countries but there are some other specific factors which affect predominantly landlocked countries such as Tajikistan, namely higher transportation costs of commodities. For example, the cost of delivering wheat and other grains from Kazakhstan to the Tajik border through the territory of Uzbekistan is significantly higher than delivering the same commodities to the Afghan or Iranian border, although the distance from Kazakhstan to the latter countries is longer (Chabot & Tondel, 2011). The border price for exported wheat at the Kazakh border is at least twice the border price for imported Kazakh wheat at the Tajik border. The reasons are the very high insurance and freight costs. The same is true for other traded agricultural commodities which pass through Uzbek territory.

Second: the decision on land use - wheat and cereal production versus cotton cultivation. In Tajikistan, the production of wheat is increasing significantly with a producer price almost double the world market price due to lower yields and input costs for irrigation. The higher producer price in domestic markets is theoretically a good incentive for production of wheat and other cereals, but not for consumers. Thus, most of the wheat production is not sold by farmers in the domestic market, instead being used for self-consumption and feed. Therefore, the producers and consumers of Tajik wheat and cereals are the farmers themselves. Interpreting the situation from such a point of view one sees that land is used with less efficiency. The higher producer price for wheat and other cereals is mainly related to lower yields and the necessity of irrigation due to climatic conditions of the country. Cereal prices influence the livestock prices. As the analysis shows, the higher price for livestock commodities in Tajikistan is mainly related to higher prices for feed crops but also to an annually growing number of livestock, while the area allocated to forage and feed crops has been decreasing or stagnating since 2011. There is no explicit governmental support for feed producers in Tajikistan. Furthermore, export or import of feed is not hampered by any Tajik measures.

The export of cotton lint is one of the most important sources of earnings of foreign exchange in the country. However, the revenues as well as the PSE fluctuate depending on the world market price for cotton. Additionally, the cotton market in Tajikistan is quite complex and often non-transparent. Until 2008, it had been a monopolized system dominated by so-called “investors” and the government who prevented competition and who still controls the export. The result was an inflation of input prices and relatively lower output prices in comparison to more competitive cotton markets like in neighbouring Kyrgyzstan. Farmers have not been interested in cultivating cotton. Even after the official removal of this monopolized system, farmers’ motivation for growing cotton has not increased. Farmers are free now to sell raw cotton to any gin but the gins still do not offer prices as high as in former times, due to fluctuating world market prices and high state tariffs and taxes (cumulatively almost 45%). The situation might change if all tariffs and taxes were removed which will serve as incentive to expand production.

Third, the main challenge in the analysis is the reliability of official data. The quality of any calculation is only as good as the quality of its data. Estimating the agricultural support in Tajikistan is a big challenge because of the data availability and reliability problem. Such data problems in transition countries are well-known and every researcher has to find a way of coping with it. This example of Tajikistan presents specific solutions for particular problems.

Border price inconsistencies were found for several internationally traded commodities. The volume of imported commodities was assessed as reliable but the CIF and FOB prices are unreliable and consistently underestimated. Official border prices (FOB or CIF) were significantly lower than the real border price (cotton and wheat are exemption) by several times or at least by 30-50%, depending on the respective commodity. This conclusion was made by comparing domestic wholesale prices, consumer prices, and producer prices for both, domestically produced and imported commodities. Therefore, the border prices FOB of trading partners of Tajikistan were taken as a reference price and adjusted to the border level.

Data analysis reveals that the prices of wheat and cotton lint reflect the real (not underestimated) border prices (respectively CIF and FOB). For maize and other grains this is not the case, as those commodities were not

internationally traded by Tajikistan in the observed time period or the volume of imports are insignificant in some years. For all other commodities of the analysis, the border price did not indicate the real price and was underestimated. This effect can be explained by misreporting of trade data by exporters and importers of agricultural commodities in order to avoid tax payments as well as by misinformation from the Customs Service under the Government of the Republic of Tajikistan that benefit from bribes. Such a situation can be observed not only for agricultural commodities but also for other commodities, where the corruption scheme is even more extensive than in agriculture.

Based on the aforementioned, it can be concluded and guesstimated that total imports outweigh exports, on average in 2007-2011, not by 2.5 times as indicated in official statistics but should exceed it by 3.5 to 4 times. The negative trade balance for the economy of Tajikistan during the last 6 years has been compensated by labour migrant remittances that have become the main source of foreign earnings. This enables the National Bank of Tajikistan to keep the official and market exchange rate at a stable level, despite the negative trade balance. Except remittances, there are nearly no other sources of financing the trade deficit, which might lead in the end to a depreciation of the national currency. This could further lead to differences between the official and market exchange rates.

5. Conclusion

The results of the PSE calculation using the OECD methodology reveal that agricultural producers in Tajikistan are supported in a certain way. Calculation of PSE and other related support indicators show that producers mainly receive support in the form of transfers from consumers to producers, transfers to producers from taxpayers, and other transfers from consumers while the budgetary payments to producers on country and regional levels within specific commodity programmes is insignificant. The transfer from consumers to producers can be explained by higher prices that consumers pay for imported commodities in comparison to world market prices due to both, higher insurance and freight costs.

The main challenge of the calculation of agricultural support indicators for Tajikistan is not related to the lack of border price data in general, but often to its unreliability. Recorded border prices were significantly lower than the real border prices for many internationally traded commodities (with the exception of cotton and wheat). The main reason is the deliberate report of incorrect trade values by exporters and importers of agricultural commodities.

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References

Agency of Statistics of Republic of Kazakhstan. (2010). *Agriculture sector in Kazakhstan*. Retrieved from http://stat.kz/digital/selskoe_hoz/Pages/default.aspx

Agency on Statistics under the President of the Republic of Tajikistan. (AS). *Prices in Tajikistan* (1995, 2005 & 2010).

Anderson, K. (Ed.) (2009). *Distortion to agricultural incentives: A Global Perspective, 1955-2007*. London: Palgrave Macmillan and Washington DC: World Bank. http://dx.doi.org/10.1596/978-0-8213-7419-1

Anderson, K., & Croser, J. (2009). *National and Global Agricultural Trade and Welfare Reduction Indexes, 1955 to 2007*. Retrieved from http://www.worldbank.org/agdistortions

Anderson, K., & Swinnen, J. (Eds.) (2008). *Distortion to Agricultural Incentives in Europe's Transition economies*. Washington DC: World Bank.

Anderson, K., & Valenzuela, E. (2008). *Global Estimates of Distortion to Agricultural Incentives, 1955 to 2007.* Retrieved from http://www.worldbank.org/agridistortions

Anderson, K., Cockburn, J., & Martin, W. (eds.) (2010). *Agricultural Price Distortions, Inequality and Poverty.* Washington DC: World Bank. http://dx.doi.org/10.1596/978-0-8213-8184-7

AS. *Agriculture sector in Tajikistan.* (2005 & 2010).

AS. *Foreign Economic Activity.* (2005 & 2010).

Chabot. Ph., & Tondel, F. (2011). *A regional view of wheat markets and food security in Central Asia* (*with focus on Afghanistan and Tajikistan)*. USAID, FEWS NET, July 2011. Retrieved from http://documents.wfp.org/stellent/groups/public/documents/ena/wfp238576.pdf

Custom Service under the Government of the Republic of Tajikistan. *FOB and CIF prices. (2000-2010)*.

Dairy Science and Technology. University of Guelph. Retrieved from http://www.foodsci.uoguelph.ca/dairyedu/butter.html

FAO. (2004). *FAO Statistical Database – Commodity Balances.* Retrieved February 2, 2012, from http://faostat.fao.org/site/614/default.aspx

FAO. (2004). *FAO Statistical Database – Producer Prices.* Retrieved February 2, 2012, from http://faostat.fao.org/site/703/default.aspx#ancor

FAO. (2004). *FAO Statistical Database – Production.* Retrieved February 2, 2012, from http://faostat.fao.org/site/339/default.aspx

Food and Agriculture Organization (FAO). (2009). *Agribusiness handbook "Milk/dairy products"* Retrieved February 2, 2012, from http://www.fao.org/docrep/012/al179e/al179e.pdf

Huang, H. (2010). *OECD Experience in using PSEs for policy analysis and monitoring.* ABARE Outlook 2010, Canberra, March 2-3.

Liefert, W., Sedik, D., Koopman, R., Serova, E., & Melyukhina, O. (1996). Producer subsidy equivalents for Russian agriculture: estimation and interpretation. *American Journal of Agricultural Economics, 78*, 792-798. Retrieved February 2, 2012, from http://www.jstor.org/stable/1243306

OECD. (2000) *Agricultural Policies in OECD Countries: Monitoring and Evaluation: Glossary of Agricultural Policy Terms.* Retrieved from http://stats.oecd.org/glossary/detail.asp?ID=864

OECD. (2009a). *Agricultural Support – how it measure and what does it mean*. Retrieved from www.oecd.org/dataoecd/23/7/44924550.pdf

OECD. (2009b). *Using the Indicators in OECD Policy Modelling (Chapter 12).* Retrieved from http://www.oecd.org/tad/agricultural-policies/41120543.pdf

OECD. (2010a). *Agricultural Outlook 2004-2013.* Retrieved from http://www.cil.org.ar/docs/32037536.pdf

OECD. (2010b). *OECD's Producer Support Estimate and related indicators of Agricultural Support – Concept, Calculation, Interpretation and Use (The PSE Manual)*. Retrieved from http://www.oecd.org/dataoecd/52/5/46193164.pdf

Organization for Economic Cooperation and Development OECD. **(**2013). Agricultural Policies and Support: Producer and Consumer Support Estimates database. Retrieved from http://www.oecd.org/agriculture/agricultural-policies/producerandconsumersupportestimatesdatabase.htm

Salathe L., & Price, J. M. (1992). Implications of raising the nonfat solids standards for beverage milk. *Southern Journal of Agricultural Economics*. December 1992, 197-209. Retrieved from http://ageconsearch.umn.edu/bitstream/29633/1/24020197.pdf

Sedik, D. (2009). *The Feed-Livestock Nexus in Tajikistan: Livestock Development Policy in Transition.* FAO. Retrieved from http://www.fao.org/docrep/017/aq338e/aq338e.pdf

Tax Committee under the Government of the Republic of Tajikistan. *Tariff rates for imported and exported agricultural commodities and other taxes for 2000-2011.*

The National Bank of Tajikistan. *Exchange Rate data*. Retrieved from http://nbt.tj/ru/kurs/?c=4&id=28&lang=ru

Thomson A., & Metz M., (1998). *Implications of Economic Policy for Food Security: A Training Manual.* Retrieved from http://www.fao.org/DOCREP/004/X3936E/X3936E14.htm

Tongeren, F. V., Kimura, Sh., & Le, Thi C., (2012). *The OECD PSE database and its use in market model.* Retrieved from https://www.gtap.agecon.purdue.edu/resources/download/5845.pdf

United States Department of Agriculture (USDA). (2003). *Raising the Minimum Nonfat Solids Standard to the National Average in Raw Milk: A Study of Fluid Milk Identity Standards*. Retrieved from http://www.ams.usda.gov/AMSv1.0/getfile?dDocName=STELDEV3013455

USDA. (2012). *Foreign Agricultural Services. Production, Supply and Distribution*. Retrieved from http://www.fas.usda.gov/psdonline/psdQuery.aspx

Notes

Note 1. The main differences between these two approaches are: WTO "Green Box" differ from General Services Support Estimate (GSSE) of OECD; WTO AMS differ from PSE of OECD; WTO calculates Market Price Support (MPS) only if "administered price" is in place, using the gap between fixed external reference price (average f.o.b. or c.i.f. price for 1986-1988) and applied producer administered price by multipliying of the latter by the quantity of production eligible to receive the applied administered price; while OECD PSE calculates MPS in any case and compares producer and border price data in the same single year, thus, the price gap calculation techniques differ; WTO calculation of MPS does not consider price levies, nor transfers from consumers and taxpayers to producers; WTO commodity specific AMS will be exempted from total AMS if it is below *de minimis*. Despite methodological differences on measuring agricultural support, depending on research needs, both approaches may serve complementary to each other.

Note 2. Australia, Canada, Chile, European Union, Iceland, Israel, Japan, Korea, Mexico, New Zealand, Norway, Switzerland, Turkey, and United States.

Note 3. Brazil, China, Indonesia, Kazakhstan, Russia, South Africa, and Ukraine.

Note 4. See the Figures in Appendix A1. These commodities are in detail: *wheat, maize, other grains (barley, rye and oats), potatoes, tomatoes, grapes, lemons, apples, onions, milk, beef and veal, sheep and goats, poultry, eggs, cotton, cotton-oil, rice.*

Note 5. In this case the "consumer" is the one who purchases commodities from the processor.

Note 6. In the case of milk, an implicit border price is calculated and compared with the same at farmgate level (producer price) because raw milk is not an internationally traded commodity.

Note 7. There is no data on support of the sector within the state budget on different levels within this period.

Note 8. There is no data on commodity balance sheets and producer prices of commodities under question.

Note 9. Representing the costs of processing, transportation, and handling.

Note 10. This analysis distinguishes between two border prices: real and actual border price. The real border price originates from the comparison of prices based on the source of trade origin and whether a commodity is imported or exported. For example, if Tajikistan is a net importer, the real border price is obtained by taking the prices of the country of origin (which are the export and producer prices in the country of origin) and adding insurance and freight costs (IF) for delivery to the Tajik border. If Tajikistan is a net exporter, the Tajik producer price is taken, added are applied taxes and tariffs which are in place, and deducted are transportation costs from farmgate to wholesalers and from wholesalers to the border. Under the actual border price this analysis understands that data available from official statistics on border prices are often (except for cotton and wheat) underestimated and unreliable.

Note 11. The commodity balance includes: production quantity, import and export quantity, stock variation, domestic supply quantity, feed, seed, waste, processed, consumption, and other utilities.

Note 12. Increase or decrease of farmer's support or taxation.

Note 13. For example, in 2007 Pakistan was the country of origin for 80% of imported potatoes to Tajikistan. In accordance with official data of the Customs Service under the Government of Tajikistan, the CIF price for imported potatoes from Pakistan to the Tajik border was 166 USD per ton, while the producer price in Pakistan was 189 USD. And the CIF price at the Tajik border for imported potatoes from Russia was recorded as 136 USD, while the producer price in the same year in Russia was 248 USD.

Note 14. One example for unreliable official data: The import price for Indian boneless frozen beef at the Tajik border in 2007 was stated at 390 USD/t while the wholesale price for imported boneless frozen beef was 3000 USD/t. Assuming a border price of 390 USD/t, adding a 10% customs tax for imported beef and a 20% VAT, plus 2% transportation costs from the border to the wholesale market,results in a border price of 507 USD which is 6 times less than the wholesale price for imported boneless frozen beef! Therefore, the stated import price is not reliable.

Annex A: Agricultural Support Indicators

Table A1. Total Support Estimate and Aggregate Single Commodity Transfers

	2000	2001	2002	2003	2004	2005	2006	2007
Total value of production (USD mn)	416	526	582	763	923	1065	1317	1174
of which, share of MPS commodities (%)	95.7	92.8	94.2	93.8	93.7	92.4	90.2	85.8
Total value of production MPS commodities (USD mn)	398	488	549	715	864	984	1187	1008
Total value of consumption (USD mn)	425	539	472	574	727	845	1033	905
of which, MPS commodities (USD mn)	408	501	438	526	669	764	904	739
Total Producer Support Estimate (USD mn)	-8	11	14	17	15	84	152	129
Total Support Estimate (%)	-0.9	1.0	1.1	1.1	0.7	3.7	5.4	-0.9
Percentage PSE	2.0	2.0	-2.3	-2.2	-1.6	7.9	11.5	11.0
Producer NAC (ratio)	1.02	1.02	0.98	0.98	0.98	1.09	1.13	1.12
Excess feed cost (USD mn)	-1.8	-0.8	0.7	6.4	-1.4	22.0	29.5	-13.0
Percentage CSE (%)	-24.3	-28.0	-29.2	-25.8	-23.0	-29.3	-27.9	-19.6
Consumer NAC (ratio)	0.3	0.4	0.4	0.4	0.3	0.4	0.4	0.3
Total Support Estimate (USD mn)	-8.4	10.8	13.6	16.6	14.9	84.7	153.0	131.0
Transfers from consumers (USD mn)	-102	-150	-139	-155	-166	-270	-317	-164
Transfers from taxpayers (USD mn)	-52.2	-35.9	-52.6	-69.2	-48.6	-42.7	-36.0	-6.9
Budget revenues (-) (USD mn)	-57.7	-104	-72.5	-68.8	-102	-143	-128	-26.4
Total Support Estimate (%)	-0.9	1.0	1.1	1.1	0.7	3.7	5.4	3.5
Gross Domestic Product (USD mn)	976	1081	1221	1556	2076	2312	2830	3744
Total Single Commodity Transfers (USD mn)	-13.1	1.4	0.2	-4.3	-12.7	46	137	74
SCT (%)	-3.2	0.3	0.03	-0.6	-1.4	4.5	10.7	6.7
Share in Total PSE (%)	156	13	1	-26	-87	55	90	57

Source: Author's calculations.

Table A2. Wheat

WHEAT	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**
Excess feed cost (USD mn)	0	0	0	0	0	11.1	15.8	5.8
Producer NPC (ratio)	2.0	2.3	2.2	2.0	2.0	1.9	1.8	1.1
Producer NRP (%)	100.1	130	120	99.8	99.5	90.5	80.6	10.1
Consumer Support Estimate (USD mn)	-89.0	-114.8	-80.1	-69.3	-100.7	-113.5	-105.8	-16.9
Percentage CSE (%)	-49.6	-57.2	-55.3	-49.6	-49.8	-51.6	-50.6	-11.5
Consumer NPC (ratio)	1.99	2.33	2.24	1.99	1.99	2.31	2.39	1.18
Consumer NAC (ratio)	1.99	2.33	2.24	1.99	1.99	2.07	2.02	1.13
Consumer NRP (%)	99	133	124	99	99	107	102	13
PSE (USD mn)	36.2	47.2	43.2	45.3	60.4	58.4	56.9	9.0
PSE (%)	50	57	55	50	50	48	46	10
Producer NAC (ratio)	2.0	2.3	2.2	2.05	2.0	1.9	1.8	1.1
Producer Single Commodity Transfers (USD mn)	36.2	47.2	43.2	45.3	60.4	58.4	56.9	9.0
SCT (%)	49.6	57.2	55.3	49.9	49.9	47.8	45.7	10.2

Source: Author's calculations.

Table A3. Maize

MAIZE	2000	2001	2002	2003	2004	2005	2006	2007
Excess feed cost (USD mn)	-0.7	-0.03	-0.005	-0.1	-0.3	2.7	-0.3	-8.9
Producer NPC (ratio)	0.72	0.73	0.57	0.57	0.91	1.18	0.92	0.49
Producer NRP (%)	-28.0	-27.0	-43.0	-43.0	-9.0	18.0	-8.0	-51.0
Consumer Support Estimate (USD mn)	1.2	5.5	3.6	6.6	1.2	1.0	1.2	-1.0
CSE (%)	32.0	36.6	71.8	73.9	7.8	8.5	6.4	-12.5
Consumer NPC (ratio)	0.67	0.73	0.58	0.57	0.91	1.18	0.92	0.50
Consumer NRP (%)	-33	-27	-42	-43	-9	18	-8	-50
Consumer NAC (ratio)	0.76	0.73	0.58	0.58	0.93	0.92	0.94	1.14
PSE (USD mn)	-1.5	-1.6	-3.7	-6.9	-1.6	1.9	-1.6	-8.4
PSE (%)	-34.0	-36.1	-71.8	-73.9	-8.2	6.1	-6.8	-49.1
Producer NAC (ratio)	0.71	0.73	0.60	0.64	0.9	1.1	0.9	0.7
Producer Single Commodity Transfers (USD mn)	-1.5	-1.61	-3.7	-6.9	-1.6	1.9	-1.63	-8.4
SCT (%)	-34.0	-36.1	-71.8	-73.9	-8.2	6.1	-6.8	-49.1

Source: Author's calculations.

Table A4. Other Grains (rye, barley, oats)

OTHER GRAINS (RYE, BARLEY, OATS)	2000	2001	2002	2003	2004	2005	2006	2007
Excess feed cost (USD mn)	-0.04	-0.003	-0.10	-0.1	-0.01	-0.3	-0.2	-2.6
Producer NPC (ratio)	0.8	1.0	0.7	0.7	1.0	0.8	1.0	0.5
Producer NRP (%)	-20	0	-30	-30	0	-20	0	-50
Consumer Support Estimate (USD mn)	0.5	0.0	1.6	2.4	0.3	0.6	0.3	2.5
CSE (%)	26	2	47	41	3	10	3	39
Consumer NPC (ratio)	0.78	0.98	0.67	0.70	0.97	0.87	0.95	0.56
Consumer NRP (%)	-22	-2	-33	-30	-3	-13	-5	-44
Consumer NAC (ratio)	0.79	0.98	0.68	0.71	0.97	0.91	0.97	0.72
PSE (USD mn)	-0.6	-0.1	-1.8	-2.6	-0.3	-1.3	-0.3	-7.5
PSE (%)	-55.7	-4.5	-100	-157	-15.3	-11.5	-1.4	-46.6
Producer NAC (ratio)	0.6	1.0	0.5	0.4	0.92	0.94	1.0	0.7
Producer Single Commodity Transfers (USD mn)	-0.6	-0.1	-1.8	-2.6	-0.3	-1.3	-0.3	-7.5
SCT (%)	-56	-4	-100	-158	-15	-11	-1	-47

Source: Author's calculations.

Table A5. Cotton lint

COTTON	2000	2001	2002	2003	2004	2005	2006	2007
Producer NPC (ratio)	0.41	0.44	0.42	0.43	0.41	0.45	0.43	0.44
Producer NRP (%)	-59	-56	-58	-57	-59	-55	-57	-56
Consumer Support Estimate (USD mn)	9	36	14	16	26	8	12	21
CSE (%)	121.9	121.7	122.1	122.3	121.8	122.2	122.4	122.1
Consumer NPC (ratio)	0.44	0.45	0.43	0.42	0.44	0.43	0.45	0.41
Consumer NRP (%)	-56	-55	-57	-58	-56	-57	-55	-59
Consumer NAC (ratio)	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
PSE (USD mn)	-58	-73	-85	-123	-115	-90	-83	-88
PSE (%)	-122.1	-122.3	-122.2	-121.9	-122.1	-122.3	-122.0	-1224
Producer NAC (ratio)	0.447	0.449	0.451	0.452	0.454	0.44.8	0.447	0.451
Producer Single Commodity Transfers (USD mn)	-58	-73	-85	-123	-115	-90	-83	-88
SCT (%)	-121.9	-122.1	-122.3	-121.9	-122.3	-122.2	-121.9	-122.1

Source: Author's calculations

Table A6. Milk

MILK	2000	2001	2002	2003	2004	2005	2006	2007
Excess feed cost (USD mn)	-0.77	-0.36	0.29	2.67	-0.60	9.25	12.40	-5.47
Producer NPC (ratio)	-0.4	-2.2	-2.7	2.1	0.5	-2.6	0.79	-0.2
Producer NRP (%)	-140	-320	-370	110	-50	-360	-21	-120
Consumer Support Estimate (USD mn)	-49	-49	-45	-52	-52	-69	-85	-105
CSE (%)	-169	-111	-110	-89	-87	-105	-78	-150
Consumer NPC (ratio)	-1.4	-9.2	-9.7	9.1	7.5	-18.6	4.4	-2.0
Consumer NRP (%)	-0.4	-2.2	-2.7	2.1	0.5	-2.6	0.79	-0.2
Consumer NAC (ratio)	-140	-320	-370	110	-50	-360	-21	-120
PSE (USD mn)	50.0	49.5	44.7	49.5	52.8	60.3	73.5	110.9
PSE (%)	159	93	84	59.9	57.0	55.7	48.7	90.9
Producer NAC (ratio)	-1.7	14.3	6.3	2.5	2.3	2.3	1.9	11.0
Producer Single Commodity Transfers (USD mn)	50.0	49.5	44.7	49.5	52.8	60.3	73.5	110.9
SCT (%)	159.5	93.0	84.2	59.9	57.0	55.7	48.7	90.9

Source: Author's calculations.

Table A7. Beef and Veal

BEEF AND VEAL	2000	2001	2002	2003	2004	2005	2006	2007
Excess feed cost (USD mn)	-0.44	-0.20	0.167	1.53	-0.35	5.22	7.09	-3.13
Producer NPC (ratio)	0.65	1.02	1.10	1.43	1.43	1.43	1.43	1.43
Producer NRP (%)	-35	2	10	43.2	42.9	43.1	43.0	43.3
Consumer Support Estimate (USD mn)	21	-1	-5	-19	-25	-25	-27	-35
CSE (%)	53	-2	-9	-30	-30	-30	-30	-30
Consumer NPC (ratio)	0.65	1.02	1.10	1.431	1.43	1.43.1	1.43.2	1.432
Consumer NRP (%)	-35	2	10	43	43	43	43	43
Consumer NAC (ratio)	0.65	1.02	1.10	1.432	1.43	1.431	1.43	1.43
PSE (USD mn)	-7.3	0.6	2.2	11.7	18.5	13.1	11.7	28.6
PSE (%)	-50.1	2.8	8.1	26.6	30.8	21.8	19.0	34.1
Producer NAC (ratio)	0.7	1.0	1.1	1.4	1.41	1.3	1.2	1.5
Producer Single Commodity Transfers (USD mn)	-7.3	0.6	2.2	11.7	18.5	13.1	11.7	28.6
SCT (%)	-50.1	2.8	8.1	26.6	30.8	21.8	19.0	34.1

Source: Author's calculations.

Table A8. Rice milled equivalent

RICE	2000	2001	2002	2003	2004	2005	2006	2007
Producer NPC (ratio)	1.5	2.3	3.8	2.5	1.4	2.3	2.5	1.1
Producer NRP (%)	50	130	280	150	40	130	150	10
Consumer Support Estimate (USD mn)	-5	-13	-21	-18	-5	-13	-20	-1
CSE (%)	-28	-56	-73	-57	-26	-45	-55	-7
Consumer NPC (ratio)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Consumer NRP (%)	-99.1	-98.9	-99	-99.2	-98.7	-99	-98.8	-99.1
Consumer NAC	1.4	2.3	3.7	2.3	1.4	1.8	2.2	1.1
PSE (USD mn)	7.8	9.0	22.1	19.2	6.8	18.9	22.7	1.2
PSE (%)	29.2	57.1	73.4	58.0	27.2	45.5	55.8	8.2
Producer NAC (ratio)	1.4	2.3	3.8	2.4	1.4	1.8	2.3	1.1
Producer Single Commodity Transfers (USD mn)	7.3	8.7	21.4	18.5	6.4	18.1	21.9	1.0
SCT (%)	23	16	40	22	7	17	15	1

Source: Author's calculations.

Table A9. Mutton and Goat

MUTTON & GOAT	2000	2001	2002	2003	2004	2005	2006	2007
Excess feed cost (USD mn)	-0.476	-0.220	0.180	1.66	-0.37	5.73	7.68	-3.39
Producer NPC (ratio)	0.8	0.9	0.8	0.9	0.9	0.9	1.2	1.5
Producer NRP (%)	-20	-10	-20	-10	-10	-10	20	50
Consumer Support Estimate (USD mn)	5.0	3.0	5.1	4.0	3.2	6.5	-13.7	-38.9
CSE (%)	29.7	14.3	19.0	9.3	5.7	9.7	-17.0	-34.7
Consumer NPC (ratio)	0.8	0.9	0.8	0.9	0.9	0.9	1.2	1.5
Consumer NRP (%)	-20	-10	-20	-10	-10	-10	20	50
Consumer NAC (ratio)	0.8	0.9	0.8	0.9	0.9	0.9	1.2	1.5
PSE (USD mn)	-4.5	-2.8	-5.3	-5.6	-2.8	-12.2	6.1	42.3
PSE (%)	-27	-13	-20	-13	-5	-18	8	38
Producer NAC (ratio)	0.8	0.9	0.8	0.9	1.0	0.8	1.1	1.6
Producer Single Commodity Transfers (USD mn)	-4.5	-2.8	-5.3	-5.6	-2.79	-12.24	6.07	42.35
SCT (%)	-26.9	-13.3	-19.7	-13.2	-5.0	-18.2	7.5	37.8

Source: Author's calculations.

Table A10. Poultry

POULTRY	2000	2001	2002	2003	2004	2005	2006	2007
Excess feed cost (USD mn)	-0.06	-0.03	0.02	0.19	-0.04	0.66	0.89	-0.39
Producer NPC (ratio)	2.0	1.9	2.0	1.9	1.8	2.0	2.1	2.3
Producer NRP (%)	100	90	100	90	80	100	110	130
Consumer Support Estimate (USD mn)	-0.6	-0.5	-1.1	-4.3	-7.4	-15.0	-6.4	-10.5
CSE (%)	-49	-47	-50	-48	-44	-49	-53	-57
Consumer NPC (ratio)	2.0	1.9	2.0	1.9	1.8	2.0	2.1	2.3
Consumer NRP (%)	100	90	100	90	80	100	110	130
Consumer NAC (ratio)	2.0	1.9	2.0	1.9	1.8	2.0	2.1	2.3
PSE (USD mn)	0.18	0.15	0.09	-0.08	0.29	-0.36	0.25	1.69
PSE (%)	72	57	41	-32	51	-58	11	74
Producer NAC (ratio)	3.5	2.3	1.7	0.8	2.1	0.6	1.1	3.9
Producer Single Commodity Transfers (USD mn)	0.18	0.15	0.09	-0.08	0.29	-0.36	0.25	1.69
SCT (%)	72	57	41	-32	51	-58	11	74

Source: Author's calculations.

Table A11. Eggs

EGGS	2000	2001	2002	2003	2004	2005	2006	2007
Producer NPC (ratio)	1.01	1.03	2.02	2.03	2.01	1.99	2.04	1.97
Producer NRP (%)	0	0	99.6	100.2	99.9	100.3	99.8	100.1
Consumer Support Estimate (USD mn)	-0.4	-1.1	-1.9	-3.8	-5.5	-8.2	-6.8	-5.4
CSE (%)	-18	-29	-41	-49	-52	-57	-48	-40
Consumer NPC (ratio)	1	1	2	2	2	2	2	2
Consumer NRP (%)	0	0	100	100	100	100	100	100
Consumer NAC (ratio)	1	1	2	2	2	2	2	2
PSE (USD mn)	0.3	0.8	1.4	2.2	3.4	5.2	4.5	3.7
PSE (%)	18	29	41	49	52	57	48	40
Producer NAC (ratio)	1.2	1.4	1.7	1.9	2.1	2.3	1.9	1.7
Producer Single Commodity Transfers (USD mn)	0.3	0.8	1.4	2.2	3.4	5.2	4.5	3.7
SCT (%)	18	29	41	49	52	57	48	40

Source: Author's calculations.

Table A12. Potato

POTATO	2000	2001	2002	2003	2004	2005	2006	2007
Excess feed cost (USD mn)	-1.3	-0.8	0.4	6.1	0.6	7.5	17.9	0.5
Producer NPC (ratio)	0.7	0.8	1.0	1.7	1.1	1.6	1.9	1.1
Producer NRP (%)	-30	-20	0	70	10	60	90	10
Consumer Support Estimate (USD mn)	10.2	4.8	-1.5	-15.0	-1.6	-16.7	-39.9	-2.6
CSE (%)	49.3	19.7	-4.6	-40.5	-5.9	-38.6	-47.2	-5.3
Consumer NPC (ratio)	0.7	0.8	1.0	1.7	1.1	1.6	1.9	1.1
Consumer NRP (%)	-30	-20	0	70	10	60	90	10
Consumer NAC (ratio)	0.7	0.8	1.0	1.7	1.1	1.6	1.9	1.1
PSE (USD mn)	-12.0	-7.9	2.5	30.4	3.6	37.0	86.6	8.7
PSE (%)	-44	-18	4	34	6	32	39	6
Producer NAC (ratio)	0.7	0.8	1.0	1.5	1.1	1.5	1.6	1.1
Producer Single Commodity Transfers (USD mn)	-12.0	-7.9	2.5	30.4	3.6	37.0	86.6	8.7
SCT (%)	-44	-18	4	33.9	5.6	32.3	39.2	5.9

Source: Author's calculations.

Table A13. Onion

ONION	2000	2001	2002	2003	2004	2005	2006	2007
Producer NPC (ratio)	0.83	0.84	0.83	0.83	0.84	0.83	0.83	0.83
Producer NRP (%)	-17.1	-16	-17.2	-17.1	-16	-17.2	-17.4	-17.1
Consumer Support Estimate (USD mn)	1.9	3.9	1.8	3.1	2.5	4.4	7.4	6.7
CSE (%)	20.2	19.7	20.6	19.8	19.6	20.4	19.9	20.1
Consumer NPC (ratio)	0.81	0.84	0.79	0.8	0.82	0.78	0.83	0.77
Consumer NRP (%)	-19.9	-20.1	-19.9	-19.8	-19.8	-19.9	-19.8	-20.0
Consumer NAC (ratio)	0.83	0.77	0.78	0.83	0.81	0.79	0.81	0.83
PSE (USD mn)	-2.4	-4.5	-2.3	-4.4	-3.4	-5.4	-9.2	-12.7
PSE (%)	-20.1	-20.4	-21.0	-20.2	-20.1	-20.3	-20.4	-20.2
Producer NAC (ratio)	0.79	0.81	0.83	0.77	0.82	0.78	0.83	0.81
Producer Single Commodity Transfers (USD mn)	-2.4	-4.5	-2.3	-4.4	-3.4	-5.4	-9.2	-12.7
SCT (%)	-20.2	-19.7	-20.6	-19.8	-19.6	-20.4	-19.9	-20.1

Source: Author's calculations.

Table A14. Tomato

TOMATO	2000	2001	2002	2003	2004	2005	2006	2007
Producer NPC (ratio)	0.83	0.77	0.78	0.83	0.81	0.79	0.81	0.83
Producer NRP (%)	-19.8	-19.9	-20.1	-20.3	-20.4	-20.2	-20.1	-19.9
Consumer Support Estimate (USD mn)	5.9	6.3	6.8	12.6	11.4	18.8	21.2	12.3
CSE (%)	19.9	20.1	19.9	19.8	19.8	19.9	19.8	20.0
Consumer NPC (ratio)	0.79	0.81	0.83	0.77	0.82	0.78	0.83	0.81
Consumer NRP (%)	-20.0	-19.8	-20.0	-19.9	-19.8	-19.8	-19.8	-18.1
Consumer NAC (ratio)	0.83	0.77	0.78	0.83	0.81	0.79	0.81	0.83
PSE (USD mn)	-6.4	-6.9	-7.0	-12.9	-15.2	-23.0	-21.6	-21.5
PSE (%)	19.8	19.9	20.01	20.1	20.4	20.0	19.9	20.1
Producer NAC (ratio)	0.81	0.84	0.79	0.8	0.82	0.78	0.83	0.77
Producer Single Commodity Transfers (USD mn)	-6.4	-6.9	-7.0	-12.9	-15.2	-23.0	-21.6	-21.5
SCT (%)	-19.9	-20.1	-19.9	-19.8	-19.8	-19.9	-19.8	-20.0

Source: Author's calculations.

Table A15. Lemon

LEMON	2000	2001	2002	2003	2004	2005	2006	2007
Producer NPC (ratio)	0.83	0.77	0.78	0.83	0.81	0.79	0.81	0.83
Producer NRP (%)	-20.0	-19.8	-20.0	-19.9	-19.8	-19.8	-19.8	-18.1
Consumer Support Estimate (USD mn)	0.3	0.3	0.1	0.1	0.2	0.2	0.2	0.3
CSE (%)	19.8	19.9	20.01	20.1	20.4	20.0	19.9	20.1
Consumer NPC (ratio)	0.81	0.84	0.79	0.8	0.82	0.78	0.83	0.77
Consumer NRP (%)	-20.0	-19.8	-20.0	-19.9	-19.8	-19.8	-19.8	-18.1
Consumer NAC (ratio)	0.81	0.84	0.79	0.8	0.82	0.78	0.83	0.77
PSE (USD mn)	-0.3	-0.3	-0.2	-0.1	-0.2	-0.2	-0.2	-0.3
PSE (%)	-19.8	-19.9	-20.1	-20.3	-20.4	-20.2	-20.1	-19.9
Producer NAC (ratio)	0.79	0.81	0.83	0.77	0.82	0.78	0.83	0.81
Producer Single Commodity Transfers (USD mn)	-0.3	-0.3	-0.2	-0.1	-0.2	-0.2	-0.2	-0.3
SCT (%)	-19.8	-19.9	-20.0	-20.0	-20.0	-20.0	-19.9	-20.0

Source: Author's calculations.

Table A16. Grape

GRAPE	2000	2001	2002	2003	2004	2005	2006	2007
Producer NPC (ratio)	0.81	0.84	0.79	0.8	0.82	0.78	0.83	0.77
Producer NRP (%)	-19.8	-20.1	-20.4	-20.3	-20.2	-19.7	-20.1	-19.9
Consumer Support Estimate (USD mn)	6.1	3.4	3.8	1.2	8.6	8.6	10.9	7.0
CSE (%)	20.0	19.8	20.0	19.9	19.9	20.01	20.0	20.03
Consumer NPC (ratio)	0.83	0.77	0.78	0.83	0.81	0.79	0.81	0.83
Consumer NRP (%)	-19.8	-19.9	-20.1	-20.3	-20.4	-20.2	-20.1	-19.9
Consumer NAC (ratio)	0.81	0.84	0.79	0.8	0.82	0.78	0.83	0.77
PSE (USD mn)	-8.3	-6.2	-6.2	-3.1	-12.4	-12.4	-15.7	-9.4
PSE (%)	-20.0	-19.8	-20.0	-19.9	-19.8	-19.8	-19.8	-18.1
Producer NAC (ratio)	0.79	0.81	0.83	0.77	0.82	0.78	0.83	0.81
Producer Single Commodity Transfers (USD mn)	-8.3	-6.2	-6.2	-3.1	-12.4	-12.4	-15.7	-9.4
SCT (%)	-20.0	-19.8	-20.0	-19.9	-19.8	-19.8	-19.8	-18.1

Source: Author's calculations.

Table A17. Apple

APPLE	2000	2001	2002	2003	2004	2005	2006	2007
Excess feed cost (USD mn)	-0.1	-0.3	-0.02	-0.04	-2.1	-1.7	-6.7	-3.3
Producer NPC (ratio)	0.81	0.84	0.79	0.77	0.82	0.84	0.78	0.83
Producer NRP (%)	-19.7	-20.1	-20.3	-19.8	-19.6	-20.4	-20.1	-20.2
Consumer Support Estimate (USD mn)	0.7	0.2	0.3	0.4	1.6	1.5	2.0	1.3
CSE (%)	20.5	20.1	20.0	19.9	20.0	19.9	20.0	19.9
Consumer NPC (ratio)	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Consumer NRP (%)	-19.8	-20.1	-20.3	-20.4	-20.1	-19.9	-20.1	-19.7
Consumer NAC (ratio)	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
PSE (USD mn)	-5.4	-3.5	-4.9	-4.7	-7.9	-9.6	-9.1	-4.9
PSE (%)	-20	-18	-20	-20	-16	-17	-11	-11
Producer NAC (ratio)	0.79	0.81	0.78	0.82	0.92	0.91	0.9	0.93
Producer Single Commodity Transfers (USD mn)	-5.4	-3.5	-4.9	-4.7	-7.9	-9.6	-9.1	-4.9
SCT (%)	-20.2	-18.4	-19.9	-19.8	-15.6	-16.8	-11.4	-10.6

Source: Author's calculations.

Table A18. Other commodities*

OTHER COMMODITIES	2000	2001	2002	2003	2004	2005	2006	2007
Producer NPC (ratio)	1.01	1.03	1.04	1.02	0.99	1.02	0.97	1.01
Producer NRP (%)	0	0	0	0	0	0	0	0
PSE (USD mn)	-0.4	0.8	0.8	1.0	0.9	6.8	16.4	20.7
PSE (%)	-2.1	2.2	2.5	2.2	1.5	8.4	12.7	12.5
Producer NAC (ratio)	0.97	1.01	1.03	1.02	1.04	1.12	1.14	1.13
Producer Single Commodity Transfers (USD mn)	-0.4	0.8	0.8	1.0	0.9	6.8	16.4	20.7
SCT (%)	-2.1	2.2	2.5	2.2	1.5	8.4	12.7	12.5

Source: Author's calculations.

Notes: *Other commodities include: Apricots, Cabbages and other brassicas, Carrots and turnips, Cucumbers and gherkins, Fruit Fresh Nes, Garlic, Honey-natural, Nuts-nes, Peaches and nectarines, Peas-dry, Plums and sloes, Pig meat, Safflower seed, Sesame seed, Sunflower seed, Tobacco-unmanufactured, Vegetables fresh nes, Watermelons.

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Field Performance of Quatity Protein Maize With Zinc and Magnesium Fertilizers in the Sub-Humid Savanna of Nigeria

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Abstract

Field experiments were conducted for three years (2006 – 2008) in Samaru (11°11'N, 7°38'E) 686m above sea level in Nigeria. The objective was to test the response of two Quality Protein Maize (QPM) varieties (SAMMAZ-14 and SAMMAZ-11) to four levels each of Zinc and Magnesium (0, 1.25, 2.5 and 5.0 kg) using their carbonates. The experiments were arranged in all possible factorial combinations and laid out as randomised complete block design (RCBD) and replicated three times. The two varieties tested did not differ significantly in all the parameters evaluated except for the number of days to 50% tasselling. Grain yield ranged between 1.9 – 2.0 t/ha when averaged over both years which was quite below the 5.0 t/ha potential. Zinc application had no significant influence on most characters evaluated except total dry matter per hectare in 2006 when the application of 1.25 kg 2n/ha produced the highest TDM. Grain yield per hectare remained unchanged with changes in Zinc rate. Magnesium application influenced grain yield on 2008 only when 1.25 kg Mg/ha increased yield compared with plots with 5.0kg mg/ha. When averaged over the three years, Mg application did not significantly influence grain yield, but increased protein yield. Grain yield correlated positively and significantly with leaf area index (r = 0.13**), plant height (r = 0.26*), TDM (r = 0.21**) and protein yield (r = 0.97**). Protein content of grain remained unchanged with changes in Zinc and Magnesium rates at 8%

Keywords: Maize, nutrients, protein

1. Introduction

Maize (*Zea mays* L.) is an important food crop in Nigeria, consumed in different forms, either roasted, boiled, made into a meal and eaten with soup or stew, or into a light poridge. It is also the basic ingredient of animal feed, particularly poultry feed. Many Nigerian diets are based on starchy staples such as sorghum (*Sorghum bicolor* L. (Moench), millet (*Pennisetum typhoides* Stapf and Huhbard), rice (*Oryza sutiva* L.) and root crops mostly yams (*Dioscorea spp*), cassava (*Manihot esculentus* Crantz) and sweet potatoes (*Ipomea batatas* L.). Protein consumption is low, particularly animal protein because of scarcity and cost. This obvious malnutrition calls for increase in protein intake to alleviate the obvious debilities casued by inadequate protein intake.

The development of Quality Protein Maize (QPM) with high content of two of the essential amino acids Lysine and Tryptophan, lacking in ordinary maize will help to ameliorate the protein – deficient diets. Badu – Apraku and Fontem – Lun (2010) reported that protein of QPM has 90% of the relative value (RV) of milk compared with 40% for ordinary maize. Akuanmo – Boateng (2003) observed that children fed QPM had signifcantly fewer sick days and grew healthier than children fed ordinary maize. Therefore, growing and consuming QPM may alleviate some of the nutritional problems of the population with predominantly starchy diets.

The easiest approach to increase QPM consumption is to increase the production. The sub-humid Savanna which is the centre of maize production in Nigeria is characterised by poor soil, low in N and P, the major nutrients for maize growth. Trials in the savanna with fertilizers have proved that application of 100 – 120 kg N, 26.4 kg P and 48 kg K per hectare was sufficient for high maize yield (Goldworthy, 1967; Jones, 1973; Balasubramanian et al., 1978; Ologunde & Ogunlela, 1984; Mbagwu, 1990; Chiezey & Shamsudeen, 2004; Jaliya, 2012). Virtually all fertilizer recommendations are based on the three major elements, N, P and K without consideration for secondary and micronutrients which are removed during crop harvesting and never replenished. This may lead to nutrient imbalance (Lombin, 1983). Trials with micronutrients in savanna are few and in some areas indicate low levels of magnesium and zinc (Osiname, 1972; Lombin, 1983). Iwuafor et al. (1991) noted that maize responded positively

to zinc application where the extractable Zn fell below 1.0 Mg Kg^{-1*} Anonymous (1989) recommended 5kg Zn per hectare for hybrid maize. Chiezey (1999) observed that soybean did not respond to zinc and magnesium applications in the northern Guinea Savanna and that grain yield declined with the application of zinc and magnesium. Cropping system has inensified in the savanna and most crop residues are removed from farms for feeding livestock, tatching, fencing and firewood, thereby reducing the levels of these nutrients without any effort at replenishment. This study was, therefore, undertaken to evaluate the response of two QPM varieties to different levels of Zn and Mg in the sub-humid savanna ecological zone of Nigeria.

2. Materials and Methods

Table 1. Rainfall data at 10 days interval in Samaru 2006 – 2009

Month	Rainfall (mm)		
	2006	2007	2008
April			
1-10	-	14.4	20.4
11-20	-	-	-
21-30	1.8	44.3	52.2
May			
1-10	47.0	96.0	23.8
11-20	83.4	4.2	32.2
21-31	72.1	69.2	52.9
June			
1-10	4.4	60.0	68.1
11-20	34.7	39.4	27.9
21-30	87.4	107.8	15.7
July			
1-10	17.8	27.4	63.4
11-20	171.8	32.4	18.2
21-31	42.6	168.8	148.2
August			
1-10	74.8	51.1	122.6
11-20	43.5	177.0	124.8
21-31	102.8	146.2	105.8
September			
1-10	76.2	16.4	114.0
11-20	126.1	3.5	58.0
21-30	78.1	12.0	45.8
Oct.			
1-10	21.8	4.9	67.2
11-20	6.7	3.4	21.8
21-31	-	-	-
Nov			
1-10	-	-	-
TOTAL	1093.0	1080.4	1183.0

Field experiments were conducted during the rainy seasons of 2006, 2007 and 2008 at the Institute for Agricultural Research (IAR), Ahmadu Bello University, Samaru, Zaria (11°11′N7°38′E), 686m above sea level. Samaru is located in the northern Guinea Savanna with an annual rainfall of 11,00 mm distributed between April and October (Kowal & Knabe, 1972). The objective was to test the response of two QPM varieties (SAMMAZ-14 and SAMMAZ-11) to four levels each of zinc and magnesium (0, 1.25, 2.5 and 5.0 kg/ha) using their carbonates in all possible factorial combinations, using a randomised complete block design with three replications.

Rainfall data were collected for the three years from the IAR metrological station (Table 1). Soil samples were randomly collected and later bulked from the sites before fertilizer application and analysed for physico-chemical properties (Table 2). The QPM varieties were released by IAR, Samaru. Both are medium maturing (100 – 120 days) and have yield potential of 5 t/ha (Ado et al., 2009). Both are resistant to striga (*Striga hermontheca*) which is endemic in the zone. Plantings were done end of June each year. Two seeds of each variety were planted on 75cm row ridges at intra-row spacing of 25cm. The crop was thinned to one plant per stand at three weeks after sowing to give a plant density of 53,333 pl/ha.

Zn and Mg using the carbonates at 0, 1.25, 2.5 and 5.0 kg/ha in all possible factorial combinations were side-banded after planting. In order to facilitate handling of the nutrients, each fertilizer rate was mixed with one kilogram of pure river sand which was thoroughly washed and rinsed with de-ionised water. A basal dose of 120 kg N, 26.4 kg P and 49 kg K per hectare were applied at planting using the compound fertilizer 20:10:10. The gross plot was 6m x 6m (36 m^2) and the net plot was 3 m x 6 m (18 m^2).

Five plants per plot were sampled to determine the effects of the treatments on leaf area index (LAI), flag leaf area (FLA), Ear leaf area (ELA), number of days to 50% tasselling, plant height at harvesting, total dry matter (TDM) per hectare at harvesting, grain weight per plant, grain yield per hectare, 100-seed weight and protein yield per hectare. N content of seed was analysed for the calculation of protein content and yield.

The data collected were analysed for individual years and the years combined using the analysis of variance (Snedecor & Cochran, 1967). The means were compared using the multple range test (Duncan, 1955).

Table 2. Physical and chemical properties of the top soil (0-30 cm) of the experimental fields for three years 2006, 2007 and 2008

Composition	Year		
	2006	2007	2008
Physical characteristics			
Clay (g/kg)	180	80	80
Silt (g/kg)	160	80	100
Sand (g/kg)	660	840	820
Textural class	Sandy loam	Sandy loam	Sandy loam
Chemical characteristics			
pH (H_20)	5.2	5.0	5.4
pH (Ca CL_2)	4.8	4.8	5.0
Organic carbon (g/kg)	0.20	0.15	0.15
Available P (mg/kg)	1.8	2.2	2.0
Total N g/kg	1.1	1.5	1.2
Exchangeable bases			
Ca (Cmol/kg Soil)	2.0	1.5	1.4
Mg “	0.2	0.3	0.3
K “	0.2	0.2	0.2
CEC “	4.1	3.8	4.0
Micronutrient			
Zn (ppm)	1.9	1.0	0.9

3. Results

The results were the obtained for three years, 2006 – 2008. Rainfall varied among the three years both in total amount and distribution and this was reflected in the gain yield (Table 1). Highest grain yield was obtained in 2006, probably because of a better rainfall distribution.

Protein content of the grain was not influenced by any of the treatments and ranged between 7.8 and 8.2% with a mean of 8.0%. Leaf area index (LAI), flag leaf area and ear leaf area at 50% silking were determined at 50% silking (Table 3,). The two varieties did not significantly differ in any of these three attributes. Similarly, neither zinc not magnesium application bad any significant effect on these parameters.

Table 3. Leaf area index, flag leaf area and ear leaf area of two Quality protein maize varieties as influenced by different rates of Zn and Mg fertilizers in Samaru (mean of three years) 2006 – 2008

Treatment	LAI	Flag leaf area (CM^2)	Ear leaf area (CM^2)
Variety			
SAMMAZ - 14	2.7	38.8	78.1
SAMMAZ - 11	2.7	39.0	78.1
Mean	2.7	38.9	78.1
SED	NS	NS	NS
Zn rate (kg/ha)			
0	2.7	39.3	78.1
1.25	2.7	39.7	78.1
2.50	2.7	38.5	78.6
5.0	2.7	38.1	77.7
SE+	NS		NS
Mg rate (kg/ha)			
0	2.7	37.8	78.5
1.25	2.7	39.1	78.1
2.50	2.7	39.1	78.2
5.0	2.7	39.6	77.8
SE+	NS	NS	NS

Means within same treatment group and column followed by same letter(s) are not significantly different at 5% level of probability using DMRT.

Number of days to 50% tasselling differed between the two varieties (Table 4). Sammaz-11 tasselled later than Sammaz-14. Neither Zinc nor Magnesium application had significant influence on the number of days to 50% tasselling.

Plant height at harvesting was similar for both varieties (Table 4). Application of 1.25 kg Zn/ha increased plant height by 4.1% compared with plots without Zn. Increasing the level of Zn above 1.25 kg/ha did not influence plant height. Application of Mg depressed plant height in 2007 only, but averaged over the three years Mg application did not significantly influence plant height.

Table 4. Number of days to 50% tasselling and plant height at harvesting of two Quality Protein Maize varieties as influenced by different rates of Zn and Mg fertilizers in Samaru (Mean of three years) 2006 - 2008

Treatment	Number of days to 50% tasselling	Plant height at harvesting (cm)
Variety		
SAMMAZ - 14	58.2b	208.9
SAMMAZ - 11	58.8a	206.3
Mean	58.5	207.6
SED	0.13	NS
Zn rate (kg/ha)		
0	58.3	202.46
1.25	58.4	210.7a
2.50	58.5	209.0ab
5.0	58.7	208.3ab
SE+	NS	2.40
Mg rate (kg/ha)		
0	58.3	206.5
1.25	58.3	210.8
2.50	58.5	206.3
5.0	58.5	206.7
SE+	NS	NS

Means within same treatment group and column followed by same letter(s) are not significantly different at 5% level of probability using DMRT.

Table 5. Total dry matter at harvesting per plant are of Quality Protein Maize varieties with varying levels of Zn and Mg fertilizers in Samaru (Mean of three years) 2006 – 2008

Treatment	Total dry matter (kg/ha)			
Variety	2006	2007	2008	Mean
SAMMAZ - 14	3041.6	2510.4	3770.7	3107.6
SAMMAZ - 11	3037.0	2073.9	3617.9	2909.6
Mean	3039.3	2292.2	3694.3	3008.6
SED	NS	NS	NS	NS
Zn rate (kg/ha)				
0	3157.4ab	2199.0	3800.8	3052.4
1.25	3240.7a	2472.0	3657.2	3123.3
2.50	2995.3ab	2530.1	3569.3	3031.6
5.0	2763.8b	1967.5	3749.9	2827.1
SE+	150.99	NS	NS	NS
Mg rate (kg/ha)				
0	3166.6	2366.0	2560.1	3030.9
1.25	3023.1	2358.8	4009.0	3130.3
2.50	2847.1	2101.6	3578.6	2842.4
5.0	3120.3	2342.5	3629.5	3030.8
SE+	NS	NS	NS	NS

Means within same treatment group and column followed by same letter(s) are not significantly different at 5% level of probability using DMRT.

Table 6. Grain weight per plant and 100 – grain weight of Quality Protein Maize as influenced by different rates of Zn and Mg fertilizers in Samaru (Mean of three years) 2006 – 2008

Treatment Variety	100 – grain wt (g)	grain wt/plant (g)
SAMMAZ - 14	21.5	108.0
SAMMAZ - 11	21.6	113.2
Mean	21.6	110.6
SED	NS	NS
Zn rate (kg/ha)		
0	21.5	115.8a
1.25	21.0	102.1b
2.50	21.8	112.4ab
5.0	21.7	112.0ab
SE+	NS	3.94
Mg rate (kg/ha)		
0	20.9	106.0ab
1.25	21.5	117.2a
2.50	21.3	104.3b
5.0	22.3	114.9ab
SE+	NS	NS

Means within same treatment group and column followed by same letter(s) are not significantly different at 5% level of probability using DMRT.

Total dry matter at harvesting was similar for both varieties (Table 5). The application of 5.0 kg Zn/ha depressed total dry matter by 14.7% compared with plots where 1.25 kg/ha of Zn was applied. Total dry matter was similar with 0, 1.25 and 2.5 kg Zn/ha. Magnesium application did not influence maize TDM/ha in any of the years and when averaged over the three years. Grain weight per plant were similar for both varieties (Table 6). Grain weight per plant ranged between 108.0 – 113.2 g. Zn application influenced grain weight per plant. Zinc application reduced grain weight when averaged over the three years. Magnesium application also influenced grain weight per plant. The application of 1.25 kg Mg/ha increased grain weight by 20.1% in 2007 when compared with plots without Mg. When averaged over the years, increasing Mg rate from 1.25 to 2.5 kg Mg/ha reduced grain weight

per plant by 11.0%. No interactions were significant. 100-seed weight was not influenced by any of the treatments (Table 6). 100-seed weight ranged between 20 and 22 g.

Table 7. Grain yield per hectare of Quality Protein Maize varieties as influenced by Zn and Mg fertilizers in Samaru (Mean of three years) 2006 – 2008

Treatment	Grain yield (kg/ha)			
Variety	2006	2007	2008	Mean
SAMMAZ - 14	2432.8	1518.0	1782.3	1911.1
SAMMAZ - 11	2609.6	1586.2	1872.9	2022.9
Mean	2521.2	1552.1	1827.6	1967.0
SED	NS	NS	NS	NS
Zn rate (kg/ha)				
0	2456.7	1350.3	1718.7	1841.9
1.25	2723.9	1521.5	1679.6	1975.0
2.50	2463.1	1583.9	1957.0	2001.3
5.0	2441.1	1752.7	1955.1	2049.6
SE+	NS	NS	NS	NS
Mg rate (kg/ha)				
0	2582.7	1414.9	1764.5ab	1920.7
1.25	2521.4	1846.6	2097.9a	2155.9
2.50	2590.8	1390.2	1624.5b	1868.5
5.0	2389.8	1554.7	1823.5ab	1922.9
SE+	NS	NS	NS	NS

Means in same treatment group and column followed by similar letter(s) are not significantly different at 5% level of probability using DMRT.

Table 8. Protein yield per hectare of QPM varieties as influenced by different rates of Zn and Mg fertilizers in Samaru (Mean of three years) 2006 – 2008

Treatment	Protein yield (kg/ha)			
Variety	2006	2007	2008	Mean
SAMMAZ - 14	194.7	121.9	140.5	152.3
SAMMAZ - 11	209.1	126.9	151.4	162.5
Mean	201.9	124.4	146.0	157.4
SED	NS	NS	NS	NS
Zn rate (kg/ha)				
0	196.5	108.9	140.5	148.7
1.25	217.9	121.7	135.3	158.3
2.50	197.1	126.7	162.2	162.0
5.0	196.0	140.2	145.9	160.0
SE+	NS	NS	NS	NS
Mg rate (kg/ha)				
0	206.6	113.2	140.5	153.4ab
1.25	201.8	148.8	168.0	172.8a
2.50	207.9	111.2	127.3	148.8b
5.0	191.2	124.4	141.8	154.5ab
SE+	NS	NS	NS	7.86

Means in same treatment group and column followed by similar letter(s) are not significantly different at 5% level of probability using DMRT.

Grain yield per hectare did not differ significantly between the two QPM varieties (Table 7). Grain yield was highest in 2006 compared with other years. Application of Zn did not influence grain yield in any of the years and when averaged over the three years. The application of 1.25 kg Mg/ha increased grain yield by 18.8% compared with plots without Mg but sifnificantly reduced grain yield by 22.5% by increasing the Mg level to 5.0 kg Mg/ha. When averaged over the three years, application of Mg had no significant influence on grain yield of QPM.

Protein yield followed same pattern with grain yield, being similar for the two varieties and not changing with Zn application (Table 8). Magnesium application influenced protein yield when averaged over the three years. Increasing Magnesium rate from 1.25 to 2.5 kg Mg/ha reduced protein yield by 13.9%.

Grain yield per hectare correlated positively and significantly with TDM (r=21**), LA (r =0.13**), plant height (r =0.26*) and protein yield (r = 0.97*).

4. Discussion

Table 1 shows the total rainfall and distribution at 10 days interval in the three years of the study. Grain yield was better in 2006 because of a more even distribution of rain. In 2007, rainfall declined during grain formation while in 2008, exersive rainfall during tasselling could have resulted in pollen wash out, both factors resulting in reduction in number of grains per cob. Maize cobs in 2006 were completely filled with grains resulting in higher yields.

The soils of the experimental fields reflected the characteristics of the savanna, low pH, low levels of N and P but high in K (Table 2). The micronutrient status of the soils ranged from medium to low. Grain yield per hectare responded positively to Magnesium in 2008 because of the low level of the nutrient in the soil (< 1.0 cmol/ha of soil).

The varieties did not significantly differ from each other in most of the parameters measured. Leaf area index, flag leaf area, ear leaf area, plant height at harvesting, total dry matter per hectare, 100-grain weight and grain yield per hectare were similar in both varieties. Protein yield per hectare were similar in both varieties. The similarities in these two QPM varieties could be attributed to their common genetic background. Both were bred from same parents. They only differed in their number of days to 50% tasselling which was not much, 58.2 and 58.8 days. This is statistically possible but in practice, these two could be assumed to tassel same period.

The application of Zinc did not influence most of the parameters such as LAI, and ear leaf area probably because of the moderate to high levels of the nutrient in the soil. Zinc application increased plant height when averaged over the three years. With 1.25kg Zn per hectare, plant height increased significantly compared with plots without Zn. Total dry matter per hectare was increased by the application of 1.25 kg Zn/ha probably because of the moderate level in the soil. This was not reflected in the grain yield. Total protein yield which correlated positively with grain yield (r = 0.9**) was also not influenced significantly by zinc application.

The application of Magnesium influenced grain weight per plant and protein yield per hectare. Although grain yield increased with the application of 1.25 kg Mg/ha, the increase was not significant probably because of the moderate levels of Mg in the soil. Soil analysis showed that the level of Mg in the soil was moderate and therefore spectacular responses were not obtained. Increasing the levels of both nutrients above 1.25kg/ha tended to reduce grain yield, implying that the nutrient may be reaching toxicity levels. Protein yield increased with Mg application because the nutrient is essential for protein synthesis (Mengel & Kirkby, 1987). The positive and significant correlations between grain yield and some growth parameters indicate their contributions to yield. Leaf area index which is a measure of total ground cover reflects the photosynthetic machinery for capturing and conversion of solar energy into assimilates for eventual translocation to grain. Therefore, increase in LAI up to the optimum eventually increased grain yield. Similarly, high TDM also translated into high grain yield as more photosynthates were eventually translocated to the sink, and in this instance the grain. Plant height has been shown also to increase grain yield in some instances. Tall plants have more or larger leaves that may promote high photosynthetic activity, therefore more assimilates for grain filling.

The study has shown that Magnesium and Zinc levels may still be adequate in the savanna. However, there is a need for constant monitoring of these nutrients with the intersification of crop production and utilisation of crop refuse for other purposes instead of returning same to the soil for soil amendment. Mg not exceeding 1.25 kgMg/ha may be applied to increase protein yield per hectare as deficiency of Mg has been shown to reduce protein synthesis.

References

Ado, S. G., Showemimo, F. A., Falaki, A. M., Alabi, S. O., & Abdullahi, U. S. (2009). *Proposal for the registration and release of Quality Protein Maize variety SAMMAZ-14 (Obatanpa)*. Department of Plant Science, Institute for Agricultural Research, Ahmadu Bello University, Zaria.

Akuamoa-Boateng, A. (2002). Quality protein maize infant feeding trials in Ghana. *Ashanti, Ghana: Ghana Health Service*.

Anonymous. (1989). Fertilizer use and management for crops in Nigeria (Series No. 2).

Badu-Apraku, B. and Fontem-Lun, A. (2010). Grain yield response of normal and Quality Protein Maize cultivars in stress and non-stress environments. *Agronomy Journal, 102*, 381-394. http://dx.doi.org/10.2134/agronj2009.0229

Balasubramanian, V., Nnadi, L. A., & Mokwunye, A. U. (1978). *Fertilizing sole crop maize for high yields*. Samaru Miscellanous paper, 76, Institute for Agricultural Research, Ahmadu Bello University, Zaria, Nigeria.

Chiezey, U. F. (1999). Effects of magnesium and zinc Fertilizers on the yield and yield components of soybean (Glycine max (L.) Merrill) in the northern Guinea Savanna of Nigeria. *Tropical Oilseeds Journal, 4*, 1-8.

Chiezey, U. F., & Shamsndeen, R. (2004). Popcorn response to plant population dynamics and nitrogen levels in the northern Guinea Savanna zone of Nigeria. *Journal of Agriculture and environment for international developments, 98*(3), 211-222.

Duncan, D. B. (1955). Multiple ranges and Multiple 'F' test. *Biometrics, 11*, 1-42. http://dx.doi.org/10.2307/3001478

Goldsworthy, P. R. (1967). Response of cereals to fertilizers in northern Nigeria. *Experimental Agriculture, 3*, 263-273. http://dx.doi.org/10.1017/S0014479700022158

Iwuafor, E. N. O., Chude, V. O., & Amapu, I. (1991). *Response of maize (Zea mays L.) to various rates of zinc fertilizer in the semi-arid zone of Nigeria*. Paper presented at SAFGRAD Network Joint Workshop, Niamey, Niger Republic 8-14 March, pp 14.

Jaliya, M. M., Falaki, A. M., & Mahmud, M. (2004). *Effects of sowing date and NPK fertilizer on growth and yield of quality protein Maize in northern Guinea savanna of Nigeria*. Unpublished M.Sc. Thesis, Department of Agronomy, Ahmadu Bello University, Zaria.

Jones, M. J. (1973). Time of application of nitrogen fertilizer to maize at Samaru, Nigeria. *Experimental Agriculture, 9*(2), 113-120. http://dx.doi.org/10.1017/S0014479700005573

Kowal, J. M., & Knabe, D. T. (1972). *An Agroclimatological Atlas of the nrothern states of Nigeria*. Zaria: Ahmadu Bello University Press.

Lombin, G. (1983). Evaluation of the micronutrient fertility of Nigeria's savanna soils. *Soil Science, 136*, 42-47. http://dx.doi.org/10.1097/00010694-198307000-00006

Mbagwu, J. S. C. (1990). Maize response to N fertilizer under two tillage and mulch treatments. *Journal of Science of Food and Agriculture, 52*(3), 365-376. http://dx.doi.org/10.1002/jsfa.2740520309

Mengel, K., & Kirkby, E. A. (1987). *Principles of plant nutrition* (No. Ed. 4). International Potash Institute.

Ologunde, O. O., & Ogunlela, V. B. (1984). Relationship of Plant density and nitrogen fertilization to maize performance in the Nigerian southern Guinea savanna. *Samaru Journal of Agricultural Research, 2*, 99-109.

Osiname, O. A. (1972). *Soil test for available copper and zinc in soils of western Nigeria*. Ph.D Thesis, University of Wisconsin, Madison.

Snodecor, G. W., & Cochran, W. G. (1962). Statistical Methods (6th ed.). Iowa State University Press, Ames.

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Effects of *Spirulina Platensis* Algae on Growth Performance, Antioxidative Status and Blood Metabolites in Fattening Lambs

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Abstract

The objective of this study was to investigate the effects of *Spirulina platensis* powder (SPP) supplementation on growth performance, antioxidative status and blood metabolites in fattening lambs. Ten healthy lambs (46.5 ± 1.06 kg BW) were randomly assigned to one of two treatments (5 lambs per treatment) and received either no supplementation or supplemented with SPP at a rate of 1 g/10 kg BW/day. The feeding experiment was conducted for 35 days with body weight recorded and blood samples collected on days 0, 17 and 35 of the experiment. The paired Student's *t*-test for means was used for statistical analysis. The results showed that SPP supplementation improved final live body weight, daily live weight gain, feed intake and feed conversion ratio, compared to the control group ($P<0.05$). Also, haemoglobin, total white blood cell count, serum globulin, vitamin A and reduced glutathione were higher ($P<0.05$), while the aspartate amino transferase, alanine amino transferase, cholesterol, glucose and serum malondialdehyde levels were lower ($P<0.05$) in SPP supplemented group compared with the control. In conclusion, the findings of the present study clearly demonstrate that the SPP could be incorporated in fattening lambs diets as an antioxidant, immune-stimulant and growth promoter feed additive.

Keywords: fattening lambs, spirulina platensis, performance

1. Introduction

Intensive livestock production systems may be associated with multiple stressful incidents that negatively impact immune response and animal performance. The high metabolic rate during intensive feeding is accompanied by an increased production of free radicals, and any imbalance between production of these molecules and their safe disposal may culminate in oxidative stress, which can damage cells and tissues (Miller et al., 1993; Lykkesfeldt & Svendsen, 2007). Therefore, under oxidative stress conditions, there is an increased demand for antioxidants to reduce the deleterious effects of free radicals on the immune system (Carroll & Forsberg, 2007). Interestingly, feeding natural, rather than synthetic, antioxidant could be advantageous to animal welfare and consumer safety (Call et al., 2008; Makkar et al., 2007). The blue-green algae, *Spirulina platensis*, have been considered as a suitable natural antioxidant and immune-stimulant to humans and animals with fewer side effects and more cost effectiveness than synthetic products (Abdel-Daim et al., 2013; Belay, 2002; Khan et al., 2005). Recently, the impact of dietary Spirulina supplementation on animal health and productivity have been reported (Holman & Malau-Aduli, 2012). However, studies on use of *Spirulina platensis* as a feed additive in ruminant feeding are still quite limited. To our knowledge, moreover, no studies have been undertaken on its usage with high concentrate diets. Therefore, the objective of this study was to test whether *Spirulina platensis* had beneficial effects when included in a high concentrate fed to fattening lambs.

2. Materials and Methods

2.1 Animals and Diets

Ten healthy lambs (46.5 ± 1.06 kg BW) were randomly allocated into two groups of 5 lambs each. Control lambs (CON) received a diet without *Spirulina*, whereas in treated group, *Spirulina platensis* powder (SPP) was incorporated daily in the concentrate of each lamb at a rate of 1 g/10 kg BW.day. The SPP was obtained from a commercial retailer in a powdered form (HERBAFORCE LTD, UNITED KINGDOM). The basal diet was formulated to meet the lamb's nutrient requirements in order to balance the body weight gain at a rate of 0.3 kg/day (NRC, 1985). The composition of the basal diet is presented in Table 1. Diet was offered twice a day in the morning and evening with free access to water. The trial period was five weeks, with a pre-trial period of one week for adaptation to diets and facilities. Animals were weighed on days 0, 17 and 35 of experiment, after fasting for twelve hours before the morning feedings.

Table 1. Ingredients and calculated chemical composition of experimental diet

Item	%
Ingredient	
Berseem hay	14.65
Wheat straw	4.88
Corn grains	57.74
Cotton seed meal	13.07
Soybean meal	7.35
Limestone	0.79
Salt	0.49
Sodium bicarbonate	0.49
Ammonium chloride	0.24
Vitamin-mineral premix	0.29
Calculated chemical composition	
DM	88.73
CP	14.17
TDN	76.17
NDF	22.76
ADF	14.92
EE	4.14
Ca	0.73
P	0.37

2.2 Sampling and Analysis

Feed and refusals were recorded daily. Body weight was recorded, and blood samples were collected from the jugular vein for each lamb on days 0, 17 and 35 of the experiment. A portion of each blood sample was used for white blood cell (WBC) counts and to measure hemoglobin (Hb) (Linne & Ringsrud, 1992) and reduced glutathione (GSH) (Beutler et al., 1963) concentrations using commercial kits (Biodiagnostic, Egypt). Remained blood samples were then centrifuged at 3000 x g for 20 min. The obtained sera were separated and stored at -20°C until assayed spectrophotometrically (Spekol 11, Carl Zeiss Jena, Germany) for total protein (TP) and albumin concentrations as well as biochemical parameters, including serum enzymes activities as aspartate amino transferase (AST) and alanine amino transferase (ALT), blood urea nitrogen (BUN), triglyceride (TG) and cholesterol (CHO) levels (Young, 2001) according to the instructions of manufacturer (Endpoint kits from Diamond Diagnostics, Egypt). Globulin was calculated by subtracting albumin values from total serum protein. The albumin/globulin (A/G) ratio was also determined. Serum malondialdehyde (MDA) concentration was measured according to (Ohkawa et al., 1979) and the instructions of manufacturer (Biodiagnostic, Egypt). Retinol concentration in plasma was determined by modifying the method described by Suzuki and Katoh (1990). In brief, 50 ml of ethanol and 150 ml of hexane were added to 50 ml of plasma, and the hexane phase was recovered after

40-min mixing and 10-min centrifugation at 6500 x *g*. Retinol concentrations were calculated based on the absorbance of hexane extracts at 325 nm and 453 nm using the equations described (Suzuki & Katoh, 1990).

3. Statistical Analysis

All data are presented as mean ± SEM. Mean comparisons were performed using Wilcoxon-Mann-Whitney test and considering $P<0.05$ as level of significance.

4. Results and Discussion

4.1 Growth Performance

The growth performance of lambs is presented in Table 2. SPP supplementation to the diets of fatting lambs significantly increased ($P<0.05$) the final live body weights, daily live weight gain and feed intake compared with the control group. Moreover, the feed conversion ratio decreased ($P<0.05$) for SPP fed lambs compared with the control group. These results are consistent with previous reports that *Spirulina* supplementation induced greater live weights in cattle (Kulpys et al., 2009) and sheep (Holman et al., 2012). The better growth performance in lambs fed SPP supplemented diet may be a subsequence of high nutrient density of *Spirulina* as well as stimulation of the secretion of extracellular enzymes by the gut microflora (Tovar-Ramírez et al., 2002). *Spirulina* contains several nutrients, especially vitamins, minerals, essential fatty acids, amino acids and other nutrients that may promote faster growth (Gershwin & Belay, 2008). Furthermore, *Spirulina* has previously been shown to decrease rumen protein degradation and produce changes in bacterial community composition with a subsequent increase the efficiency of rumen microbial crude protein production in steers (Panjaitan et al., 2010).

Table 2. Effect of Spirulina platensis powder on growth performance of fattening lambs

Item	Treatments	
	CON	SPP
Initial weight (kg)	46.0 ± 0.50	47.0 ± 2.25
Final weight (kg)	50.4 ± 0.92^{a}	55.3 ± 2.10^{b}
Daily weight gain (kg/day)	0.127 ± 0.012^{a}	0.236 ± 0.015^{b}
Daily feed intake (kg/day)	1.64 ± 0.002^{a}	1.72 ± 0.010^{b}
Feed conversion ratio	13.2 ± 1.38^{a}	7.35 ± 0.41^{b}

CON = Control. SPP = Spirulina platensis powder.

Mean values in the same row with different superscripts differ ($P<0.05$).

4.2 Haematology

The Hb concentration and total WBC count of fattening lambs during the experiment are shown in Table 3. The Hb concentration and total WBC count were higher ($P<0.05$) in SPP fed group compared to the control. Leucocytes play an important role in non-specific or innate immunity and their count can be considered as an indicator of relatively lower disease susceptibility (Matanović et al., 2007). The increased WBC production may be due to the presence of phycocyanin and polysaccharides components in *Spirulina* (Zhang, 1994). The WBC counts and Hb concentration were increased with supplementation of polysaccharide of *Spirulina* in mice (at a dose of 30-60 mg/kg) and dogs (at a dose of 12 mg/kg) (Zhang et al., 2001). Similarly, *Spirulina* was found to enhance immunity in chickens fed 10 g/kg of *Spirulina platensis* (Qureshi et al., 1996) and fish (Watanuki et al., 2006).

Table 3. Effect of Spirulina platensis powder on Hb concentration (g/dl) and total WBC counts (per cmm) of fattening lambs

Item	1st Sampling (0 day)		2nd Sampling (17^{th} day)		3rd sampling (35^{th} day)	
	CON	SPP	CON	SPP	CON	SPP
Hb	12.0 ± 0.55	13.5 ± 0.60	10.7 ± 0.50^{a}	12.9 ± 0.27^{b}	12.2 ± 0.20^{a}	13.0 ± 0.09^{b}
WBC	11383 ± 1490	10883 ± 1482	8217 ± 784	9783 ± 683	7600 ± 891^{a}	9900 ± 908^{b}

CON = Control. SPP = Spirulina platensis powder.

Hb = Haemoglobin. WBC = White blood cells.

Mean values in the same row with different superscripts differ ($P<0.05$).

4.3 Biochemical Parameters

Plasma TP, albumin, globulin, A/G ratio, AST, ALT, BUN, TG, CHO and glucose concentrations are presented in Table 4. Significant differences (P<0.05) in the serum globulin, AST, ALT, CHO, TG, BUN and glucose were found between the treatment groups (Table 4). There was no significant difference in the concentrations of TP, albumen and A/G ratio between treatment groups. Supplementation with SPP induced significant elevation (P<0.05) in plasma globulin while significantly reduced (P<0.05) the AST, ALT, CHO and blood glucose concentrations. Furthermore, there was a significant increase in BUN with an unexpected increase in TG concentrations in lambs fed SPP supplemented diets.

Table 4. Effect of Spirulina platensis powder on biochemical parameters of fattening lambs

Item	1st Sampling (0 day)		2nd Sampling (17th day)		3rd sampling (35th day)	
	CON	SPP	CON	SPP	CON	SPP
TP (g/dl)	4.34 ± 0.21	4.52 ± 0.05	4.71 ± 0.09	4.96 ± 0.16	5.37 ± 0.11	5.80 ± 0.28
Albumin (g/dl)	2.08 ± 0.05	2.16 ± 0.12	2.50 ± 0.15	2.33 ± 0.21	2.99 ± 0.17	2.86 ± 0.17
Globulin (g/dl)	2.36 ± 0.10	2.29 ± 0.08	2.26 ± 0.06[a]	2.62 ± 0.09[b]	2.37 ± 0.09[a]	2.93 ± 0.21[b]
A/G ratio	1.02 ± 0.05	0.91 ± 0.08	1.09 ± 0.10	0.89 ± 0.11	1.27 ± 0.12	0.98 ± 0.09
AST (U/ml)	51.2 ± 2.00	45.3 ± 4.91	50.0 ± 2.55[a]	40.5 ± 1.50[b]	85.7 ± 14.5[a]	39.3 ± 1.86[b]
ALT (U/ml)	21.3 ± 0.67	22.3 ± 1.45	30.7 ± 2.73[a]	20.3 ± 0.95[b]	65.3 ± 12.8[a]	25.7 ± 4.84[b]
Urea (mg/dl)	19.3 ± 0.39	23.8 ± 3.37	25.3 ± 3.75[a]	40.0 ± 1.42[b]	19.4 ± 2.05	25.7 ± 1.92
Triglycerides (mg/dl)	78.4 ± 3.94	76.5 ± 2.42	68.3 ± 4.41[a]	80.1± 1.34[b]	79.4 ± 1.0	73.6 ± 5.54
Cholesterol (mg/dl)	45.9 ± 2.46	50.1 ± 6.70	62.8 ± 1.73[a]	49.6 ± 2.48[b]	81.2 ± 10.4	77.2 ± 12.5
Glucose (mg/dl)	40.2 ± 1.13	35.4 ± 5.78	68.65 ± 2.85 [a]	52.72 ± 6.15 [b]	54.9 ± 10.6	58.33 ± 2.90

CON = Control. SPP = Spirulina platensis powder.

TP = Total protein. A/G ratio = Albumin/globulin ratio. AST = Aspartate amino transferase. ALT = Alanine amino transferase.

Mean values in the same row with different superscripts differ (P< 0.05).

In this study, higher globulin concentration was found in the SPP supplemented group. The increased concentrations of plasma globulin may be related to the high protein contents in *Spirulina* (Gershwin & Belay, 2008). Increased plasma globulin levels are thought to be associated with a stronger innate response in lambs and indicate higher resistance (Matanović et al., 2007). This result is supported by increased total leukocytic count in SPP fed group.

Supplementation with SPP reduced the total plasma CHO. This is in consistent with previous findings in rats (Kato et al., 1984) hamsters (Riss et al., 2007) rabbits (Cheong et al., 2010) and human (Ruitang & Chow, 2010). Although the mechanism by which the SPP reduces CHO has not been fully examined, the hypocholesterolemic actions of SPP involve reducing plasma and liver CHO levels due to the increase in lipoprotein lipase and hepatic triglyceride lipase activity (Karkos et al., 2008), inhibition of both jejunal CHO absorption and ileal bile acid resorption (Nagaoka et al., 2005), in addition to modifying lipoproteins metabolism (decrease of low density lipoprotein and increase of high density lipoprotein; Torres-duran et al., 2007). Alternatively, the hypocholesterolemic activity of *Spirulina* is related to the large amount of cystine found in the C-phycocyanin protein of *Spirulina* (Nagaoka et al., 2005). A negative correlation was reported between the blood CHO concentrations and the level of cystine in dietary protein in rats fed a high CHO diet (Sugiyama et al., 1986).

Spirulina has been reported to have a hypolipidemic effect due to the C-phycocyanin protein which inhibits the pancreatic lipase activity in a dose-dependent manner (Torres-Duran et al., 2007). Controversy, the unexpected increase in the TG in SPP fed lambs in this study may imply that the *Spirulina* dose might be not enough to affect plasma TG or the supplementation period was not long enough for *Spirulina* to exert its lipid-modulating properties. Ishimi et al. (2006) reported that *Spirulina* may affect plasma lipids only in hyperlipidemic conditions. Further trails are required to characterize the efficacy of *Spirulina* in lowering blood lipid in ruminants.

The activity of AST and ALT are indicators of hepatotoxicity (Azab et al., 2013). In the present study, treatment with *Spirulina* showed a significant decrease in AST and ALT indicating that *Spirulina* may play a protective role against liver dysfunctions (Bhattacharyya & Mehta, 2012).

4.4 Antioxidative Status

Vitamin A, blood GSH and serum MDA concentrations are presented in Table 5. There was a significant increase ($P<0.05$) in vitamin A and GSH and a significant decrease ($P<0.05$) in MDA levels in SPP supplemented diets compared with control. Increased vitamin A and GSH and decreased MDA concentrations are indicators of improved oxidative defense of animal tissues (Celli, 2010). These results are in an agreement with previous reports stated that treatment with *Spirulina* could reduce oxidative stress with a consequent decrease in lipid peroxidation (Reddy et al., 2004; Riss et al., 2007). Reddy et al. (2004) suggested that *Spirulina* supplementation resulted in significantly higher activities of superoxide dismutase and catalase in the erythrocytes with a concomitant increase in reduced tripeptide glutathione content in broiler chickens. The antioxidative effect of *Spirulina* is related to several active ingredients, notably phycocyanin, polysaccharides, α-tocopherol and ß-carotene that have potent antioxidant activities working, individually or in synergy, directly on free radicals (Riss et al., 2007). Gershwin and Belay (2008) reported that the antioxidant activity of phycocyanin is about 20 times more efficient than vitamin C. In addition, *Spirulina* contains superoxide dismutase that acts indirectly by slowing down the rate of oxygen radical generating reactions (Belay, 2002).

In conclusion from the above results it can be concluded that SPP increased body weight gain, total WBC count, plasma globulin, vitamin A and reduced glutathione concentration while decreased liver enzymes activities, cholesterol, glucose and plasma malondialdehyde concentration. The potential application of *Spirulina* in fattening lambs diet as antioxidants to protect against free radicals cellular damage from stress, to enhance growth, and as an immunomodulator is worth exploring. Further experiments with implementation of different levels of *Spirulina* with higher replications and varying feeding practices are worthwhile to evaluate the nutritional value of *Spirulina* more accurately and precisely.

Table 5. Effect of Spirulina platensis powder on Vitamin A, GSH and MDA concentrations of fattening lambs

Item	1st Sampling (0 day)		2nd Sampling (17th day)		3rd sampling (35th day)	
	CON	SPP	CON	SPP	CON	SPP
Vitamin A (μg/dL)	60.9± 2.5	61.5± 1.8	69.0± 5.1^{a}	106.3± 3.8^{b}	69.1± 0.35^{a}	71.4± 0.84^{b}
Blood GSH (μM/L)	2.8± 0.64	3.0± 0.74	5.2± 0.75^{a}	9.6± 0.73^{b}	8.9± 0.69^{b}	14.2± 1.7^{b}
Serum MDA (nM/ml)	4.8± 0.87	4.1± 0.93	12.4± 5.9	8.0± 2.6	99.1± 27.4^{a}	15.7± 3.6

CON = Control. SPP = Spirulina platensis powder.

GSH = Reduced glutathione. MDA = Reduced malondialdhyde.

Mean values in the same row with different superscripts differ ($P<0.05$).

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References

Abdel-Daim, M. M., Abuzead, S. M. M., & Halawa, S. M. (2013) Protective Role of Spirulina platensis against Acute Deltamethrin-Induced Toxicity in Rats. *PLoS ONE, 8*(9), e72991. http://dx.doi.org/10.1371/journal.pone.0072991

Azab, S., Abdel-Daim, M., & Eldahshan, O., (2013) Phytochemical, cytotoxic, hepatoprotective and antioxidant properties of Delonix regia leaves extract. *Med Chem Res, 22*, 4269-4277. http://dx.doi.org/10.1007/s00044-012-0420-4

Belay, A. (2002). The potential application of Spirulina (Arthrospira) as a nutritional and therapeutic supplement in health management, Review. *J. Am. Nutraceut. Assoc., 5*, 27-48.

Beutler, E., Duran, O., & Kelly, M. B. (1963). Improved method for the determination of blood glutathione. *J. Lab. Clin. Med., 61*, 882-888.

Bhattacharyya, S., & Mehta, P. (2012). The hepatoprotective potential of Spirulina and vitamin C supplementation in cisplatin toxicity. *Food Funct, 3*, 164-169. http://dx.doi.org/10.1039/c1fo10172b

Call, D. R., Davis, M. A., & Sawant, A. A. (2008). Antimicrobial resistance in beef and dairy cattle production. *Anim. Health Res. Rev., 9*, 159-167. http://dx.doi.org/10.1017/S1466252308001515

Carroll, J. A., & Forsberg, N. E. (2007). Influence of stress and nutrition on cattle immunity. *Vet. Clin. North Am. Food Anim. Pract., 23*, 105-149. http://dx.doi.org/10.1016/j.cvfa.2007.01.003

Celli, P. (2010). The role of oxidative stress in small ruminants' health and production. *Rev. Bras. Zootec., 39*, 348-363. http://dx.doi.org/10.1590/S1516-35982010001300038

Cheong, S. H., Kim, M. Y., Sok, D. E., Hwang, S. Y., Kim, J. H., Kim, H. R., … Kim, M. R. (2010). Spirulina prevents atheroschelerosis by reducing hypercholesterolemia in rabbits fed a high-cholesterol diet. *J. Nutr. Sci. Vitaminol., 56*, 34-40. http://dx.doi.org/10.3177/jnsv.56.34

Gershwin, M. E., & Belay, A. (2008). *Spirulina in human nutrition and health*. CRC Press- Boca Raton, FL, USA.

Holman, B. W. B., Kashani, A., & Malau-Aduli, A. E. O. (2012). Growth and body conformation responses of genetically divergent Australian sheep to Spirulina(Arthrospira platensis) supplementation. *Am. J. Exp. Agr., 2*, 160-173.

Holman, B. W., & Malau-Aduli, A. E. O. (2012). Spirulina as a livestock supplement and animal feed. *J Anim Physiol Anim Nutr., 97*(4), 615-623. http://dx.doi.org/10.1111/j.1439-0396.2012.01328.x

Ishimi, Y., Sugiyama, F., Ezaki, J., Fujioka, M., & Wu, J. (2006). Effects of Spirulina, a blue-green alga, on bone metabolism in overiectomized rats and hindlimb-unloaded mice. *Biosci. Biotechnol. Biochem., 70*, 363-368. http://dx.doi.org/10.1271/bbb.70.363

Karkos, P. D., Leong, S. C., Karkos, C. D., Siraji, N., & Assimkapoulos, D. A. (2008). Review of spirulina in clinical practice: Evidence-Based human applications. *Evid. base Compl. Alternative Med., 14*, 1-4.

Kato, T., Takemoto, K., Katayama, H., & Kuwabara, Y. (1984). Effects of *Spirulina (Spirulina platensis)* on dietary hypercholesterolemia in rats. *J. Jpn. Soc. Nutr. Food Sci., 37*, 323-332. http://dx.doi.org/10.4327/jsnfs.37.323

Khan. Z., Bhadouria, P., & Bisen, P. S. (2005). Nutritional and therapeutic potential of Spirulina. *Curr. Pharm. Biotechnol., 6*, 373-379. http://dx.doi.org/10.2174/138920105774370607

Kulpys, J., Paulauskas, E., Pilipavicius, V., & Stankevicius, R. (2009). Influence of cyanobacteria Arthrospira (Spirulina) platensis biomass additive towards the body condition of lactation cows and biochemical milk indexes. *Agron. Res., 7*, 823-835.

Linne, J., & Ringsrud, K. (1992). *Basic techniques in clinical laboratory science* (3rd ed.). Mosby Year Book. http://dx.doi.org/10.1016/j.tvjl.2006.06.005

Lykkesfeldt, J., & Svendsen, O. (2007). Oxidants and antioxidants in disease: Oxidative stress in farm animals. *Vet. J., 173*, 502-511.

Makkar, H. P. S., Francis, G., & Becker, K. (2007). Bioactivity of phytochemicals in some lesser-known plants and their effects and potential applications in livestock and aquaculture production systems. *Animal, 1*, 1371-1391. http://dx.doi.org/10.1017/S1751731107000298

Matanović, K., Severin, K., Martinkovic, F., Šimpraga, M., Janicki, Z., & Barišic, J. (2007). Hematological and biochemical changes in organically farmed sheep naturally infected with Fasciola hepatica. *J. Parasitol. Res., 101*, 1657-1661.

Miller, J. K., Brzezinska-Slebodzinska, E., & Madsen, F. C. (1993). Oxidative stress, antioxidants, and animal function. *J. Dairy Sci., 76*, 2812-2823. http://dx.doi.org/10.3168/jds.S0022-0302(93)77620-1

Nagaoka, S., Shimizu, K., & Kaneko, H. (2005). A novel protein C-phycocyanin plays a crucial role in the hypocholesterolemic action of Spirulina platensis concentrate in rats. *J. Nutr., 135*, 2425-2430.

National Research Council. (1985). *Nutrient requirement of sheep* (6th ed.). Washington, DC: National Academy Press.

Ohkawa, H., Ohishi, W., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem, 95*, 351-358. http://dx.doi.org/10.1016/0003-2697(79)90738-3

Panjaitan, T., Quigley, S. P., McLennan, S. R., & Poppi, D. P. (2010). Effect of the concentration of Spirulina (Spirulina platensis) algae in the drinking water on water intake by cattle and the proportion of algae bypassing the rumen. *Anim. Prod. Sci., 50*, 405-409. http://dx.doi.org/10.1071/AN09194

Qureshi, M. A., Garlich, J. D., & Kidd, M. T. (1996). Dietary Spirulina platensis enhances humoral and cell-mediated immune functions in chickens. Immunopharmacol. *Immunotoxicol, 18*, 465-476. http://dx.doi.org/10.3109/08923979609052748

Reddy, B. S., Yuvaraj, N. Babitha, V. Ramnath, V., Philomina, P. T., & Sabu, M. C. (2004). Antioxidant and hypolipidemic effects of Spirulina and natural carotenoids in broiler chicken. *Indian Vet. J., 81*, 383-386.

Riss, J., ecord´e, K. D., & Sutra, T. (2007). Phycobiliprotein C-phycocyanin from Spirulina platensis is powerfully responsible for reducing oxidative stress and NADPH oxidase expression induced by an atherogenic diet in hamsters. *J. Agric. Food Chem., 55*, 7962-7967. http://dx.doi.org/10.1021/jf070529g

Ruitang, D., & Chow, T. J. (2010). Hypolipidemic, antioxidant, and antiinflammatory activities of microalgae Spirulina. Cardiovasc. *Therapeutics, 28*, 33-45. http://dx.doi.org/10.1111/j.1755-5922.2010.00200.x

Sugiyama, K., Ohkawa, S., & Muramatsu, K. (1986). Relationship between amino acid composition of diet and plasma cholesterol level in growing rats fed a high cholesterol diet. *J. Nutr. Sci. Vitaminol., 32*, 413-423. http://dx.doi.org/10.3177/jnsv.32.413

Suzuki, J., & Katoh, N. (1990). A simple and cheap method for measuring serum vitamin A in cattle using only a spectrophotometer. *Jpn. J. Vet. Sci., 52*, 1281-1283. http://dx.doi.org/10.1292/jvms1939.52.1281

Torres-Durán, P. V., Ferreira-Hermosillo, A., & Juárez-Oropeza, M. A. (2007). Antihyperlipemic and antihypertensive effects of Spirulina maxima in an open sample of Mexican population: a preliminary report. *Lipids Health Dis., 6*, 33. http://dx.doi.org/10.1186/1476-511X-6-33

Tovar-Ramírez, D., Zambonino, J., Cahu, C., Gatesoupe, F. J., Vázquez-Juárez, R., & Lésel, R. (2002). Effect of live yeast incorporation in compound diet on digestive enzyme activity in sea bass (Dicentrarchus labrax) larvae. *Aquaculture, 204*, 113-123. http://dx.doi.org/10.1016/S0044-8486(01)00650-0

Watanuki, H., Ota, K., Malina, A. S., Kato, T., & Sakai, M. (2006). Immunostimulant effects of dietary Spirulina platensis on carp, Cyprinus carpio. *Aquaculture, 258*, 157-163. http://dx.doi.org/10.1016/j.aquaculture.2006.05.003

Young, D. S. (2001). Effects of disease on clinical laboratory tests (4th ed.). *Am. Assoc. Clin. Chem., 1504*, 82-106.

Zhang, C. (1994). *The effects of polysaccharide and phycocyanin from Spirulina platensis variety on peripheral blood and hematopoietic system of bone marrow in mice*. Second Asia Pacific conference on Alga Biotechnology.

Zhang, H. Q., Lin, A. P., & Sun, Y. (2001). Chemo and radio protective effects of polysaccharide of spirulina platensis on haematopoietic stem cells in dog and mice. *Acta. Pharmacologica. Sinica, 22*, 1121-1124.

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Observations on Zambia's Crop Monitoring and Early Warning Systems

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Abstract

A good early warning system is one that provides timely planning information to a diverse set of stakeholders. While policy makers need very concise messages for quick decisions, aid and development agencies need very specific and detailed information which can help them in programming at grass-roots level. This paper reviews Zambia's crop monitoring and early warning systems and suggests practical ways to improve its efficiency and effectiveness, taking advantage of existing and potential synergistic and institutional opportunities.

Keywords: early warning systems, crop monitoring, crop production forecasts, Zambia

1. Introduction

Impacts of disasters on society often are huge and tend to be exacerbated by society's inability to fully adapt their livelihoods and frameworks of development to the environment around them. Inability to fully anticipate and deal with such crises can be partly attributed to the paucity of early warning information. It is argued that the combined effect of inadequate information, inadequate response mechanisms, and policy weaknesses account for frequent food shortages in much of sub-Saharan Africa. This is so even though the precursors are largely the same - adverse weather.

In much of southern Africa, existing early warning information systems often are inadequate and not timely enough. The observed levels of vulnerability to weather-related shocks are also very high and tend to be exacerbated by a number of underlying factors, including poor access to basic inputs such as seed and fertilizer; loss of livestock, draught power and other production inputs; the devastating effects of HIV/AIDS on the households; and inadequate extension services. Food shortages are frequent and, quite often, very serious.

The need for a quicker and cost-effective season and crop monitoring system is fully recognized by the governments and their cooperating partners. Donor-supported efforts in early warning systems of ministries of agriculture, such as Zambia's FAO-supported Crop Monitoring Survey Project (CMSP), have helped fill some of the information gaps. However, it is recognized that such arrangements are of limited value unless they are part of and facilitate strengthening of existing institutions.

These and other such initiatives present a unique opportunity to draw lessons for building national and regional capacities to develop effective early warning systems. This paper summarizes the experiences associated with past and existing early warning initiatives with emphasis on lessons for improvement. In the rest of the paper, we first review conceptually the need for an effective early warning system (Section 2), followed by a summary of the existing crop monitoring systems in Zambia (Section 3) and suggestions to improve existing systems (Section 4). Section 5 presents some concluding remarks and recommendations.

2. The Need for an Effective Early Warning System

An effective early warning system needs to have four inter-related elements (Figure 1): i) Continuous monitoring of the precursors (or indicators), which is critical for understanding the risks from both the hazards and people's vulnerabilities; ii) forecasting of a probable event, and, if the probability is high enough; iii) appropriate measures

need to be taken to warn all the relevant stakeholders; and iv) an effective and highly collaborative response mechanism by the various stakeholders.

As Figure 1 illustrates, continuous monitoring (Phase I) and forecasting (Phase II) are continuous processes that are supposed to be carried out throughout the relevant period (December through May) regardless of the character of the agricultural season. If the forecast indicates a normal season (Answer is 'No' in Phase II), then the first two phases are all that the early warning system (EWS) will do. The important thing to bear in mind is that an effective EWS is not a reactive process but one that is continuous and designed to support preparedness. Continuous monitoring and measurement of the precursors and vulnerability trends and dynamics will make it possible for the bad seasons to be identified early enough for contingency plans and stakeholder response mechanisms to take effect in a timely manner. The key word in Phase IV (Figure 1) is *anticipated*, made possible only if Phases I and II are comprehensive enough. In general, an effective EWS requires substantial amounts of resources and a strong technical base (human capital), both arguably requiring serious attention in the Zambian crop monitoring system (Mukhala & Kwendakwema, 2005). At no stage is this more the case than in the execution of phases I and II.

For an agriculture-related EWS, precursors would include weather-related hazards (rainfall pattern, floods, drought, etc) and agricultural and livelihood vulnerability indicators (land area planted to crops, access to inputs, livestock asset base, and pest and disease incidences, sources of income, etc). The diversity of these elements identifies the need for a multi-disciplinary and multi-sectoral approach, taking into account the relative strengths of the various departments and organizations (Masdar, 2004; Mukhala & Kwendakwema, 2005; Tembo, 2006).

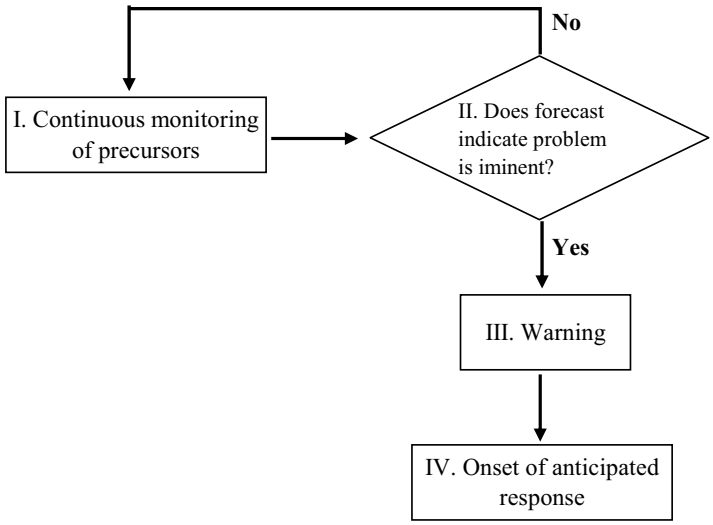

Figure 1. Phases and elements of an effective early warning system

Source: Adapted from De Leon, Bogardi, Dannenmann & Baster (2006).

3. Materials and Methods

This study relied on desk review and stakeholder consultations, involving partners at national, provincial and district levels. These stakeholder consultations were done within Lusaka; through visits to selected districts and provincial centres (Mongu, Senanga, Chipata, Mambwe, Livingstone, and Kazungula); and two stakeholder workshops held in Lusaka. A checklist was used to guide the stakeholder interviews. Emphasis was placed, among other things, on assessing the usefulness of the existing crop monitoring systems, how they can be revised to make them even more relevant to the needs of the stakeholders, and how best to fit the Crop Monitoring System (CMS) with other existing information systems, such as the crop forecast survey (CFS), National Vulnerability Assessment Committee (NVAC) and Agromet models. Perceptions about the reliability of the estimates provided by the district agricultural offices, especially those on the area planted, were also discerned.

Several documents were reviewed in the process, including dekadal crop weather bulletins from the Zambia Meteorological Department (ZMD), previous CMSP monthly reports, policy briefs by other organizations such as USAID's FEWS NET, the Food Security Research Project (FSRP), and several other publications on early warning systems in Zambia (for example Masdar, 2004).

4. Existing Crop Monitoring Systems

In Zambia, crop monitoring and forecasting has traditionally been the responsibility of the ministry of agriculture. Prior to 1990 the ministry performed this function exclusively through crop monitoring surveys (CMS) implemented by its field staff in the districts and camps. Since 1990, the ministry has been implementing two parallel monitoring systems: district-level CMS, as before, and through crop forecast surveys (CFS). Unlike CMS, implementation of the CFS is largely contracted out to the Central Statistical Office (CSO) (Note 1). The CFS is also based on probability sampling (see Megill, 2000; 2004) and is designed to produce valid production forecasts at national level.

However, at least three constraints render the CFS inadequate for early warning and intervention programming: i) inherent delays do not leave much time for planning and reaction, ii) sample size is not large enough to produce reliable estimates at district or lower levels (Note 2), and iii) as a one-time annual activity, it is useful only as a source of the final production forecasts (Note 3). Owing to these limitations, among other things, district offices have continued and/or have re-initiated their own monthly crop monitoring systems though less comprehensive and on much smaller budgets.

In recent years, district-level CMS activities have received support under the overall umbrella of the National Early Warning Unit (NEWU) facilitated by donor funding through the FAO/DfID crop monitoring survey project (Food and Agriculture Organization [FAO]/Government of the Republic of Zambia[GRZ], 2006). One advantage of a district-based monitoring system is that it can be inexpensive – as it uses staff who are based within the districts and camps – and is more useful for generating disaggregated data and qualitative information suitable for district-level planning (Note 4). Also, by collecting data at several points during the season (on a monthly basis), the CMS provides the means to monitor the season as it progresses. However, in practice, district-level crop monitoring is also not constraint-free. In the rest of this section, we highlight the key features of the CMS system with the view to identifying areas that need improvement.

4.1 Agricultural Inputs Monitoring

The existing CMS system collects district-level information on input prices and availability from the Department of Marketing (DoM). Types and quantities of seeds and fertilizers, and numbers of farmers in the district are also monitored. The District Agricultural Coordinators (DACOs) are generally confident with the figures obtained from government programmes, whereas activities of private traders and non-governmental input support programmes present greater challenges. Private supplies are especially difficult to document due to the fact that there are usually many private suppliers, including some hard-to-trace small-scale ones. Consideration of potential tax repercussions on the part of the trader further diminishes the prospects for accurate information on private stocks and activities.

Seed retentions and intra-community exchanges are not captured in the CMS. Yet, for the majority of the smallholder farmers, these constitute the primary sources of seed. Therefore, availability statements based on observations at the district central market might not be exactly accurate. Moreover, using retail prices at the central market to judge scarcity of the commodity at community level assumes that the two markets are adequately integrated. For many remote areas, this may not be the case. Thus, prices in local communities would be more relevant to the households in them than prices from central district markets. In some markets, such as border towns, central market prices reflect more the extent of trade with the neighboring districts and countries.

4.2 Area Planted

Land area planted is a very important variable for the crop monitoring process. Yet it is arguably one of the least reliable data generated by the CMS. The most traditional way to estimate area planted is to ask the farmers directly and aggregate over all of them (Note 5). This approach, however, is possible only if one has the luxury of complete enumeration or a scientifically designed sample survey. In the event that neither is possible, as has been the case so far, the districts have had to rely on their own best guesses, in most cases with the aid of information from some baseline period. For example, some districts estimate the area planted by first estimating the size of the departure from the baseline figure. This is done by *guestimating* the proportion of farmers that have broken new land and the proportion that have cultivated less than their long-term average. The difference between the two proportions is then used to estimate the land area planted:

$$Area = \mathrm{A}\left(1 + \{\beta - \alpha\}\right) \tag{1}$$

where *Area* is land area planted to the crop of interest in hectares, α is the proportion of the interviewed farmers whose area has gone down, $\beta$is the proportion that have increased their hectarage, and A is the baseline hectarage.

The reliability of the estimates based on Equation (1) depends on the reliability of the three unknowns –α, β and A. Unfortunately, in most cases, almost all the three are of questionable credibility. First, the proportions α and β are estimated based on a conveniently drawn sample and are, thus, not a representation of the district as a whole. Second, the baseline hectarage, A, often is based on very old data and some other crude estimation processes. Some districts still use information from a baseline survey that was done in the early 1980s (Note 6).

Different approaches have been used by different districts to try and update this old and outdated baseline cultivated land area estimates, some more systematic than others. Livingstone and Kazungula, for example, use the latest household census figures to update the baseline cultivated land area. Using a population of 75,000 people, and assuming an average household size of 6, and an average cultivated area of just under one hectare per household, they estimate the baseline area planted to be about 11,000 hectares (Note 7). Recent attempts to use Camp Extension Officers (CEOs) have not been very successful owing to the CEOs' limited mobility (Shibulo, 2006).

Other districts have employed varying but equally crude strategies to estimate area planted, most limited by the amount of travel that they can possibly do within the available resources and a poor sampling strategy. One approach that has been considered but not really implemented is to estimate area planted using quantity of seed planted and known average plant population (see examples of recommended plant populations for selected common crops in Figure 2).

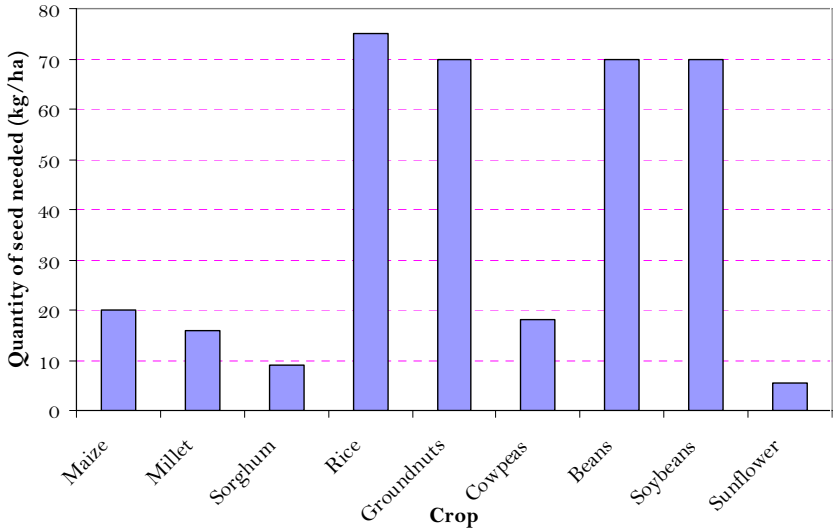

Figure 2. Average plant population for selected crops

Source: Data from the DACO's office for Livingstone and Kazungula, September 2006.

Under this approach, one has to ensure that all sources of seed are fully accounted for, including commercial purchases, input support programmes (ISPs), friends and relatives, and own retained seed. Two problems make this daunting task nearly impossible:

i) With the exception of government ISPs, all other ISPs are not easy to trace fully. Even if they are traced, private traders have an incentive to under-declare their sales.

ii) There is no way of fully accounting for own retentions and intra-community sharing of seed unless through a census or a scientifically valid sample survey. The post-harvest survey (PHS) for the previous season could be considered but its small sample size limits its usefulness at district level.

It should be noted that a comprehensive assessment of the reliability of these different methods will require quantitative comparative analysis. However, although post-harvest and agricultural census data may be available and used for this purpose, comparable data on the methods actually employed by the district agricultural offices are not consistently available. Gathering this across the districts and sifting through PHS and census data requires more effort and resources than were available at the time this paper was prepared.

4.3 Rainfall

Much of Zambia's agriculture is rain-fed. This makes rainfall one of the most important determinants of yields and rural welfare. However, the existing crop monitoring systems do not fully factor rainfall in. The qualitative

assessments often included in crop monitoring questionnaires are inadequate. While the MD already monitors rainfall and other weather variables, these are not fully incorporated in the crop forecasts.

4.4 Sample Design

All the districts that are still undertaking the CMS, with or without the support of the CMS Project, collect their data from purposively selected camps with accessibility and convenience as the key selection criteria. This is at variance with the kinds of conclusions that most interested stakeholders would like to be drawing on the basis of these data.

In most of the CMS documentation, it is implicitly assumed that the District Agricultural Coordinator's (DACO's) office is able to employ complete enumeration. With the network of camp officers and the infrastructure already in place, it is compelling to think that complete enumeration is a possibility. However, in practice, this has not been possible due, mainly, to poor staffing levels and inadequate logistical support. Logistical support includes, among other things, reconstructing camp offices and houses, both of which have been run down over the years (Note 8).

Some districts have tried some form of sampling, stratifying the camps by agro-ecological region. Livingstone and Kazungula, for example, collect monthly information from 9 of the 21 agricultural camps drawn from the three agro-ecological regions (three camps per region) (Note 9). Apparently, Livingstone and Kazungula are able to do this in part because the distances are relatively short, compared to most other districts. Even then, the selection of the sampled camps and of the interviewed farmers is at best purposive. Moreover, the district team also goes to the same camp from month to month and year to year (Note 10). Both leave the sampling-related concerns pretty much active, even for these 'role model' districts.

The relative ease of sampling and field visits observed with respect to the Livingstone/ Kazungula team cannot be generalized to many other districts with relatively more difficult physical terrains. Mongu, for example, has some of the most inaccessible camps owing to a poor road network and deep Kalahari sands. In general, smaller districts with relatively more accessible camps are able to accomplish their tasks more comprehensively as they require less fuel and fewer days to cover a reasonable sample of camps. This disparity in the sizes of the districts and in the accessibility of the camps across districts implies that the logistical requirements and associated costs will also differ. This fact is, however, not taken into account in existing budgetary allocations.

Another sampling-related issue is the fact that the CMS does not include large-scale farmers in its sample (Note 11). Although, by design, the CFS is meant to interview all the large-scale farmers in the country, this has never been achieved in practice. There is need for a more detailed review of the performance of the CFS with respect to the large-scale category of farmers and whether probability sampling should be considered.

5. Suggestions for Improving Crop Monitoring

5.1 Input Demand Surveys

In the past, the district agricultural office used to conduct input demand surveys, which were used to project types and approximate quantities of inputs expected to be demanded by the farmers in the approaching season. It is believed that the input demand estimates from these surveys were reasonably accurate, and perhaps has the protential to avert some of the existing input monitoring challenges. The input demand survey typically used to be done just before the season began, around August/September. However, this is considered a little too late. It appears June/July would be more appropriate for such a survey as it would allow more time to prepare the input demand report and inform the government, private players and other stakeholders of expected input demand.

5.2 Synergies with Existing Information Systems

The usefulness and sustainability of any EWS are functions, among other things, of the quality, timeliness and accuracy of its messages, and its ability to build on the strengths of existing information systems. One of the weaknesses of the CMS is that its reports are excessively descriptive and qualitative even on variables that are inherently quantitative. In many cases, this over-reliance on qualitative and perception-based analyses does not necessarily reflect scarcity of information but rather merely inadequate collaboration with organizations whose strength and preserve is precisely generation and interpretation of such kinds of information.

In Zambia, it is important that any crop monitoring system builds upon and benefits from existing and potential synergies with the Meteorological Department's (MD's) AgroMetShell (AMS) and the national vulnerability assessment and analysis (NVAC).

5.2.1 The AgroMet System and AgroMetShell

The Meteorological Department (MD) of the ministry of agriculture collects rainfall data on a daily basis and reports it on a dekadal (10-day) basis in Crop Weather Bulletins (MCT, 2003-2006). The crop monitoring system

needs to take such information into account in developing judgments about the season and production forecasts. Moreover, the MD also runs its own national early warning card system, which, if not integrated in the CMS, amounts to duplication of effort.

The advent of the AgroMet System (AMS), a water balance crop growth model developed by the FAO (Note 12), presents a real opportunity to extend the usefulness of the MD by transforming observed weather conditions through well-established agronomic relationships into yield forecasts. The AMS model uses historical rainfall data (over 30 years of data), soil conditions, and other agro-ecological conditions to estimate 16 indices that are relevant for understanding crop performance at the various stages of crop development (Note 13). Principal components analysis is then used to further narrow the number of variables/indices to a few most important ones (Note 14). This information is then used, in combination with observed rainfall conditions, to forecast expected yield, which could in turn be used to forecast production:

$$\text{Production} = \text{Yield} \times \text{Area} \quad (2)$$

Thus, with the AMS, the challenge of forecasting production reduces to accurately estimating area planted. Because estimating area planted is one of the key functions of both the CMS and the MD's national early warning card system, implementation of Equation (2) demands stronger collaboration between the CMS and the MD. This could entail optimizing the use of field staff from the two departments to estimate area planted and to interpret crop condition.

This AMS model has been used with some success in several countries in the region under the overall coordination of the Southern African Development Community (SADC). The SADC has been organizing training of national experts on the mechanics of the AMS model.

It should be stated that, like most government departments, the MD also faces a number of operational challenges, including inadequate and dilapidated equipment in some districts. There are strong sentiments that the MD has not received the attention it deserves. As a result, although meteorological data are generally supposed to be very reliable, in Zambia the predicting models have not been fully developed and applied. Moreover, such models' predicting strength depends heavily on agricultural data (planted area, etc), which are themselves unreliable. However, recent acquisition of rain gauges by the Department of Water Affairs (DWA) and the Zambia National Service (ZNS), and recent support to the MD by the EDRP project are some of the positive developments that need to be exploited (Mukhala and Kwendakwema 2005). The MD also supplements the data collected from its stations with data from voluntary stations, littered all over the country.

5.2.2 The NVAC System

The National Vulnerability Assessment Committee (NVAC), under the overall coordination of the Disaster Management and Mitigation Unit (DMMU), is part of a regional initiative to monitor the dynamics of vulnerabilities and to identify priority areas and modes of intervention if need be. An effective and continuous EWS such as the CMS would, therefore, be a valuable source of information to support the activities of the NVAC and to identify areas that need following up and more detailed vulnerability assessment.

The Zambia NVAC, like other NVACs in the region, does from time to time carry out annual vulnerability assessments. The information from the CMS could be combined with the NVAC livelihood zonal information to refine the stratification and sampling process for such assessments. It should not be difficult to align the needs of the NVAC with the CMS and vice versa as the two have quite a sizable intersection set of members. The NVAC's comprehensive vulnerability assessment and analysis (CVAA) survey presents great opportunities for crop monitoring as it could serve as a source of baseline information. The advantage of the CVAA, unlike CSO's annual CFSs, is that it is planned to have a large enough sample to warrant district-level inferences.

5.3 Opportunities to Optimize Resource Use

5.3.1 Existing Farmer Organizations

In addition to existing information systems, the crop monitoring system could optimize the use of available resources by recognizing and taking advantage of existing organizational structures and farmer groupings. The Zambia National Farmers's Union's (ZNFU's) network of District Farmer Associations (DFA) and Area Farmer Associations (AFAs), for example, could be utilized to reduce the cost of collecting early warning information. Some of these organizations already collaborate with the DACO's office and perform duties similar and/or relevant to what the crop montitoring system is doing.

Mongu District Farmers' Association (MDFA), for example, takes weather and market information, inputs, and extension services to the farmers through the AFAs and with the support of the DACO's office and some NGOs

like Concern Worldwide, World Vision International (WVI), and Programme Against Malnutrition (PAM). The DFAs also do needs assessments, which could be synchronized with the qualitative information gathering for early warning purposes. However, it should be recognized that, for most farmer organizations, the capacity to integrate all these information sources may be a real constraint. Thus, there may be need to assess capacity needs and develop capacity-building initiatives.

5.3.2 Operational Efficiency and Role of NGOs

To improve operational efficiency and reduce the costs of data collection and transmission, the early warning data collection mechanisms need to be designed on improved coordination with existing development activities in the district. Several innovative models can be considered. Models that have worked in other places in the past and/or new ones that seem to be supported by existing conditions may be considered and experimented.

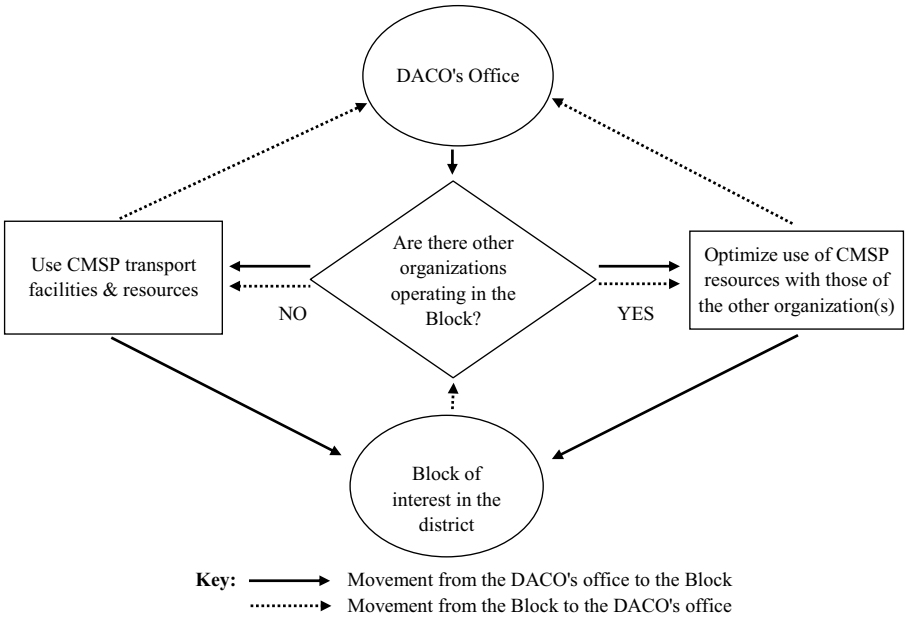

Figure 3. A highly synergistic district-level early warning model

Source: Author's own analysis.

For example, if another department or organization operates in and makes regular visits to a particular agricultural block, the DACO's office could have a standing arrangement to have an officer join that organization's team on crop monitoring duties in those areas (see Figure 3).

Some DACOs already have the responsibility and facilities with which to implement projects on behalf of NGOs and/or bilateral and multilateral development agencies. But often the financing agencies demand that use of such facilities be restricted to their own projects. With increased coordination, lobbying and collective bargaining at district level, through fora such as the District Development Coordinating Committee (DDCC) meetings, it is possible that the crop monitoring activities could be allowed to use such facilities. A well-coordinated schedule could be developed, which is flexible enough to permit adjustment whenever the stakeholder composition and spatial focus change. Moreover, in some cases, the DACO is also the DDCC chairperson. A few other natural experiments for increasing collaboration should be identified on a case by case basis.

Perhaps one more practical model could be to explicitly task the NGOs to collect crop monitoring data and submit regular reports. Internalizing this activity in the NGOs' work plans could help to minimize interference from the crop monitoring demands. The NGOs can be appraised on the nature and importance of the data that they collect on a regular basis. This can serve as a launch-and-report-back session, to bring all district stakeholders together and, with their participation, define roles and set targets. This arrangement can be formalized as a DDCC sub-committee.

However, the presence of NGO activity should not be used as a basis for selecting operational areas for crop monitoring. Such an approach would be especially detrimental in districts where NGOs operate only in camps that

satisfy certain characteristics, such as accessibility. Although using NGOs as a link between the DACOs' offices and Lusaka has worked relatively well in the past, the real future and potential for substantial gains in operational efficiency lie in developing an electronic communication network.

6. Conclusions and Recommendations

This study has reviewed the existing crop monitoring systems with the view to identifying areas for improvement. While the CFS is a valuable mechanism for generating national production forecasts, owing to a statistically valid sampling scheme, its small sample size renders it unsuitable for generating estimates for district-level planning. District-level agricultural monitoring survey is generally regarded as one of the means for filling this gap.

Several challenges impede effective collection and delivery of early warning information in Zambia. First, all crop monitoring efforts could benefit greatly from increased and reliable public funding during the critical months of December through March. Late and unreliable funding are some of the reasons the CFS is often late. The situation is worsened by the fact that the critical period for crop monitoring (December through March) coincides with the period during which the government is switching from one financial year to the next.

Suggestions have been made for the government to create special provisions that will ensure CFS funds are available at the time they would make the most impact. Similarly, district funding for crop monitoring activities should be activity-based and responsive to the size of the district both in terms of the numbers of households and the distances. For the field staff to cover the entire camp in 30 days, they need to be well equipped with transport facilities and other operating resources.

Because it is costly and practically impossible to attain 100 percent enumeration of all households in the district, sampling should be an important component of the crop monitoring system. The sampling process should recognize and take into account inherent variations in farming systems and agro-ecological conditions. The exact stratification scheme is likely to differ from district to district, depending on each district's specific spatial characteristics.

Emphasis needs to be placed on optimizing resource utilization by, among other things, identifying and utilizing synergies with existing systems, including other early warning information systems such as the AgroMetShell, and the NVAC systems. AgroMetShell analysis, which could be done on a continuous basis during the planting period, could facilitate not only point forecasts but also indications of the time path of the season. Remote sensing is also appropriate if high-resolution data for Rada sat are available.

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References

De Leon, J. C. V., Bogardi, J., Dannenmann, S., & Baster, R. (2006). *Early Warning Systems in the Context of Disaster Risk Management*. United Nations University – Institute for Environment and Human Security, and Platform for the Promotion of Early Warning (UN/ISDR), Bonn, Germany, February 2006.

FAO/GRZ. (2006). *Project Agreement for FAO/GRZ Cooperative Programme GCP/ZAM/062/UK*. FAO/GRZ, 2006.

Masdar. (2004). *Zambia: Improving Vulnerability, Food Security, and Nutrition Assessment, Phase 1 Scoping Report*. DfID/GRZ/MTL Consult. December 2004.

MCT (2003-2006). *Crop Weather Bulletin*. MD/MCT. Various Issues, 2003-2006.

Megill, D. J. (2000). *Review of Sample Design for Zambia Post-Harvest Survey (1997/98) and Recommendations for Improving the Sampling Strategy and Estimation Procedures*. Working Paper No. 2, Food Security Research Project, Lusaka, Zambia. 2000.

Megill, D. J. (2004). *Recommendations on Sample Design for Post-Harvest Surveys in Zambia Based on the 2000 Census*. Working Paper No. 11, Food Security Research Project, Lusaka, Zambia, 2004.

Mukhala, E., & Kwendakwema, N. (2005). *Review of the Agricultural Production Information Systems and Assessment of the Crop Monitoring Project and its Institutionalization*. MACO/FAO/DfID, August 2005.

Shibulo, S. (2006). *Personal communication*. September 2006.

Tembo, G. (2006). *Methodology review for the Crop and Livestock Monitoring Survey Project*. Ministry of Agriculture and Cooperatives (MACO) and the Food and Agriculture Organization of the United Nations (FAO), Lusaka, Zambia. 2006.

Notes

Note 1. CSO implements the CFS in close collaboration with the early warning unit of the Ministry of Agriculture but much to the exclusion of staff in the provincial and district agricultural offices.

Note 2. District agricultural coordinators (DACOs) justify their continued implementation of district-level monitoring (alongside the CFS) based on the argument that the CFS almost always produces wrong production estimates for their districts.

Note 3. With a sample of 8,000, the CFS is designed to accurately draw inferences at provincial and national levels whereas district-level estimates would be characterized with large margins of error (Megill, 2000).

Note 4. However, its accuracy is sometimes doubted due to over-dependence on historical trends to the exclusion of other relevant covariates, such as variations in input use, production technology, and policy thrust.

Note 5. Even if the respondent may not be able to provide the area, the personal interview environment allows for several other alternatives. For example, if the respondent presents an estimate of the size of the field in terms of 'number of lines', the enumerator may probe to get a feel of the length of each line/row and the number of such lines/rows. The enumerator can also attempt to get a feel of the distance between rows (inter-row spacing). Using all this information the enumerator can then estimate the area of the field. For another example, the farmer could present the size of the field in terms of "number of steps", e.g. 40 steps by 80 steps. If this is the case, the enumerator can estimate roughly the length of the farmer's step in metres and multiply this by the number of steps to get the distance in meters, which then can be used to compute the area in meter squared.

Note 6. With donor support during the first half of the 1980s, a comprehensive survey was conducted throughout the country in which actual area cultivated was measured (Shibulo, 2006).

Note 7. This is almost double the figure in the 20 year old baseline (5,700 hectares).

Note 8. The lack of accommodation at camp level is one of the key constraints that have hampered staff recruitment efforts. It was learnt that in some instances, staff meant for the camps have been housed at the district office, greatly defeating the idea of reducing field costs by having staff in the camps. However, MACO has recently received support from the ADB and International Monetary Fund (IMF) to facilitate recruitment and infrastructure rehabilitation and construction.

Note 9. Livingstone and Kazungula, still being administered by one DACO office, are together divided into three agro-ecological regions – 1, 2, and a transitional region that demarcates the two. The DACO's office samples three camps in each of these regions and interviewing 5 farmers per camp. This is an impressive coverage, given the resources

Note 10. However, the DACO's office argues that, despite not re-sampling, the estimates obtained are a lot more reliable than those obtained through CEOs.

Note 11. The CSO defines large scale farmers as those that cultivate more than 20 hectares per year.

Note 12. The AgroMetShell (AMS) was initially known as the FAO Index.

Note 13. The AgrometShell model identifies and uses four crop growth stages to make judgments about crop performance: i) Initial stage, ii) Vegetative stage, iii) Flowering stage, and iv) Ripening stage.

Note 14. In most cases, only three of the 16 indices (3 per crop development stage), are used.

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Heavy Metals in Oysters, Shrimps and Crabs from Lagoon Systems in the Southern Gulf of México

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Abstract

Lagoon systems in the southern Gulf of Mexico are highly productive. These aquatic systems have been severely negatively impacted by anthropogenic and industrial activities. The objective of this research was to estimate the concentration of heavy metals Pb, Cu, Cd and Zn in oysters, shrimp and crabs from the Carmen-Pajonal-Machona and Mecoacán lagoon systems in Tabasco, México. Samples were collected from fishing zones within these lagoon systems and included oysters *Crassostrea virginica*, and crustaceans such as *Litopenaeus setiferus* (shrimp) and *Callinectes sapidus* (crab). Concentrations of Pb, Cu, Cd and Zn were determined by atomic absorption using flame spectrophotometry. The heavy metal concentration pattern in oysters, shrimp and crab in the Carmen-Pajonal-Machona system was Cu > Pb > Cd. The maximum average concentration of Cu was 259.12 ± 12.312 in oyster; 0.516 ± 0.154 in shrimp, and in crab 0.907 ± 0.273 μg g^{-1}. Pb had a maximum concentration of 1.37 ± 0.77 in oyster, in shrimp was 0.059 ± 0.044, and for crab was 0.0055 μg g^{-1} ($p>0.05$), while in the Mecoacán lagoon system the pattern showed Pb > Cd > Zn. The maximum average concentration of Pb was 321.15 ± 28.828 μg g^{-1}, the minimum was 84.70 ± 8.612 μg g^{-1}. The highest concentration of Cd was 63.74 ± 8.446 μg g^{-1}, and the minimum 13.00 ± 0.64 μg g^{-1}. For Zn the maximum average concentration obtained was 24.42 ± 2.665 μg g^{-1}.

Keywords: contaminants, toxicological effects, public health, fisheries resources, bioaccumulation

1. Introduction

Lagoon systems in the southern Gulf of Mexico are key parts of agroecosystems and economic development in the country due to the diversity of habitats, dynamic interactions between water bodies, abundant fishery resources, contribution to biogeochemical cycles, and supply of minerals and products used in the pharmaceutical industry (Botello & Páez-Osuna, 1986; Contreras & Castañeda, 2004). In particular, these systems are areas of shelter, food and reproduction for many species of great importance to coastal fisheries (Toledo, 2005; Rivera & Borges, 2006). All of the above has resulted in industrial and urban development in these regions (Castañeda, Lango, & Landeros, 2011).

Among the coastal agroecosystems in the southern Gulf of México, Carmen-Pajonal-Machona, Mecoacán and Términos are known for high levels of fish production, as well as agriculture, livestock and forestry. However, these systems also contain oil wells that pose a high risk of contamination of the inherent natural resources (Contreras & Castañeda, 2004; Toledo, 2005).

Previous studies in these lagoon systems have shown environmental impacts from improper management and use of natural resources, agricultural production activities and the generation of waste pollutants. Within the latter category are included biological and chemical contaminants such as bacteria and viruses, pesticides, hydrocarbons and heavy metals (Cruz, 2011; Carrillo et al., 2012). Heavy metals are among the most studied contaminants and pollutants in the coastal environment because some are toxic (Páez, 2005). This study is focused on estimating the concentrations of the heavy metals Pb, Cd, Cu and Zn in these systems and comparing them with the permissible limits established by the Food and Drug Administration (FDA/OMS).

2. Materials and Methods

2.1 Study Area

2.1.1 Carmen-Pajonal-Machona Lagoon System

This system is located on the western edge of the coastal plain of Tabasco, between 18°14' and 18°18' N and 93°45' and 93°53' W in the municipality of Cárdenas, Tabasco (Gutiérrez & Galaviz, 1983). The system covers 8,800 ha (Carta Nacional Pesquera, 2004) (Figure 1).

2.1.2 Mecoacán Lagoon System

This system is located on the east coast of the municipality of Paraíso, Tabasco, between 18°16' and 18°26' N and 93°04' and 93°14' W (Galaviz, Gutierrez, & Castro, 1986; Carta Nacional Pesquera, 2004) and covers 5,168 ha (Figure 2).

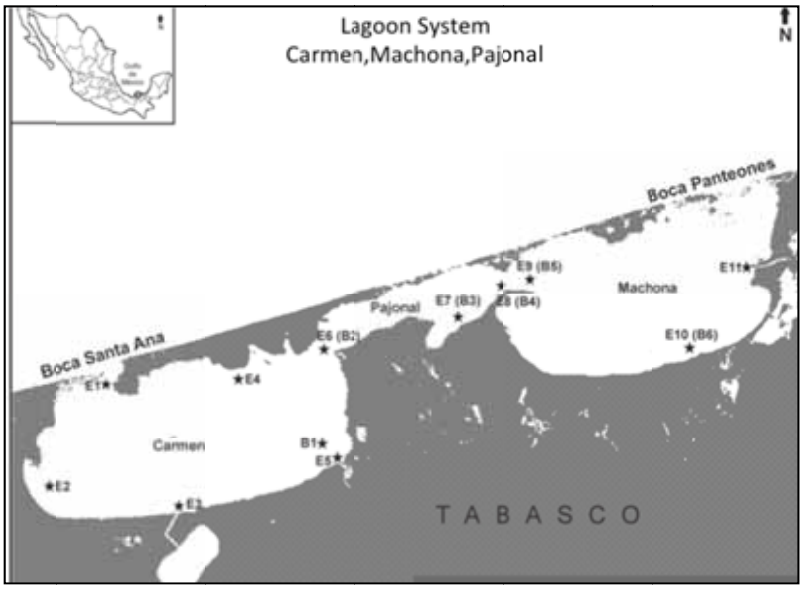

Figure 1. Location of sampling stations (E) and oyster banks (B) in the Carmen-Machona-Pajonal lagoon system

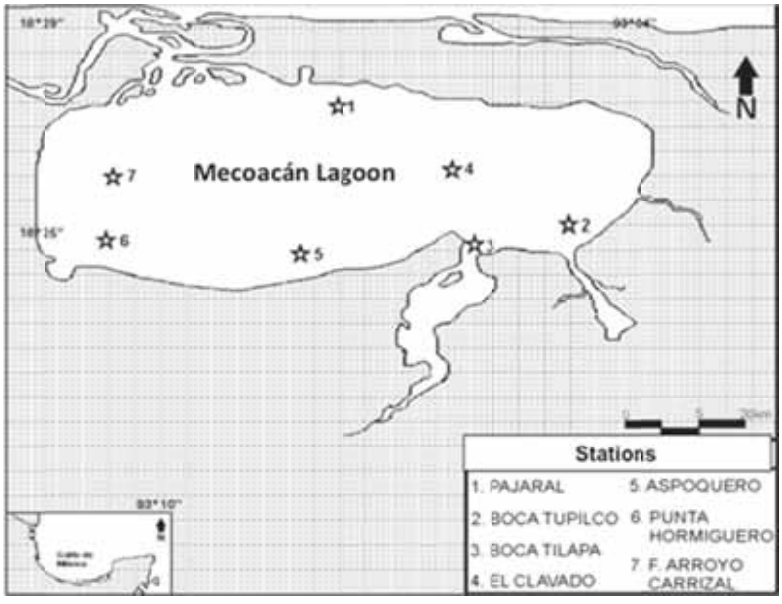

Figure 2. Location of sampling stations in the Mecoacán lagoon system

2.2 Sample Collection and Treatment

Sampling for collection of organisms was performed during the windy season (January and February), dry season (March to June) and rainy season (July to October). Oyster (*Crassostrea virginica*) samples were collected in 2007 from the Mecoacán lagoon system and in 2011 from the Carmen-Machona-Pajonal lagoon system. Samples of crab (*Callinectes sapidus*) and shrimp (*Litopenaeus setiferus*) were collected only in 2011 from the Carmen-Machona-Pajonal lagoon system.

Each oyster sample consisted of 100 oysters collected by free diving at established locations in each lagoon system, from which 30 oysters of commercial size were selected (7.0 ± 3.0 cm) to be washed and remove loose particles or bonded materials, then they were packed in Ziplock® polyethylene bags. For shrimp and crab 1 kg was collected of commercial sized individuals of 10 to 15 g each for shrimp and 100 ± 10 mm for crabs, the necessary amount to reach 1 kg of pulp per sample and they were placed in labeled Ziplock® polyethylene bags and transported to the laboratory in coolers at 5±1°C according to the protocol established by the Mexican National Standard NOM-109-SSA1-1994 (Diario Oficial de la Federación, 1994).

2.3 Laboratory Analysis of Samples

Oysters samples were removed from their shell and the soft tissues were separated by dissection and placed in triplicate in labeled Ziploc® polyethylene bags. Each one of the samples (oyster, shrimp and crab) was frozen at -40 °C in a Thermo Model 726 ultra-freezer (Thermo Fisher Scientific Inc., San Fernando, CA, USA). The frozen samples were lyophilized in a Thermo Savant MODULYOD-115 for 72 hours at -49 °C and a vacuum pressure of $36x10^{-3}$mbars. The samples were then ground in an Osterizer blender to obtain a fine particle size and then homogenized using a No. 30 sieve with a mesh size of 595 µm. To avoid contamination with humidity the samples were then stored in hermetically sealed bags in a desiccator with silica gel. To determine heavy metal concentrations, the samples were subjected to an acid digestion process in a microwave oven CEM model *Mars 5* (CEM Corporation, Matthews, NC) according to the Oyster Pure method (EPA, 1996).

2.3.1 Determination of Heavy Metals

The glassware used for the digestion process was previously washed with a 10% neutral soap solution free of phosphates to prevent ionic interference when reading the spectrophotometer. The glassware was then rinsed with potable water and immersed in a solution of distilled water with 20% nitric acid for 24 hours to ensure complete removal of the acid. The glassware was immersed in water free of heavy metals *Milli-Q* quality for 24 hours. At the end the glassware was drained and dried in a forced-air oven (Riossa CF-102) at 100 °C for 24 hours. For digestion, 0.5 g of each sample were placed in a Teflon beaker (HP-500) to which was added 9 ml of 70% reagent grade nitric acid. This process was performed at 190 °C. Each group of samples was accompanied by a blank sample and a control. After digestion, the samples were vacuum-filtered into a Nalgene bottle using 0.45 µm Millipore® nitrocellulose filters. The filtrate was diluted in a volumetric flask to a volume of 25 ml with water free of heavy metals *Milli-Q* quality. The diluted samples were transferred into polypropylene bottles and stored at 4 °C for further reading. To ensure the quality of the data a white reference was analyzed in each matrix. Certified standards were used to prepare the standard curve for each item tested (Pb, Cu, Cd and Zn) and the reading was performed by atomic absorption through flame spectrophotometry on a Thermo Scientific 3500 Model AA Ice System (Thermo Scientific®, China). The results for each metal concentration were expressed in µg g^{-1} dry weight.

2.4 Statistical Analysis

Single factor analyses of variance (ANOVAs) were conducted on heavy metal concentrations in oyster, crab and shrimp samples. Data of heavy metal in oyster were transformed to natural logarithms to achieve normality and homogeneity. In addition, a Tukey multiple comparisons test was performed. Data were analyzed using Statistica 7.0 software (StatSoft, Inc., Tulsa, OK, USA).

3. Results

3.1 Carmen-Pajonal-Machona Lagoon System

Crassostrea virginica showed the highest concentrations of heavy metals compared to *Litopenaeus setiferus* and *Callinectes sapidus*. The average concentration of the aquatic organisms (*Crassostrea virginica*, *Litopenaeus setiferus* and *Callinectes sapidus*) is indicated in Table 1. The concentration pattern recorded in all species analyzed was Cu > Pb > Cd, with the exception of Cu in crabs, where higher values were obtained with respect to shrimp.

The maximum average concentration of Cu recorded was 259.12 ± 12.312 in oysters; 0.516 ± 0.154 in shrimp, and 0.907 ± 0.273 μg g^{-1} in crabs ($p<0.05$), (Table 1). The maximum concentration of Cu in oysters was recorded from Bank 4 (Table 2); however, there was no significant difference among the banks. Respect to crustaceans, crabs had a higher mean concentration versus shrimp, 0.907 ± 0.273 and 0.516 ± 0.154 respectively. Cu was the metal with the highest bioaccumulation and significantly different to Pb and Cd ($p<0.05$), (Table 1).

Pb registered a maximum concentration of 1.37 ± 0.77 in *C. virginica* ($p<0.05$). On the other hand, shrimp had an average concentration of 0.059 ± 0.044. Pb in *C. Sapidus* was only detected during the windy season with a concentration of 0.0055 μg g^{-1} (Table 1).

Table 1. Average concentration of heavy metals among the three species analyzed (x ± SD). Different superscript indicates significant difference for the same metal (*=unique value; [§]=not tested due to single site presence; ND=not detected)

Species analyzed	Heavy Metals (μg g^{-1} dry weight)		
	Pb	Cu	Cd
Oyster	1.001±0.714 [a]	259.12±12.312 [a]	2.766±0.394 [§]
Shrimp	0.059±0.044 [b]	0.516±0.154 [b]	ND
Crab	0.0055 *	0.907±0.273 [b]	ND

Cd was not detected in shrimp and crab. The maximum concentration in oysters recorded was 2.91 ± 0.09 μg g^{-1}. Comparison of the same metal between sampling sites showed no significant difference either between the contents of Pb and Cd in banks 4, 5 and 6; which was not the case of banks 1, 2 and 3 where there was a difference (Table 2).

Table 2. Average concentration of Pb, Cu and Cd (x ± S.D.) in oyster muscle *C. virginica* from samples collected in the Carmen-Pajonal-Machona lagoon system, Tabasco, during the three sampling seasons

Banks	Heavy Metals in Oyster (μg g^{-1} dry weight)		
	Pb	Cu	Cd
B1	0.56 ± 0.46 [a]	228.32 ± 12.519 [b]	2.91 ± 0.09 [ab]
B2	1.37 ± 0.77 [a]	277.08 ± 13.945 [b]	2.89 ± 0.64 [ab]
B3	1.08 ± 0.85 [a]	271.94 ± 15.286 [b]	2.50 ± 0.19 [ab]
B4	1.00 ± 0.44 [a]	157.09 ± 8.962 [b]	2.16 ± 0.54 [a]
B5	0.65 ± 0.12 [a]	136.53 ± 8.957 [b]	1.96 ± 0.28 [a]
B6	0.45 ± 0.26 [a]	130.25 ± 15.421 [b]	1.59 ± 1.38 [a]

3.2 Mecoacán Lagoon System

The concentration pattern in oyster *C. virginica* registered in the Mecoacán lagoon system was Pb > Cd > Zn. Pb showed the highest concentration among the rest of the metals. The maximum average concentration of Pb was recorded for Bank 3 with 321.15 ± 28.828 μg g^{-1}, while the lowest concentration was observed for Bank 6 with 84.70 ± 86.12 μg g^{-1}. The highest mean concentration of Cd was 63.74 ± 84.46 μg g^{-1} recorded from Bank 5 and the lowest was 13.00 ± 0.64 μg g^{-1} from Bank 4. For Zn, the maximum mean concentration was 24.42 ± 26.65 μg g^{-1} recorded from Bank 2 (Table 3).

Table 3. Mean concentrations (x ± SD) of heavy metals in *Crassostrea virginica* from the Mecoacán lagoon system, Tabasco, México

Banks*	Heavy Metals in Oyster (μg g^{-1} dry weight)		
	Pb	Cd	Zn
1	108.50 ± 146.37	53.647 ± 63.077	13.60 ± 3.66
2	151.95 ± 192.40	32.33 ± 43.93	24.42 ± 26.65
3	321.15 ± 288.28	63.35 ± 32.44	11.69 ± 1.90
4	284.30 ± 118.08	13.00 ± 0.64	22.02 ± 6.62
5	164.70 ± 181.16	63.74 ± 84.46	14.49 ± 7.14
6	84.70 ± 86.12	50.73 ± 7.32	11.89 ± 0.52
7	213.20 ± 254.41	18.81 ± 6.30	13.19 ± 8.05

*No significant differences were observed among banks during the study.

4. Discussion

The Carmen-Pajonal-Machona and Mecoacán lagoon systems have the most important oyster banks of the area due to their contribution to the diversity of macrofauna associated with the banks (Susan & Aldana, 2008). However, the oyster, shrimp and crab fisheries are exposed to heavy metal pollution, which was evidenced by the high concentrations of heavy metals found in the muscle tissue of oyster, shrimp and crab samples. This condition may be related to the upward trend in metal pollution in coastal areas of the Gulf of México, particularly Pb and Cd. These metals can produce adverse toxicological effects on aquatic animals inhabiting these areas when exceeding natural or trace concentrations in lagoon systems such as Carmen-Pajonal-Machona (Botello, Villanueva, & Rosales, 2004). In this article all concentration data used to discuss is on dry basis.

Bivalves are considered indicators of *in situ* contamination because of their ability to bioaccumulate heavy metals (Rodríguez de la Rúa, Arellano, González, Blasco, & Sarasquete, 2005). The highest concentration of heavy metals during the study period was in oysters, confirming their bioaccumulative abilities that can have adverse effects on consumers (Vázquez, Aguirre, Pérez, Rábago y Genaro, 2005). However, according to the FDA the concentrations of Pb and Cd were found below the maximum allowable limits. In contrast, the values obtained in the Mecoacán lagoon system exceeded the limits established by the FDA, averaging 189.78 μg g^{-1} for Pb and 42.23 μg g^{-1}, for Cd.

For crustaceans, the WHO (1989) sets a maximum allowable value of 10 μg g^{-1} for Pb and Cd. Concentrations in the analyzed species *L. setiferus* and *C. sapidus* were lower and undetectable (ND) levels in both species (Table 1).

Cu is a metal that has no national legislation for commercially important such as molluscs, crustaceans or fish. Therefore, international laws must be used to compare the measured concentrations. The FDA provides a maximum permissible concentration 32.5 μg g^{-1} dry weight (1993), these values were only surpassed by oysters while crustaceans obtained concentrations below the limit.

Variations in the Cu, Pb, Cd and Zn concentrations measured from the Carmen-Pajonal-Machona and Mecoacán lagoon systems are associated with a range of factors such as absorption, excretion, storage and efficiency in the regulation and detoxification of organismal systems (Bryan, 1971), although such physiological and biochemical strategies may differ among species (Gerlach, 1981). This leads to variation in the concentrations of metals among species and tissues, depending on age, sexual maturity, feeding habits, migration, metabolism, and particularly by the different affinity of the metals for specific organs (McFarlane & Franzis, 1980; Márquez et al., 2008).

Furthermore, different ecological and feeding habits among species can affect the route of uptake of metals, such as in fish (Márquez et al., 2008). In crustaceans, there is a relationship between the concentration of heavy metals and the feeding habits of omnivorous penaeid shrimp (Scelzo, 1997). Márquez et al. (2008) recorded higher levels of metals in species that feed on lagoon sludge in Unare, Venezuela.

According to Frias, Osuna, Sandoval, and López (1999), there is a relationship between the bioaccumulation of metals and their physicochemical properties, as well as with the metabolic needs of the organisms and the availability of food in the water column. Villanueva and Botello (2005) associated this bioavailability among

others, with the type of sediment and physicochemical characteristics of water. Laws (1993) reported that benthic organisms, due to their direct interaction with sediments, are among the most affected by heavy metal concentrations.

Pb showed the highest concentrations in the species analyzed, with a maximum of 525 μg g^{-1} in the Mecoacán lagoon system. The differences in the concentration of Pb between the lagoon systems can be attributed to nearby oil extraction operations in Dos Bocas. According to Botello et al. (2004), high concentrations of Pb may be related to the direct disposal of wastewater and air emissions from urban and industrial areas of Villahermosa. Due to the volatile nature of Pb, it tends to settle in areas other than its source. The values obtained from the Mecoacán lagoon system in the present study are higher than those reported by Hernández, Hernández, Botello and Villanueva (1996) for the Mandinga lagoon of 11.55 μg g^{-1}, while Vázquez, Aguilera, and Sharma (1993) registered 5.81 μg g^{-1} for San Andrés lagoon. Also, these values were not in agreement with those reported by Vázquez et al. (2005), with 0.86 μg g^{-1} for the same location.

In the Carmen-Pajonal-Machona lagoon system, Pb had the highest concentration in oysters followed by Cu. The trend in concentration among species was oysters>shrimp>crab, with 1.00 μg g^{-1}, 0.059 μg g^{-1} and 0.0055 μg g^{-1} values reaching the permissible limits established by national and international laws for *C. virginica* oysters. These results differ from those reported by Sosa (2005) and Lango et al. (2010) for *C. virginica* from Tamiahua lagoon with a maximum of 0.56 ± 0.30 μg g^{-1} and 0.43 ± 0.17 μg g^{-1} of Pb. However, the values obtained in the present study were lower than those reported by Botello (1996) who registered 51.80 μg g^{-1} for Carmen lagoon and 22.38 μg g^{-1} for Machona lagoon. All levels in oysters from the Mecoacán lagoon system exceeded the maximum permissible limit for human consumption established by the FDA (Table 4). Meanwhile, the levels obtained in the lagoon system-Carmen-Pajonal-Machona were within the limits provided by the FDA (1993).

Table 4. Permissible limits of heavy metals in aquatic organisms of commercial importance

Reference	**Group**	**Permissible limits (μg g^{-1} dry weight)**		
		Pb (μg g^{-1})	**Cd** (μg g^{-1})	**Cu** (μg g^{-1})
FDA, 1993.	Molluscs	1.7	3.7	32.5
WHO, 1989.	Molluscs Crustaceans	5	0.20	32.5
	Crustaceans	2.0	5.0	-

In shrimp and crab the levels of Pb were significantly low, similar to those obtained by Mendoza (2010) for *F. aztecus*, where concentrations of Pb were not detectable (ND). However, Botello (1996) reported average concentrations of 12.13 μg g^{-1} in the crab *Callinectes rathbunae*. The low concentrations obtained in this study are for shrimp 0.05 μg g^{-1} and for crab 0.005 μg g^{-1}. These concentrations are lower than those reported by Palomarez, Castañeda, Lango, and Landeros (2009) for *F. aztecus* 0.119 μg g^{-1}, suggesting low bioavailability of Pb. According to Kargin, Donmez, and Cogun (2001) recorded high levels of Pb in crustaceans when there were high levels of metals in sediments.

Copper was the second most concentrated metal in oysters from the Carmen-Pajonal-Machona lagoon system with a maximum average concentration of 259.12 μg g^{-1}. This value was similar to those reported by Guzman, Villanueva, and Botello (2005) in *C. virginica* for the Alvarado with 278.00 ± 26.43 μg g^{-1}, Mandinga with 165.75 ± 13.37 μg g^{-1} and Tamiahua with 202.43 μg g^{-1}. Frias et al. (2009) reported a value of Cu concentration of 166.36 ± 38.70 μg g^{-1} in *C. corteziensis*. According to Rodríguez de la Rúa et al. (2005) the high concentrations of Cu are due to the capacity of oysters to accumulate the metal in different cellular compartments, neutralizing its effects and excreting the contaminant using different physiological strategies. Increases in the accumulation of these metals have been observed with respect to exposure time and environmental concentration. According to Laws (1981), Cu is bioaccumulated by filtering organisms reaching a concentration of several orders of magnitude compared with other macroinvertebrates. Its assimilation involves the formation of complexes with organic substances, which are not easily excreted.

In crustaceans such as shrimp, the Cu concentration showed differences according to the species analyzed. Boada, Moreno, Gil, Marcano, and Maza (2007), registered in different species of wild shrimp from the east coast of Venezuela maximum concentration of Cu of 24.00 ± 9.48 μg g^{-1} in *F. notialis*, 18.76 ± 8.28 μg g^{-1} in *L.*

schmitii, 16.98 ± 10.40 μg g^{-1} in *F. subtilis* and 11.98 ± 7.25 μg g^{-1} in *F. brasiliensis*. The present data also differed from those reported by Mendoza (2010) for *F. aztecus,* where the Cu presented a maximum concentration of 18.62 μg g^{-1}. According to Scelzo (1997), there is a relationship between these values and Cu metabolism in the hepatopancreas of crustaceans, where this metal is transported and carried out through semi-permeable biological membranes such as gills and other similar epithelia using specific transport proteins. Thus, organisms such as shrimp can maintain constant levels of Cu through ionic regulatory processes in their tissues.

The highest values of Cd in oysters were from the Mecoacán lagoon system, above the limits set by the FDA with an average concentration of 42.235±3.64 μg g^{-1}, while in the Carmen-Pajonal-Machona was 2.766 ± 0.394 μg g^{-1}, below the maximum limit set by the FDA. Its high concentration in oysters and lagoon systems along the Gulf of Mexico is due to increased industrial use in these areas over the past 30 years, resulting in its release into the environment; since it is not found naturally in the earth's crust (Jaramillo, 2009; Maldonado, González, & Jaramillo, 2009). The concentrations recorded in the present study from the Carmen-Pajonal-Machona lagoon system were similar to those reported by Botello (1996) 3.29 μg g^{-1} and 2.94 μg g^{-1} from the Carmen and Machona lagoons, respectively However, the values from the Mecoacán lagoon were lower than those reported by Sosa (2005) and Lango et al. (2010), 13.54 ± 1.96 μg g^{-1} and 11.01 ± 4.05 μg g^{-1}, respectively for oysters from the Tamiahua lagoon.

Cd was not detected in crustaceans, in contrast to Palomarez et al. (2009) who reported an average concentration of 0.03 μg g^{-1} for *F. aztecus*. However, for the same species, Mendoza (2010) reported a maximum concentration of 1.55 μg g^{-1} and Botello (1996) reported 0.71 μg $g^{-1}$for the crab *Callinectes rathbunae* from the Yucateca lagoon, Tabasco. Peerzada, Nojok, & Lee (1992), reported that Cd was found in low concentrations in muscle and up to 18 times more in hepatopancreas and digestive gland of decapod crustaceans. Similarly, Kargin et al. (2001) noted the hepatopancreas is the main storage and detoxification site of heavy metals in crustaceans.

National legislation does not stipulate Zn concentrations in aquatic organisms for human consumption. However, concentrations in samples from the Mecoacán lagoon were lower than concentrations recorded for other systems. Gold et al. (2007) reported that Zn, a bioessential metal, had a maximum concentration of 514.97 μg g^{-1} and a minimum of 327.16 μg g^{-1} from Terminos Lagoon. Zn is one of the most common elements in the earth's crust and can be found in the air, soil and water. It is present in all foods, although its presence can also be attributed to its wide range of use in industrial activities. Due to its high concentration in the environment there are concerns regarding health effects on consumers of oysters because it can be assimilated and accumulated by organisms from the sediment or water they process for food (ATSDR, 2005).

The differences in heavy metal concentrations reported for coastal lagoons performed by other researchers generally reflect the bioavailability of such metals in these environments (Frias et al., 2005). Other studies carried out by Vázquez et al. (1993) and Vázquez et al. (2005) associated the heavy metal content in oysters from coastal lagoons with periods of drought and the entry of river water into the sea. Galaviz, Lango and Castañeda (2013), reported that contributions of heavy metals into coastal lagoon systems during the rainy season were due to runoff associated with the hauling of sugarcane from cultivation areas, as well as pineapple, tamarind and watermelon where chemical fertilizers are used.

5. Conclusions

The pattern of concentration of heavy metals in oyster, shrimp and crab from Carmen-Pajonal-Machona was Cu > Pb > Cd; while Mecoacán lagoon showed Pb > Cd > Zn. According to the result previously mentioned, it is inferred that the highest concentrations of Pb and Cd were found in the Mecoacán Lagoon system. This is due to the presence of great industrial activity, as well as oil spills caused by the petrochemical industry. Reflecting that oyster consumption is a risk to public health. Concentrations of Pb and Cd in oysters reported in this study show that in the Carmen-Pajonal-Machona the levels are below the limits set by the FDA, which is a low risk to the health of consumers of these organisms. According to the concentrations obtained in crustaceans such as shrimp and crab, they were both within the limits stipulated by the FDA. Therefore, the consumption of these crustaceans is no risk to public health.

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References

ATSDR (Agency for Toxic Substances and Disease Registry). (2005). Toxicological profile for Zinc. Department of Health and Human Services, Public Health Service. Atlanta, Georgia, USA.

Boada, M., Moreno, M. A., Gil, H., Marcano, J., & Maza, J. (2007). Metales pesados (Cu^{+2}, Cd^{+2}, Pb^{+2}, Zn^{+2}) en músculo y cefalotórax de camarones silvestres *Litopenaeus schmitti, Farfantepenaeus subtilis, F. notialis* y *F. brasiliensis* de la región oriental de Venezuela. *Revista Científica, FCV-LUZ, 17*(2), 186-192. Retrieved from http://www.saber.ula.ve/bitstream/123456789/28585/2/art12.pdf

Botello, V. A. (1996). Hidrocarburos tóxicos en cuerpos acuáticos y su bioacumulación. Informe Final presentado a Petróleos Mexicanos. Instituto de Ciencias del Mar y Limnología, UNAM (Eds.), México.

Botello, V. A., & Páez-Osuna, F. (1986). El problema crucial: la contaminación. In Serie *Medio Ambiente en Coatzacoalcos. Centro de Ecodesarrollo, México, 1*, 62-85.

Botello, V. A., Villanueva, F. S., & Rosales, H. L. (2004). Distribución y contaminación de metales en el Golfo de México. In M. Caso, I. Pisanty, & E. Ezcurra (Eds.), *Diagnostico Ambiental del Golfo de México* (pp. 683-710). SEMARNAT, INE, Instituto de Ecología and Harte Research Institute for Gulf of Mexico Studies. Vol. II. 1104 p.

Bryan, G. W. (1971). The effects of heavy metals (other than mercury) on marine and estuarine organisms. *Proceeding of Royal Society of London Biological Sciences, 177*, 389-410. http://dx.doi.org/10.1098/rspb.1971.0037

Carrillo, A. P., Quiroga, B. C., Castañeda, C. M. R., Wakida, K. T. A, Márquez, G. E., Loran, N. R. M., ... Zárate, N. V. (2012). *Plan de Manejo Pesquero del Sistema Lagunar Carmen-Pajonal-Machona, Tabasco*. Editorial SAGARPA, México.

Carta Nacional Pesquera. (2004). *Ecosistemas lagunares costeros: El Carmen-Machona*. Retrieved May 25, 2011, from http://www.conapesca.sagarpa.gob.mx/wb/cona/cona_parte_4

Castañeda, Ch. M. R., Lango, R. F., & Landeros, S. C. (2011). DDT in Crassostrea virginica (Gmelin, 1791) of coastal lagoons in the Gulf of Mexico. *Journal of Agriculture Science, 3*(1), 183-193. Retrieved from http://ccsenet.org/journal/index.php/jas/article/viewFile/9765/7034

Contreras, E. F., & Castañeda, L. O. (2004). Biodiversidad de las lagunas costeras. *Ciencias, 76*, 46-56.

Cruz, A. M. J. (2011). *Determinación de metales pesados (Cd, Hg, Pb) en tilapia y camarón de la laguna de Alvarado, Veracruz. Tesis de Maestría en Ciencias en Acuacultura.* Instituto Tecnológico de Boca del Río, Boca del Río, Veracruz, México.

Diario Oficial de la Federación. (1994). *Norma Oficial Mexicana NOM-109-SSA1-1994*. Bienes y servicios. Procedimientos para la toma, manejo y transporte de muestras de alimentos para su análisis microbiológico. Secretaria de Salud. D.F. México.

EPA (Environmental Protection Agency). (1996). *Method 3052*. Microwave assisted acid digestion of siliceous and organically based matrices. United States.

FDA (Food and Drug Administration). (1993). *Guidance documents for trace elements in seafood*. Guidance documents for arsenic, cadmium, chromium, nickel (January), lead (August). Washington, D.C., USA.

Frías, E. M. G., Izaguirre, F. G., Valenzuela, Q. F., Osuna, L. J. I., Voltolina, G. D., López, L. G., ... Rubio C. W. (2009). Metal content of the Gulf of California blue shrimp *Litopenaeus stylirostris* (Stimpson). *Bulletin of Environmental Contamination and Toxicology, 79*, 214-217. http://dx.doi.org/10.1007/s00128-007-9165-z

Frías, E. M. G., Osuna, L. I., Bañuelos, V. I., López, L. G., Muy, R. M. D., Izaguirre, F. G., ... Voltolina, D. (2009). Cadmium, copper, lead and zinc contents in the mangrove oyster, *Crassostrea corteziensis*, of seven coastal lagoons of NW Mexico. *Bulletin of Environmental Contamination and Toxicology, 83*, 595-599. http://dx.doi.org/10.1007/s00128-009-9828-z

Frías, E. M. G., Osuna, L. J. I., Estrada, T. F. J., López, L. G., & Izaguirre, F. G. (2005). Heavy metals in the edible muscle of shrimp from coastal lagoons located in northwest México. *Bulletin of Environmental Contamination and Toxicology, 74*, 1098-1104. http://dx.doi.org/10.1007/s00128-005-0694-z

Frías, E. M. G., Osuna, L. J. I., Sandoval, S. G., & López, L. G. (1999). Distribution of trace metals in different tissues in the rock oyster *Crassostrea iridescens*: seasonal variation. *Bulletin of Environmental Contamination and Toxicology, 63*, 73-79. http://dx.doi.org/10.1007/s001289900950

Galaviz, S. A., Gutiérrez, E. M., & Castro del Río, A. (1986). Morfología, sedimentos e hidrodinámica de las lagunas de Dos Bocas y Mecoacán, Tabasco, México. *Anales del Instituto de Ciencias del Mar y Limnología, 14*(2), 109-124. http://dx.doi.org/10.1007/978-3-642-68182-0

Galaviz-Villa, I., Lango, R. F., & Castañeda, C. M. R. (2013). *Estudio de la calidad sanitaria del ostión. Mediante el método de análisis de riesgos, identificación y control de puntos críticos (ARICPC o HACCP)*. Saarbrücken, Alemania: Editorial Académica Española.

Gerlach, S. A. (1981). *Marine Pollution: Diagnosis and Therapy*. Springer-Verlag: Berlin. VIII.

Gold, B. G., Zapata, P. O., Ceja, M. V., Rodríguez, F. G., Simá, A. R., Aguirre, M. L., & Vidal, M. V. M. (2007). Biological effects of environmental pollutants in the American oyster, *Crassostrea virginica*: a field study in Laguna de Terminos, Mexico. *International Journal of Environment and Health, 1*(2), 171-184. Retrieved from http://inderscience.metapress.com/content/E32666Q81X307535

Guzmán, A. P., Villanueva, F. S., & Botello, V. A. (2005). Metales en tres lagunas costeras del estado de Veracruz. In V. A. Botello, J. Rendón von Osten, G. Gold-Bouchot, & C. Agraz Hernández (Eds.), *Golfo de México Contaminación e Impacto Ambiental: Diagnostico y Tendencias* (2nd ed., pp. 361-372). Universidad Autónoma de Campeche, Universidad Nacional Autónoma de México, Instituto Nacional de Ecología.

Hernández, A. E., Hernández, V. E., Botello, A. V., & Villanueva, F. S. (1996). Deterioro por metales del sistema lagunar de Mandinga, Veracruz. In Instituto Nacional de Geoquímica (Ed.). *6° Congreso Nacional de Geoquímica, México* (pp. 307-312).

Jaramillo, L. A. M. (2009). *Estudio de la biología trófica de cinco especies de peces bentónicos de la costa Cullera. Relaciones con la acumulación de metales*. Tesis Doctoral. Universidad Politécnica de Valencia, Valencia, España.

Kargin, F., Donmez, A., & Cogun, H. (2001). Distribution of heavy metals in different tissues of the shrimp *Penaeus semiculatus* (sic) and *Metapenaeus monocerus* (sic) from the Iskenderun Gulf, Turkey: seasonal variations. *Bulletin of Environmental Contamination and Toxicology, 66*, 102-109. http://dx.doi.org/10.1007/s0012800211

Lango, R. F., Landeros, S. C., & Castañeda, C. M. R. (2010). Bioacumulation of cadmium (Cd), lead (Pb) and arsenic (As) in *Crassostrea virginica* (Gmelin, 1791) from Tamiahua lagoon system, Veracruz, México. *Revista Internacional de Contaminación Ambiental, 26*(3), 201-210. Retrieved from http://scielo.unam.mx/pdf/rica/v26n3/v26n3a3.pdf

Laws, E. A. (1981). *Aquatic Pollution. An Introductory Text*. NY: J. Wiley and Sons.

Laws, E. A. (1993). *Aquatic Pollution*: *An Introductory Text* (2nd ed.). NY: J. Wiley and Sons, Inc.

Maldonado, V. M., González, R. D., & Jaramillo, J. F. (2009). Toxicología de los metales. In J. F. Jaramillo, S. A. R. Rincón, & M. R. Rico (Eds.), *Toxicología Ambiental. Universidad Autónoma de Aguascalientes, Universidad de Guadalajara* (pp. 261-285).

Márquez, A., Senior, W., Fermín, I., Martínez, G., Castañeda, J., & González, J. (2008). Cuantificación de las concentraciones de metales pesados en tejidos de peces y crustáceos de la laguna de Unare, estado Anzoátegui, Venezuela. *Revista Científica, FCV-LUZ, 18*(1), 1-14. Retrieved from http://www.saber.ula.ve/bitstream/123456789/23618/2/articulo11.pdf

McFarlane, G. A., & Franzis, W. G. (1980). An examination of Cd, Cu and Hg concentrations in livers of Northern Pike, *Esox lucius*, and White Sucker, *Catostomus commersoni*, from Five Lakes near a base metal smelter at Flin Flon, Manitoba. *Canadian Journal of Fisheries and Aquatic Sciences, 37*(10), 1573-1578. http://dx.doi.org/10.1139/f80-203

Mendoza, D. F. (2010). Determinación de metales, Cd, Cr, Cu y Pb en *Farfantepenaeus aztecus* (Ivis, 1891) colectados en la laguna de Tampamachoco, Veracruz. Tesis de Maestría. Universidad Veracruzana. México.

Palomarez-García, J. M., Castañeda-Chávez, M. R., Lango-Reynoso, F., & Landeros-Sánchez, C. (2009). Niveles de metales pesados en camarón café *Farfantepenaeus aztecus* de la Laguna de Tamiahua, Veracruz, México. *Revista de Investigaciones Marinas, 30*(1), 63-69. Retrieved from http://www.cim.uh.cu/rim/pdf/2009/1/2009-63.pdf

Peerzada, N., Nojok, M., & Lee, C. (1992). Distribution of heavy metals in prawns from northern territory, Australia. *Marine Pollution Bulletin, 24*(8), 416-418. http://dx.doi.org/10.1016/0025-326X(92)90504-Y

Rivera, A. E., & Borges, S. G. (2006). El gran ecosistema marino del Golfo de México: perspectivas para su manejo. *Jaina Boletín Informativo, 16*(1), 30-48.

Rodríguez de la Rúa, A., Arrellano, J. M., González de Canales, M. L., Blasco, J., & Sarasquete, C. (2005). Acumulación de cobre y alteraciones histopatológicas en el ostión *Crassostrea angulata*. *Ciencias Marinas, 31*(003), 455-466. Retrieved from http://hdl.handle.net/10261/43319

Scelzo, A. M. (1997). Toxicidad del cobre en larvas nauplii del camarón comercial *Artemesia longinaris* Bate (Crustacea, Decapoda, Penaeidae). *Investigaciones Marinas, 25*, 177-185. Retrieved from http://www.scielo.cl/pdf/imar/v25/art13.pdf

Sosa, B. E. (2005). Evaluación de la concentración de metales pesados en tejidos de hembras y machos de ostión *Crassostrea virginica* (Gmelin, 1791), en el sistema laguna Tamiahua, Veracruz. Tesis de Maestría en Ciencias en Acuacultura. Instituto Tecnológico de Boca del Río, Boca del Río, Veracruz, México.

Susan, T. P. V., & Aldana, P. D. (2008). Macrofauna bentónica asociada a bancos ostrícolas en las lagunas costeras Carmen, Machona y Mecoacán, Tabasco, México. *Revista de Biología Tropical* (*Int. J. Trop. Biol.), 56*(Suppl. 1), 127-137. Retrieved from http://www.revistas.ucr.ac.cr/index.php/rbt/article/view/5582/5326

Toledo, O. A. (2005). Marco conceptual: caracterización ambiental del Golfo de México. In: A. V. Botello, J. Rendón-von Osten, G. Gold-Bouchot, & C. Agraz-Hernández (Eds.), *Golfo de México Contaminación e Impacto Ambiental: Diagnostico y Tendencias* (2nd ed., pp. 25-52). Universidad Autónoma de Campeche, Universidad Nacional Autónoma de México, Instituto Nacional de Ecología. 696 p.

Vázquez, G. F., Aguilera, G. L., & Sharma, K. V. (1993). Metals in sediments of the San Andres Lagoon, Tamaulipas, Mexico. *Bulletin of Environmental Contamination and Toxicology, 52*, 382-387. http://dx.doi.org/10.1007/BF00197825

Vázquez, S. M. L., Aguirre, G. G., Pérez, C. R., Rábago, C. J., & Genaro, S. J. (2005). Contenido de cadmio y plomo en agua, ostión y sedimento de la laguna de San Andrés, Aldama, Tamaulipas. *Ciencia y Mar, 9*(27), 3-9. Retrieved from http://www.umar.mx/revistas/27/cadmioyplomo.pdf

Villanueva, F. S., & Botello, V. A. (2005). Vigilancia y presencia de metales tóxicos en la laguna El Yucateco, Tabasco, México. In A. V. Botello, J. Rendón-Von Osten, G. Gold-Bouchot, C. Agraz-Hernández (Eds.), *Golfo de México Contaminación e Impacto Ambiental: Diagnostico y Tendencias* (2nd ed., pp. 407-430). Universidad Autónoma de Campeche, Universidad Nacional Autónoma de México, Instituto Nacional de Ecología.

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Variety and N-Fertilizer Rate Influence the Growth, Yield and Yield Parameters of Baby Corn (*Zea mays* L.)

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Abstract

Four baby corn varieties viz. Hybrid baby corn-271, Shuvra, Khoibhutta and BARI sweet corn-1 were planted at five N fertilizer rates viz. 0, 80, 120, 160 and 200 kg N ha^{-1} in the experiment to find out the suitable variety and N fertilizer rate for baby corn production. The experiment was carried out at the Regional Station under Bangladesh Agricultural Research Institute at Jamalpur, Bangladesh during *rabi* season of 2008-09. Hybrid baby corn-271 and Shuvra took about 85 days and Khoibhutta and BARI sweet corn-1 took about 71 days to first silking. The results revealed that the highest values was recorded in variety Shuvra with 200 kg N ha^{-1} in most of the growth parameters which was statistically similar to 160 kg N ha^{-1}. Number of ear $plant^{-1}$, length of ear, baby corn yield without husk and with husk varied significantly due to interaction of variety and N-rates. Baby corn yield without husk increased significantly with 160 kg N ha^{-1} and beyond this rate yield increment was not significant in Hybrid baby corn-271 and Shuvra while N-rate increased baby corn yield without husk significantly not beyond 120 kg ha^{-1} in Khoibhutta and BARI sweet corn-1. Number of cob $plant^{-1}$ and length of cob were found the main yield parameters attributed to increased baby corn yield without husk.

Keywords: crop growth rate, ear yield, fodder yield, leaf area index, total dry matter

1. Introduction

Baby corn (*Zea mays* L.) the "Queen of Cereals" is grown almost throughout the world is an off shoot of maize which is grown for its young, fresh, finger like green ears, harvested at the time of silk emergence and before pollination and fertilization (Ramachandrappa, Nanjappa, & Shivakumar, 2004). Baby corn has short growth duration offers an intensive rotation cultivation system which is an excellent solution for promoting economic and poverty alleviation in countries with high populations like Bangladesh, Vietnam, Thailand and the Philippines. The other advantage of growing baby corn is its remaining biomass after harvesting. These green products can be used as feed for animal and aquaculture raising (Bindhani, Barik, Garnayak, & Mahapatra, 2007). Ears are ideal for baby corn if they are bite size, 5-10 cm long and 0.85-1.70 cm diameter at the base (Bar-zur & Saadi, 1990). Expected yield is approximately 8500 pounds of unhusked baby corn ears $acre^{-1}$, or 1140 pounds of husked baby corn ears $acre^{-1}$. The baby corn has many uses. It is being used by Chinese as vegetables and this practice has spread to other Asian countries. Recently it is becoming popular very rapidly as vegetables, salad, pasta, soup, pakora, chutney, cutlets chat, dry vegetable, kofta curry, masala, manchurian, chilly, raita, pickle, candy, jam, murabba, burfi, halwa, kheer, laddo and other favorite dishes for different chinese hotels and restaurants in Bangladesh. Recently with the establishment of new dairy and beef fattening farms in our country, the demand of maize plants as a fodder are increasing day by day. Moreover stover, dry

leaves and cob covering can be used as good fuel (Ahmed, 1994). Foreign exchange can be earned by exporting baby corn and its products (Das et al., 2008).

In Bangladesh, it is not commercially grown yet in different locations. At present, baby corn is growing some areas of Chittagong hill tracts. The soil and climate of our country is suitable for baby corn production. It can be grown all the year round (Salahuddin & Ivy, 2003). It has short growth duration of about 70-80 days, thus the farmer can grow baby corn three or four times a year and thus farmers can earn money in the shortest possible time by cultivating baby corn even it can be fitted for any cropping pattern. Although the production and marketing started since 1992-93 in our country with the co-operation of IFDC but its uses, area and marketing facility have not yet been increased considerably. Eventually to meet the demand of baby corn it is imported from foreign countries like Thailand, Taiwan etc. and costing about Tk 10 (ten) crores per year (BARI, 2004). The yield of baby corn of our country is 0.99-1.1 t ha^{-1} (BARI, 2008). But its potentiality is 5 t ha^{-1} (BARI, 2004). Nevertheless it is not cultivated all over the country due to the lack of production technology knowledge. Growth of baby corn are affected by cultural management practices especially fertilizer application. The different levels of nutrition on corn plants greatly affected. Maximum and minimum nitrogen content differed in plants and also in different parts of the individual plant. The amount of nitrogen is generally much higher in leaves than in stems, leaf sheaths and roots, and it changes with plant age. More than a minimum level of nitrogen supply is necessary for N from vegetative parts to contribute to the formation of seed protein (Venekamps, Vries, & Koot, 1985). Maize is an exhaustive crop and requires high quantities of nitrogen during the period of efficient utilization, for higher productivity. Nitrogen is indispensable for increasing crop production as a constituent of protoplasm and chlorophyll and is associated with the activity of every living cell. An increased response to applied nitrogen was observed in baby corn by Pandey, Mani, Prakash, Singh, and Gupta (2002). The application of 150:75:40 kg NPK ha^{-1} + 10 t FYM was found to be optimal for obtaining high baby corn and fodder yields with good quality (Ramchandrappa et al., 2004). Application of NPK at 150:75:40 kg ha^{-1} +10 ton farm yard manure (FYM) was found to be optimum for obtaining high baby corn and green fodder yields with good quality. The need to increase food production is one of the major world problems, where physical areas under cultivation cannot be increased. The only way is to increase the productivity per unit area per unit time. This can be achieved by changes of N-fertilizer doses and by selecting suitable variety. But the information is meager on baby corn production in Bangladesh till now.

The present research work was, therefore, undertaken to evaluate the effect of N fertilizer rates for higher yield on the different varieties to be used for baby corn production.

2. Method

2.1 Experimental Site and Climatic Condition

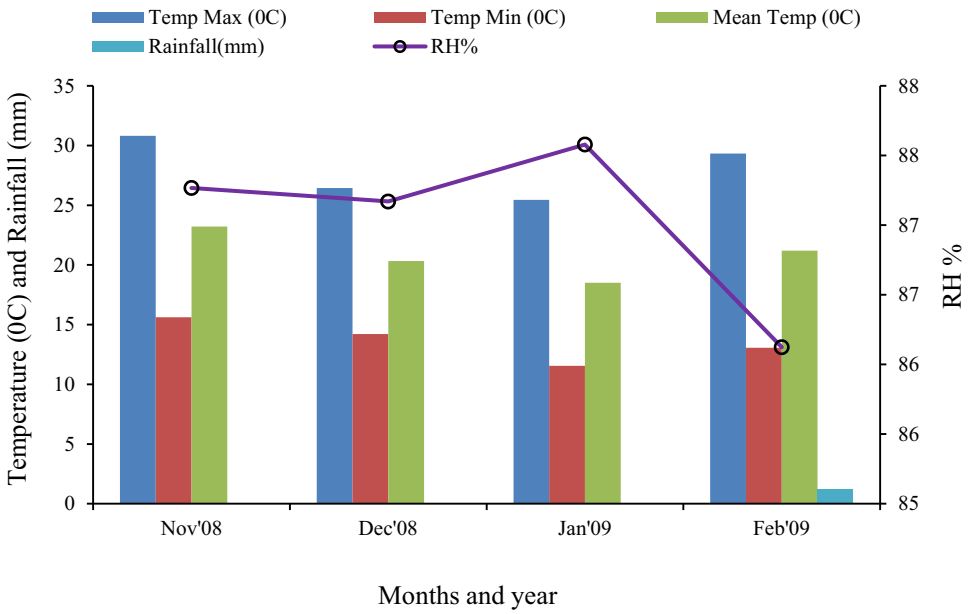

Figure 1. Temperature, rainfall and relative humidity (RH%) during the crop growing season from November 2008 to February 2009

The experiment was conducted in 2008-09 at the Regional Station under Bangladesh Agricultural Research Institute (BARI) at Jamalpur in Bangladesh which is located at the Agro Ecological Zone-9 (AEZ-9) (UNDP & FAO, 1988). The texture of the soil was silty loam. The land remained fallow before initiation of the experiment. The climate of the site was sub-tropical having the characteristics of scanty rainfall, low humidity, low temperature and short days during the *rabi* season. The average maximum (23.2 °C) temperature was found in the month of November at early stage of crop establishment and minimum (18.5 °C) in the month of January during the crop growing season (Figure 1). The total rainfall was only 1.25 mm occurred in the month of February 2009 and there was no rainfall in the months of November, December and January during the growing season.

2.2 Experimental Treatment and Cultivation Procedure

There were four baby corn varieties viz. Hybrid baby corn-271(V_1), BARI sweet corn-1 (V_2), BARI Khoibhutta (V_3) and Shuvra (V_4) and five N fertilizer rates viz. 0 kg N ha^{-1} (N_0), 80 kg N ha^{-1} (N_1), 120 kg N ha^{-1} (N_2), 160 kg N ha^{-1} (N_3) and 200 kg N ha^{-1} (N_4) in the experiment. Fertilizers @ 125-80-125-8 kg ha^{-1} of TSP, MOP, Gypsum and Zinc Sulphate, respectively (BARI, 2004) were applied as blanket dose for all treatments during final land preparation. One-third N was applied as basal during final land preparation and the rest of N was top dressed in two equal splits at 25 and 45 days after emergence (DAE), respectively. The experiment was laid out in a split-plot design with three replications having unit plot size 4.5 m × 3 m and assigning variety in the main plot and levels of N in the sub-plot. Seeds used in the experiment had more than 90% germination. Seeds were sown November 18, 2008 at the rate of 25 kg ha^{-1} maintaining 50 cm × 25 cm spacing. At first furrow was made with a tine and seeds were placed at 3-4 cm depth and then covered by light soil properly to ensure germination. Three irrigations were applied at 20, 40 and 55 DAE. Weeding and thinning was done within 20 days of sowing before first irrigation. Plots were thinned to get desired stand at the seedling stage according to the principles of Vafias & Ipsilandis (2005). Earthing-up was done within 45-50 days after second irrigation.

2.3 Data Recorded

Data were recorded on growth parameters like dry matter accumulation, crop growth rate (CGR), leaf area index (LAI), days to first tasseling and days to first silking.

CGR (g cm^{-2} day^{-1}), was calculated using equation as suggested by Yellamanda Reddy & Sankara Reddi (2005) as follows.

$$CGR = \frac{W_2 - W_1}{t_2 - t_1} g\, cm^{-2}\, day^{-1}$$

Where, W_1 is the previous weight of plant, W_2 is the final weight of plant, t_1 is the time when previous weight of the plants was recorded, t_2 is the time when final weight of the plants was recorded.

The Leaf Area Index (LAI) was found by the equation as follows.

$$LAI = \frac{Total\ leaf\ area\ of\ the\ crop}{Total\ ground\ area\ under\ the\ crop}$$

Data were also collected on yield and yield parameters. Baby corn was harvested within 2-3 days of silking and corn yield was recorded with and without husk.

2.4 Statistical Analysis

Statistical analysis were done to compare the treatment means by using computer program MSTAT-C and mean separation was done at 5% level of significance following Duncan's Multiple Range Test (DMRT).

3. Results

3.1. Growth Parameters of Baby Corn

3.1.1 Effects of Variety on the Growth Parameters of Baby Corn

The plant height was significantly different among the varieties at all growing stages (Figure 2). It increased sharply up to 80 DAE. Finally, Shuvra produced the tallest plant (179.1 cm) and BARI sweet corn-1 produced the shortest plant (149.3 cm). Days to first tasseling of different baby corn varieties also differed significantly (Table 1). Minimum days required for both tasseling and silking were found in Khoibhutta (66.7) which was statistically similar to BARI sweet corn-1 while maximum values were found in Hybrid baby corn-271. Total dry matter (TDM) accumulation $plant^{-1}$ varied significantly among the varieties at all the growth periods (Figure 3). Shuvra produced the highest DM at all stages of growth except at 80 DAE. At 80 DAE Hybrid baby corn-271

produced the highest DM. Khoibhutta had the lowest DM accumulation at all growth stages. Significant effect on CGR value at all growth periods was found among the varieties (Figure 4). A gradual increasing trend was recorded in CGR value with the advancement of growth periods. Hybrid baby corn-271 produced the highest CGR value at the period 0-20 and 50-65 DAE followed by Shuvra and Khoibhutta produced the lowest. On the other hand at 20-35 DAE Shuvra produced the highest CGR (1.28 g m^{-2} day^{-1}) and the lowest (0.993 g m^{-2} day^{-1}) was produced by BARI sweet corn-1 at the same period. Shuvra gave the highest LAI and the lowest was noted for BARI sweet corn-1 at all growth stages except at 0-20 DAE (Figure 5). At 0-20 DAE Khoibhutta gave the lowest LAI.

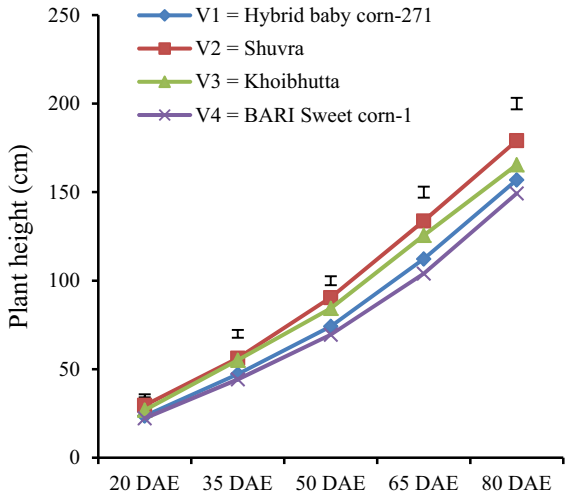

Figure 2. Plant height of different baby corn varieties at 15 day intervals

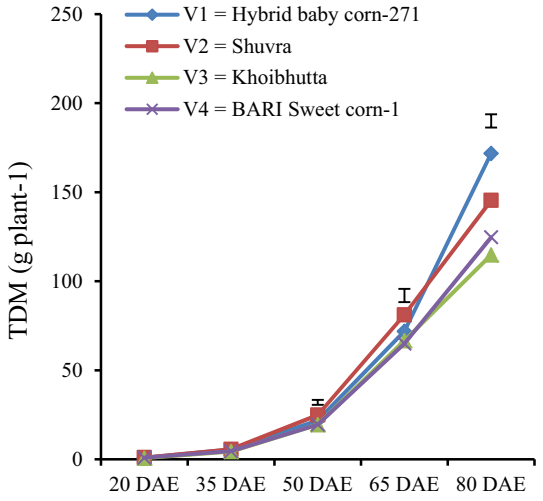

Figure 3. TDM of different baby corn varieties at 15 day intervals

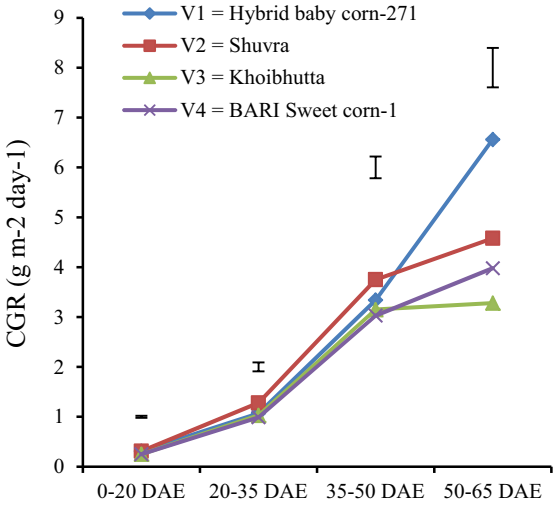

Figure 4. CGR of different baby corn varieties at 15 day intervals

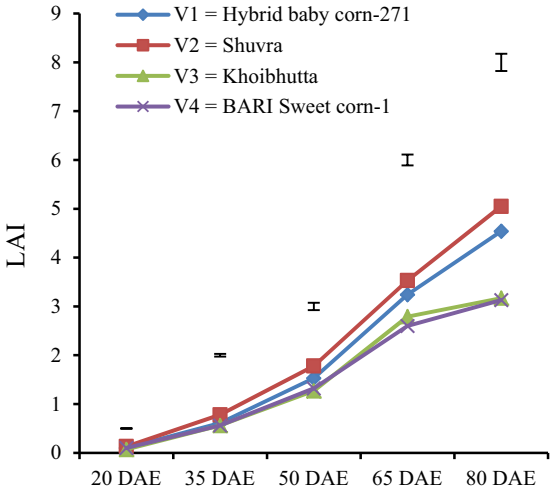

Figure 5. LAI of different baby corn varieties at 15 day intervals

Table 1. Plant population m^{-2} at 10 DAS and at harvest, days to first tasseling and days to first silking and period of harvest of different baby corn varieties as influenced by different N fertilizer doses during rabi, 2008-09

Treatment	Plant population m^{-2} at harvest	Days to first tasseling	Days to first silking	Period of harvest (days)
Variety (V)				
V_1	6.9	82.1 a	86.3 a	5.3 c
V_2	6.7	80.4 b	84.9 b	5.6 c
V_3	6.4	66.7 c	71.2 c	9.3 a
V_4	6.9	66.7 c	71.3 c	7.8 b
Significance	ns	**	**	**
CV%	8.8	1.5	1.3	22.7
N fertilizer (N)				
N_0	6.5 b	73.9	78.3	7.5
N_1	6.7 ab	74.0	78.3	6.8
N_2	6.9 a	73.9	78.3	7.1
N_3	6.6 b	73.7	78.3	6.7
N_4	7.0 a	74.3	78.8	7.0
Significance	**	ns	ns	ns
CV%	5.2	1.1	1.0	12.0

Note: DAE=Days after emergence, CV=Coefficient of variation, ns=not significant, **Significant at 1% level. Figures in a column followed by same or no latter do not differ significantly at 5% level.V_1= Hybrid baby corn-271, V_2= Shuvra, V_3= Khoibhutta, V_4= BARI sweet corn-1, N_0= 0 kg N ha^{-1}, N_1= 80 kg N ha^{-1}, N_2= 120 kg N ha^{-1}, N_3= 160 kg N ha^{-1}, N_4= 200 kg N ha^{-1}.

3.1.2 Effects of N-Level on the Growth Parameters of Baby Corn

N-fertilizer had significant effect on plant height at all growth stages (Figure 6). At 65 DAE the maximum plant height was recorded at 160 kg N ha^{-1} which was statistically similar to 200 kg N ha^{-1}. In general, the tallest plant was observed at 200 kg N ha^{-1} and the shortest at 0 kg N ha^{-1} at all growth stages. There was no significant difference for days to fist tasseling and silking due to N fertilizer rates (Table 1). Significant variation was observed in respect of DM accumulation due to different N fertilizer rates at all growth stages (Figure 7) irrespective of varieties. Results revealed that dry matter accumulation increased sharply with the increase of N-rates. There were no significant differences between 160 and 200 kg N ha^{-1} in terms of DM accumulation except at 80 DAE. The lowest DM was recorded in 0 kg N ha^{-1} at all growth stages and the differences of DM accumulation was higher in later than early stages. N-fertilizer exerted significant influence on CGR values and it was inconsistent at all growth periods (Figure 8). The highest CGR value (0.432 g m^{-2} day^{-1}) was recorded at 200 kg N ha^{-1} and the lowest was in 0 kg N ha^{-1} at 0-20 DAE. At 20-35 and 35-50 DAE the highest CGR values were recorded in 160 kg N ha^{-1} and the lowest in 0 kg N ha^{-1}. The highest CGR value was recorded with 200 kg N ha^{-1} and the lowest in 0 kg N ha^{-1}at 50-65 DAE. Significant variation was recorded in case of LAI due to N fertilizer doses (Figure 9). At all growth stages the highest LAI was obtained with 200 kg N ha^{-1}. On the contrary, 0 kg N ha^{-1} recorded the lowest LAI at all growth stages.

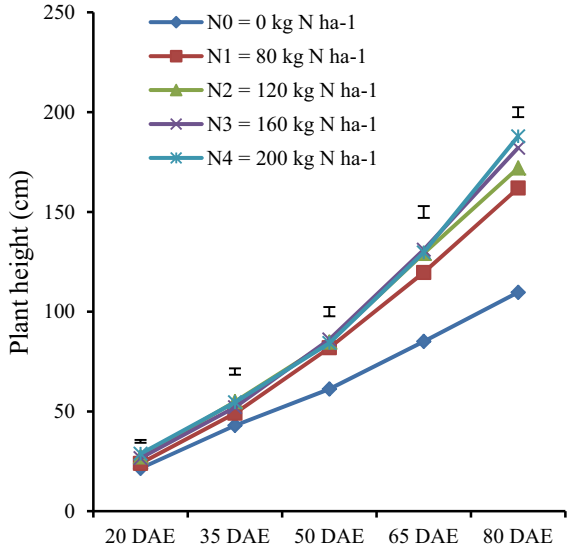

Figure 6. Plant height of different baby corn varieties as influenced by N-fertilizer at 15 day intervals

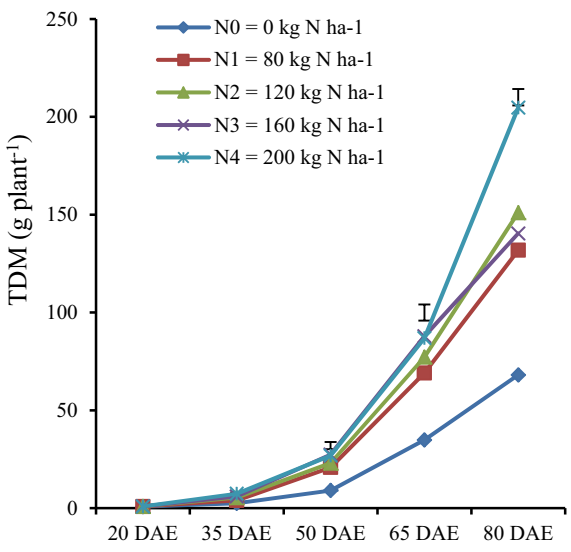

Figure 7. TDM of different baby corn varieties as influenced by N-fertilizer at 15 day intervals

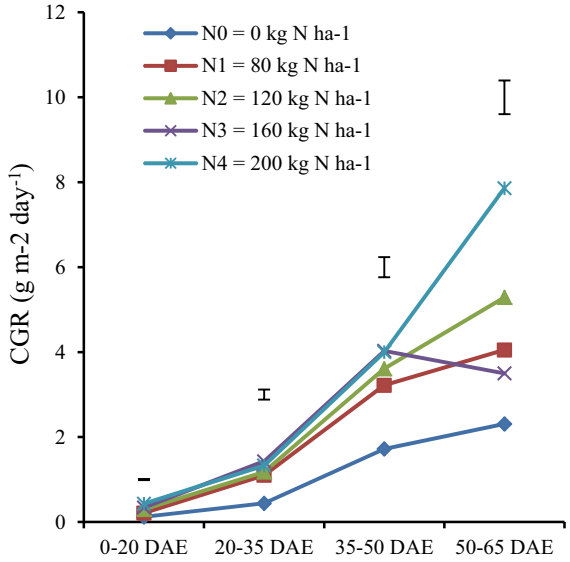

Figure 8. CGR of different baby corn varieties as influenced by N-fertilizer doses at 15 day

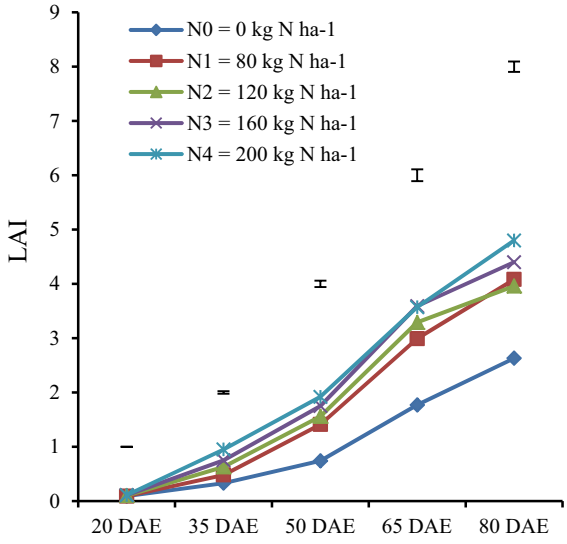

Figure 9. LAI of different varieties as influenced by N-fertilizer doses at 15 day intervals

3.1.3 Interaction of Variety and N-Level on the Growth Parameters of Baby Corn

Interaction effects of variety and N fertilizer was found significant for plant height at all growth stages (Figure 10). The tallest plant was obtained from Shuvra at 200 kg N ha^{-1} and the shortest from BARI sweet corn-1 at 0 kg N ha^{-1} at all growth stages. Interaction between variety and N fertilizer rates was found significant for days to first tasseling and first silking (Table 2). Hybrid baby corn-271 took maximum days (83.0) for tasseling with 200 kg N ha^{-1} whilst BARI sweet corn-1 at 160 kg N ha^{-1} took the minimum (65.3). Hybrid baby corn-271 took maximum days (87.7) for silking with 200 kg N ha^{-1} and BARI sweet corn-1 with 160 kg N ha^{-1} took the minimum days (70.0). In general, less period of harvest (5-6 days) was needed in Hybrid baby corn-271 and Shuvra compared to Khoibhutta and BARI sweet corn-1 (7-10 days) at all rates of N-fertilizer. Interaction of

variety and different N fertilizer rates exerted significant variation on DM accumulation at all growth periods (Figure 11). Shuvra produced the maximum DM at all growing stages except at 35 and 80 DAE with the application of 120- 200 kg N ha^{-1}. At 35 and 80 DAE Hybrid baby corn-271 had the highest TDM with 160 and 200 kg N ha^{-1}, respectively. The minimum DM accumulation was found with 0 kg N ha^{-1} in all varieties at different growth stages but the values were inconsistent. The lowest DM accumulation at 20 and 35 were obtained in Khoibhutta and BARI sweet corn-1 while at 50 and 65 DAE in Baby corn-271 and at 80 DAE in Khoibhutta with 0 kg N ha^{-1}. Interaction of variety and N-fertilizer produced significant influence on CGR values at all growth periods (Figure 12). CGR values were minimum at all growth periods with 0 kg N ha^{-1} in all varieties. At 0-20 DAE the highest CGR value was recorded with 200 kg N ha^{-1} in Shuvra and the lowest was recorded in BARI sweet corn-1 with 0 kg N ha^{-1}. At 20-35 and 35-50 DAE the highest CGR values were recorded with 160 kg N ha^{-1} in Shuvra and the lowest in Baby corn-271 at 0 kg N ha^{-1}.The highest CGR value was recorded in Baby corn-271 at 50-65 DAE followed by Shuvra with 200 kg N ha^{-1} while the lowest in Khoibhutta at 0 kg N ha^{-1}. Interaction effects of variety and N fertilizer rates on LAI were found highly significant at all plant growth stages (Figure 13). Shuvra gave the highest LAI values with 200 kg N ha^{-1} at all growth stages but not at 65 DAE. At 65 DAE, Shuvra gave the highest LAI with 160 kg N ha^{-1}. From the Figure 13 it was observed that all varieties showed the lowest LAI values with 0 kg N ha^{-1} at all growth stages.

Table 2. Plant population m^{-2} at 10 DAS and at harvest, days to first tasseling and days to first silking and period of harvest of different baby corn varieties as influenced by different N fertilizer doses during rabi, 2008-09

Interaction (V×N)	Plant population m^{-2} at harvest	Days to first tasseling	Days to first silking	Period of harvest (days)
V_1N_0	6.9	82.3 ab	86.3 ab	5.0 h
V_1N_1	6.5	81.3 bc	85.3 bc	5.7 f-h
V_1N_2	7.2	81.3 bc	85.7 b	5.3 gh
V_1N_3	6.8	82.3 ab	86.3 b	5.3 gh
V_1N_4	7.1	83.0 a	87.7 a	5.3 gh
V_2N_0	6.4	80.7 cd	85.0 bc	5.7 f-h
V_2N_1	6.9	81.0 b-d	85.3 bc	5.7 f-h
V_2N_2	6.3	79.7 d	84.0 c	5.3 gh
V_2N_3	6.7	80.3 cd	85.0 bc	5.7 f-h
V_2N_4	7.2	80.3 cd	85.0 bc	5.7 f-h
V_3N_0	6.1	66.0 gh	70.3 ef	9.0 a-c
V_3N_1	6.4	66.3 f-h	71.0 d-f	8.7 b-d
V_3N_2	6.9	66.7 e-h	71.3 d-f	10.0 ab
V_3N_3	6.2	66.7 e-h	71.7 de	8.7 b-d
V_3N_4	6.6	67.7 ef	71.7 de	10.3 a
V_4N_0	6.5	66.7 e-h	71.7 de	10.3 a
V_4N_1	7.2	67.3 e-g	71.3 d-f	7.3 de
V_4N_2	7.2	68.0 e	72.3 d	7.7 c-e
V_4N_3	6.6	65.3 h	70.0 f	7.0 ef
V_4N_4	7.2	66.0 gh	71.0 d-f	6.7 e-g
Significance	ns	**	**	**
CV%	5.2	1.1	1.0	12.0

Note: DAE=Days after emergence, CV=Coefficient of variation, ns=not significant, **Significant at 1% level. Figures in a column followed by same or no latter do not differ significantly at 5% level.V_1 = Hybrid baby corn-271, V_2 = Shuvra, V_3= Khoibhutta, V_4 = BARI sweet corn-1, N_0 = 0 kgN ha^{-1}, N_1 = 80 kgN ha^{-1}, N_2 = 120 kgN ha^{-1}, N_3 = 160 kgN ha^{-1}, N_4 = 200 kgN ha^{-1}.

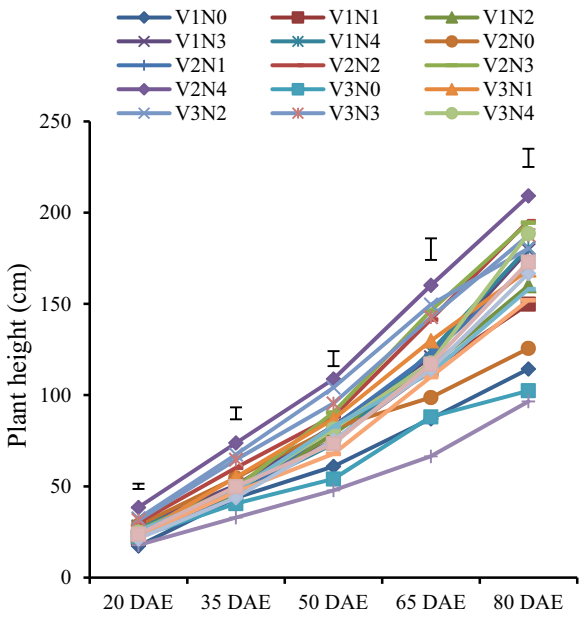

Figure 10. Interaction effect of different varieties and N-fertilizer on plant height at 15 day intervals

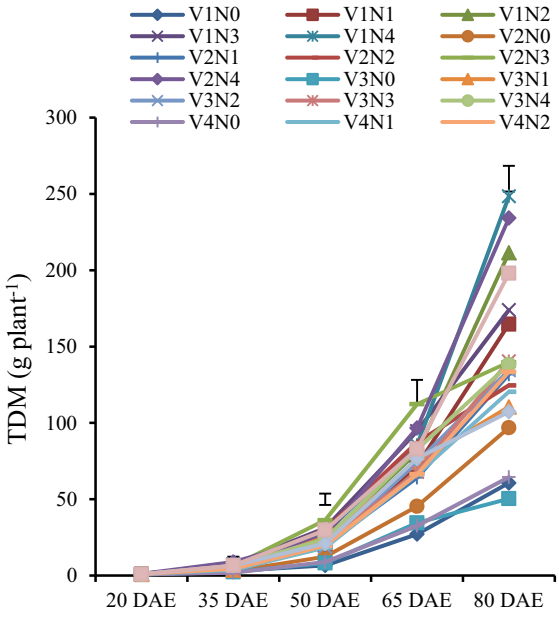

Figure 11. Interaction effect of varieties and N-fertilizer on TDM at 15 day intervals

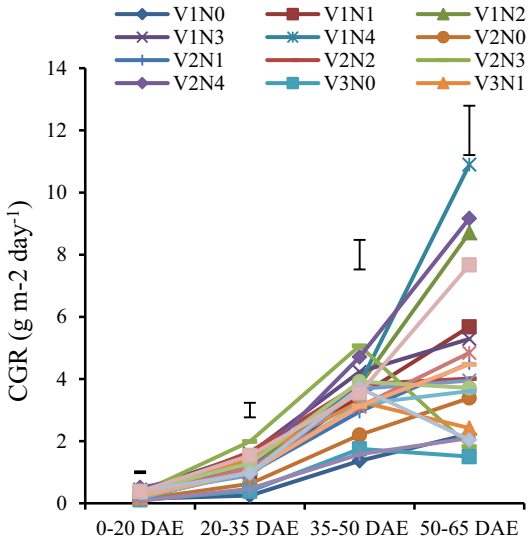

Figure 12. Interaction effect of varieties and N-fertilizer doses on CGR at 15 day intervals

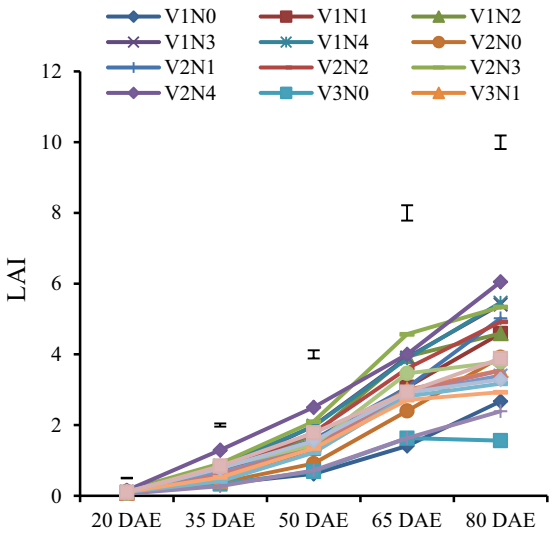

Figure 13. Interaction effect of varieties and N-fertilizer doses on LAI at 15 day intervals

3.2 Yield and Yield Components of Baby Corn

3.2.1 Effects of Variety on the Yield and Yield Components of Baby Corn

Number of ear plant^{-1} of baby corn varieties differed significantly among the varieties (Table 3). The maximum ears plant^{-1} (2.2) was recorded in Hybrid Baby Corn-271 and the minimum (1.4) was in Shuvra. Length of ear also varied significantly having the longest ear (10.8 cm) in Shuvra and the shortest (9.6 cm) in BARI sweet corn-1 (Table 3). Performance of baby corn varieties was statistically significant in case of ear diameter (Table 3). The maximum ear diameter (1.7 cm) was found in Shuvra and minimum (1.4 cm) was found in Khoibhutta which was statistically similar to Hybrid Baby Corn-1. Significantly highest corn yield without husk (1.9 t ha^{-1}) was obtained from BARI sweet corn-1 which was statistically similar to Hybrid Baby Corn-271 (1.8 t ha^{-1}) and Khoibhutta (1.8 t ha^{-1}) and the minimum ear yield (1.7 t ha^{-1}) without husk was recorded in Shuvra. The ear yield

with husk of baby corn was statistically significant (Table 3) and it was found that the maximum ear yield with husk (12.8 t ha^{-1}) was recorded in Hybrid Baby Corn-271 and the minimum (9.7 t ha^{-1}) was recorded in Shuvra. Varieties Khoibhutta and BARI sweet corn-1 produced significantly different corn yield with husk from those of Hybrid baby corn-271 and Shuvra. BARI sweet corn-1 produced the second highest baby corn yield with husk (12.1 t ha^{-1}). Significant difference was observed (Table 3) in respect of fodder yield and the highest fodder yield (32.1 t ha^{-1}) was found in Shuvra followed by Hybrid Baby Corn-271 (29.5 t ha^{-1}). The lowest fodder yield (25.4 t ha^{-1}) was recorded in BARI Sweet Corn-1.

3.2.2 Effects of N-Rate on the Yield and Yield Components of Baby Corn

The N fertilizer rates showed significant difference in case of ear plant^{-1}, ear length, corn yield without husk and with husk, and fodder yield (Table 3). Number of ear plant^{-1} increased sharply with the increment of N-fertilizer rates and the highest number of ear plant^{-1} (2.3) was recorded at 200 kg N ha^{-1} which was significantly different from those of others N-fertilizer rates. The lowest number of ear plant^{-1} (1.3) was recorded at 0 kg N ha^{-1}. The maximum ear length (10.5 cm) was found at 200 kg N ha^{-1} similar to all other N-rates but except 0 kg N ha^{-1}. The minimum ear length (9.5 cm) was found at 0 kg N ha^{-1}. The effect of N fertilizer rates on ear diameter was found non-significant. Baby corn yield without husk increased gradually with higher rates of N but the increment was not significant beyond 160 kg N ha^{-1}. Statistically similar baby corn yield (2.1 t ha^{-1}) was obtained from 160 and 200 kg N ha^{-1}. The lowest baby corn yield without husk (1.0 t ha^{-1}) was found at 0 kg N ha^{-1}which was significantly different from others. The highest ear yield (14.6 t ha^{-1}) with husk was recorded at 200 kg N ha^{-1} which was significantly different from other rates and the lowest was found at 0 kg N ha^{-1} (5.7 t ha^{-1}). The results revealed that fodder yield increased with increased rates of N and finally 200 kg N ha^{-1} produced the highest fodder yield (32.6 t ha^{-1}) whilst 0 kg N ha^{-1} produced the lowest (19.5 t ha^{-1}).

Table 3. Ear yield, yield attributes and fodder yield of different baby corn varieties as influenced by different N fertilizer rates

Treatment	Ear plant^{-1}	Ear length (cm)	Ear diameter (cm)	Yield without husk (t ha^{-1})	Yield with husk (t ha^{-1})	Fodder yield (t ha^{-1})
Variety (V)						
V_1	2.2 a	10.0 bc	1.5 b	1.8 a	12.8 a	29.5 b
V_2	1.4 c	10.8 a	1.7 a	1.7 b	9.7 d	32.1 a
V_3	2.0 b	10.3 b	1.4 b	1.8 a	10.8 c	27.0 c
V_4	2.1 ab	9.6 c	1.5 ab	1.9 a	12.1 b	25.4 d
Significance	**	**	*	*	**	**
CV%	10.6	4.9	11.2	8.4	6.2	6.0
N fertilizer (N)						
N_0	1.3 e	9.5 b	1.5	1.0 d	5.7 e	19.5 e
N_1	1.8 d	10.2 a	1.5	1.8 c	11.2 d	28.9 d
N_2	2.0 c	10.4 a	1.5	2.0 b	12.0 c	30.3 c
N_3	2.1 b	10.2 a	1.5	2.1 a	13.2 b	31.2 b
N_4	2.3 a	10.5 a	1.5	2.1 a	14.6 a	32.6 a
Significance	**	**	ns	**	**	**
CV%	5.5	4.4	6.2	6.6	5.0	2.3

Note: DAE=Days after emergence, CV=Coefficient of variation, ns=not significant, *Significant at 5% level, **Significant at 1% level. Figures in a column followed by same or no latter do not differ significantly at 5% level. V_1= Hybrid baby corn-271, V_2= Shuvra, V_3= Khoibhutta, V_4= BARI sweet corn-1, N_0= 0 kgN ha^{-1},, N_1= 80 kgN ha^{-1}, N_2= 120 kgN ha^{-1}, N_3= 160 kgN ha^{-1}, N_4= 200 kgN ha^{-1}.

3.2.3 Interaction of Variety and N-Level on the Yield and Yield Components of Baby Corn

The interaction effect of baby corn varieties and N fertilizer rate was statistically significant in case of all parameters studied except ear diameter (Table 4). In general, higher number of ear plant^{-1} was found in the varieties Hybrid baby corn-271, Khoibhutta and BARI sweet corn-1 compared to Shuvra with all rates of

N-fertilizer. The maximum ear plant^{-1} (2.6) was recorded in Hybrid Baby Corn-271 at 200 kg N ha^{-1} and the minimum ear plant^{-1} (1.0) was recorded in Shuvra at 0 kg N ha^{-1}. Ear length increased significantly with the application of increased rates of N-fertilizer. The longest ear (11.3 cm) was observed in Shuvra at 160 kg N ha^{-1} and the shortest (8.8 cm) in BARI Sweet Corn-1 at 0 kg N ha^{-1}. The highest baby corn yield (2.23 t ha^{-1}) without husk was recorded in Hybrid Baby Corn-271 at 160 kg N ha^{-1} and the lowest baby corn yield (0.84 t ha^{-1}) was recorded in Shuvra similar to that of Hybrid Baby Corn-271 at 0 kg N ha^{-1}. Baby corn yield without husk did not increase significantly beyond 160 kg N ha^{-1} in Hybrid baby corn-271 and Shuvra. Contrary on this, baby corn yield without husk did not increase significantly beyond 120 kg N ha^{-1} in Khoibhutta and BARI sweet corn-1. The highest baby corn yield (18.04 t ha^{-1}) with husk was recorded in Hybrid baby corn-271 at 200 kg N ha^{-1} and the lowest (4.26 t ha^{-1}) in Shuvra at 0 kg N ha^{-1}. Significantly highest fodder yield (36.10 t ha^{-1}) was recorded in Shuvra with 200 kg N ha^{-1} whilst the lowest fodder yield (16.0 t ha^{-1}) was recorded in BARI sweet corn-1 with 0 kg N ha^{-1}.

Table 4. Ear yield, yield attributes and fodder yield of different baby corn varieties as influenced by different N fertilizer doses during rabi, 2008-09

Interaction (V×N)	Ear plant^{-1}	Ear length (cm)	Ear diameter (cm)	Yield without husk (t ha^{-1})	Yield with husk (t ha^{-1})	Fodder yield (t ha^{-1})
V_1N_0	1.4 h	9.0 ef	1.5	0.94 i	5.89 j	20.83 l
V_1N_1	2.1 d-f	10.1 cd	1.4	1.78 ef	11.39 g	29.80 g
V_1N_2	2.2 c-e	10.3 b-d	1.5	1.96 c-e	13.00 de	31.43 f
V_1N_3	2.5 ab	10.2 cd	1.4	2.23 a	15.79 b	32.27 ef
V_1N_4	2.6 a	10.4 b-d	1.5	2.20 ab	18.04 a	33.00 de
V_2N_0	1.0 j	10.4 b-d	1.6	0.84 i	4.26 k	21.30 l
V_2N_1	1.3 hi	10.6 a-c	1.7	1.53 g	9.44 h	33.80 cd
V_2N_2	1.4 hi	10.6 a-c	1.7	1.69 fg	9.77 h	34.43 bc
V_2N_3	1.3 hi	11.3 a	1.8	2.12 a-c	11.45 g	35.00 b
V_2N_4	1.8 g	11.1 ab	1.8	2.14 a-c	13.46 c-e	36.10 a
V_3N_0	1.5 g	9.8 c-e	1.4	1.18 h	7.02 i	19.33 m
V_3N_1	2.0 f	10.5 b-d	1.4	1.81 d-f	11.13 g	26.73 j
V_3N_2	2.2 c-e	10.6 a-c	1.4	2.05 a-c	11.68 fg	28.13 hi
V_3N_3	2.2 c-e	10.3 b-d	1.4	2.01 a-d	11.76 fg	29.17 gh
V_3N_4	2.3 c	10.3 b-d	1.4	2.05 a-c	12.61 ef	31.53 f
V_4N_0	1.2 i	8.8 f	1.5	1.01 hi	5.73 j	16.60 n
V_4N_1	2.0 ef	9.7 de	1.5	1.98 b-e	13.01 de	25.07 k
V_4N_2	2.1 d-f	10.4 b-d	1.6	2.14 a-c	13.59 c-e	27.07 ij
V_4N_3	2.2 cd	9.0 ef	1.5	2.15 a-c	13.69 cd	28.50 h
V_4N_4	2.3 bc	10.2 cd	1.5	2.13 a-c	14.43 c	29.83 g
Significance	**	*	ns	**	**	**
CV%	5.5	4.4	6.2	6.59	4.98	2.29

Note: DAE=Days after emergence, CV=Coefficient of variation, ns=not significant, *Significant at 5% level, **Significant at 1% level. Figures in a column followed by same or no latter do not differ significantly at 5% level. V_1= Hybrid baby corn-271, V_2= Shuvra, V_3= Khoibhutta, V_4= BARI sweet corn-1, N_0= 0 kgN ha^{-1},' N_1= 80 kgN ha^{-1}, N_2= 120 kgN ha^{-1}, N_3= 160 kgN ha^{-1}, N_4= 200 kgN ha^{-1}.

3.3 Plant Population and Period of Harvest of Baby Corn

3.3.1 Effects of Variety on the Plant Population and Period of Harvest of Baby Corn

Variation in plant population m^{-2} at harvest was statistically non-significant among the varieties but significant in case of N fertilizer rates (Table 1). The harvesting period of all varieties was statistically significant (Table 1). The maximum period of harvest (9.3) was recorded in Khoibhutta while the minimum harvesting period (5.3) was recorded in Hybrid Baby Corn-271 and it was statistically similar (5.6) to Shuvra.

3.3.2 Effect of N-Level on the Plant Population and Period of Harvest of Baby Corn

Maximum plant population m^{-2} (7.0) at harvest was found in 200 kg N ha^{-1} and the minimum plant stand (6.5) was recorded in 0 kg N ha^{-1} (Table 1). Harvesting period was not statistically significant for N fertilizer rates and it ranged from 7-8 days.

3.3.3 Interaction of Variety and N-Level on the Plant Population and Period of Harvest of Baby Corn

The interaction effect of plant population at harvest was not statistically significant (Table 2). There were no consistency for different combinations of varieties and N-rates. Maximum plant population m^{-2} at harvest (7.2) was found in BARI sweet corn-1 at 200 kg N ha^{-1} which was similar to Hybrid baby corn-271 and Shuvra at 160 kg N ha^{-1}. Khoibhutta had the lowest (6.1) plant population m^{-2} at harvest with 0 kg N ha^{-1}. Interaction effect of variety and N fertilizer was found significant for period of harvest. The maximum harvesting period (10.3) was recorded in BARI sweet corn-1 at 0 kg N ha^{-1} followed by Khoibhutta (10.3) at 200 kg N ha^{-1}. The minimum harvesting period (5.0) was recorded in Hybrid Baby Corn-271 with 0 kg N ha^{-1}. The results revealed that generally, harvesting period was higher in Hybrid baby corn-271 and Shuvra but lower in Khoibhutta and BARI sweet corn-1 varieties at all rates of N fertilizer.

4. Discussion

Interaction effects of variety and N fertilizer was significant for plant height of different baby corn varieties at all growing stages. The tallest plant was obtained from Shuvra at 200 kg N ha^{-1} and the shortest from BARI Sweet Corn-1 at 0 kg N ha^{-1} at all growth stages. Plant height was significantly different among the varieties at all growth stages and it increased sharply onward 35 DAE indicating proper amount of nutrients and water supply need to be ensured in the crop field at this time. Finally, Shuvra produced the tallest plant (179.1 cm) and BARI Sweet Corn-1 produced the shortest plant (149.3 cm). The variation observed among the varieties was mainly due to variation of varietal characters. The result was corroborated with Kgasago (2006) who reported significant variation among the varieties of baby corn in terms of plant height. Plant height differed significantly at all growth stages for N-levels. Usually N-fertilizer enhances the growth of a crop plant synthesizing more protein and chlorophyll. This helps to increase the plant height and other growth parameters. Thakur and Sharma (1999) reported that plant height of baby corn was found significantly increased up to 200 kg N ha^{-1}. Plant height increased significantly with the increase of N-levels was observed by the other scientists also (Thakur, Prakash, & Kharwara, 1997; Sahoo & Panda, 1999; Sunder Singh, 2001).

Interaction between variety and N fertilizer was found significant for days to first tasseling and days to silking. Comparatively both the varieties Hybrid baby corn-271 and Shuvra took about 15 days more for days to tasseling and days to silking than the varieties Khoibhutta and BARI Sweet Corn-1 with all levels of N fertilizers. The results indicated that Khoibhutta and BARI Sweet Corn-1 were the early varieties while Hybrid Baby Corn-271 and Shuvra were the late varieties. The results also indicated that initiation of reproductive stages was not influenced by the application of N fertilizer.

Interaction of variety and different N fertilizer doses exerted significant variation on DM accumulation and CGR values at all growth periods. Since the variety Shuvra had the tallest plant, in most cases it produced the highest DM and CGR values with the application of 160 kg and 200 kg N ha^{-1}. It was observed that 0 kg N ha^{-1} produced the lowest DM at all growth stages but not in conjunction with same variety while 0 kg N ha^{-1} had the lowest CGR values at all periods in conjunction with same variety of Khoibhutta. These findings expressed that growth parameters studied in the experiment increased not significantly beyond 160 kg N ha^{-1}. Dry matter accumulation was lower in low rates of N and it was minimum in 0 kg N ha^{-1} at all growth stages but the differences of DM accumulation at advanced stages were higher than early stages. This indicated that more N required with the progress of plant growth but when N became a limiting factor at later stages it affected adversely on DM accumulation. Lee-Joung, Park, Chung, and Kim (2005) reported similar results. Sunder Singh (2001) reported that in baby corn, increasing nitrogen levels recorded significant increase in dry matter production in maize up to 150 kg ha^{-1} but it was comparable with 180 kg ha^{-1} both in kharif and summer seasons. CGR was influenced significantly by different levels of N-fertilizer at all growth periods. Haque, Hamid, and Bhuiyan (2001) reported that nitrogen is a component of protein and nucleic acids and lower nitrogen reduces the crop growth. Rasheed, Ali, and Mahmood (2004) and Alwony and Hasson (1997) reported similar results. Mian, Ahmed, and Matin (2002) reported that CGR value increased with the increase of nitrogen fertilizer. CGR values were increased with the progress of the growth and development of the crop. The findings are in agreement with Khaleque (2005). Hossain and Shahjahan (2008) opined that CGR value was slow at early growth because of incomplete cover and low percentage of sunlight interception which corroborated with the present study. LAI was found highly significant at all growth periods. Shuvra gave significantly highest LAI with 200 kg N ha^{-1} at all growth

stages except 65 DAE. At 65 DAE, the highest LAI was recorded in Shuvra with 160 kg N ha^{-1}. It was observed that all varieties showed the lowest LAI with 0 kg N ha^{-1}. Sorensen, Stone, & Rogers (2000) stated that synchrony between maximum LAI and solar radiation had a great influence on yield of maize. The results revealed that short duration variety like Khoibhutta and BARI Sweet Corn-1 had less LAI than the long duration variety might be due to small plant stature along with decreased number of leaf and size. Modarres, Hamilton, Dwyer, Dijak, and Smith (1998) reported similar results. LAI is important in determining radiation interception up to a value of about 4.0 in maize, after that additional leaf area has little effect on light interception (Tollenaar, Aguilera, & Nissanka, 1997). Bindhani et al. (2007) stated that in baby corn, application of 120 kg N ha^{-1} resulted in tallest plant with maximum dry matter yield and leaf area index, which were significantly higher than those at remaining lower levels of nitrogen. Thakur et al. (1997) studied the response of baby corn to different levels of nitrogen and found that growth parameters viz., plant height, leaf area and dry matter accumulation were increased with increasing levels of nitrogen application up to 150 kg N ha^{-1}.

Interaction effects of baby corn varieties and N fertilizer rate were statistically significant in case of ear $plant^{-1}$, ear length, ear yield without husk, ear yield with husk and fodder yield. Thakur et al. (1997) noticed increased number of baby corn cobs $plant^{-1}$ with 200 kg N ha^{-1} compared to no nitrogen. Length of baby corn, weight of ear and number of ears $plant^{-1}$ were found to be the highest with 120 kg N ha^{-1} (Sahoo & Panda, 1999). Bindhani et al. (2007) observed that in baby corn a significant increase in baby corns $plant^{-1}$, their fresh weight, length and girth were also recorded up to 120 kg N ha^{-1}. Lee-Joung et al. (2005) opined that ear length increased with the increase of N-levels. Thakur and Sharma (1999) registered higher number of baby corn $plant^{-1}$ and length of baby corn with 200 kg N ha^{-1} as compared to 100 kg N ha^{-1}. Contrary to this, significant differences were not observed in the weight of cob when nitrogen was applied at 100, 150 and 200 kg ha^{-1} to baby corn (Thakur & Sharma, 1999). Similar result was obtained in this study being baby corn yield without husk did not differ significantly at 120, 160 and 200 kg N ha^{-1} in Khoibhutta and BARI sweet corn-1. Baby corn yield without husk also did not differ significantly in Hybrid baby corn-271 and Shuvra with 160 and 200 kg N ha^{-1}. Higher baby corn yield without husk was ascribed to higher of ear $plant^{-1}$ and ear length mainly. Pandey, Ved, Mani, & Singh (2000.) reported that the number of baby corn cobs $plant^{-1}$ and cob weight were highest with 120 kg N ha^{-1} than at 60 and 90 kg N ha^{-1} but did not observe any significant difference in the length of baby corn with increased levels of nitrogen from 60 to 120 kg N ha^{-1}. The increased availability of photosynthates might have enhanced number of flowers and their fertilization resulting in higher number of yield attributes. Further, in most of cereals, greater assimilating surface at reproductive developments results in better green cob formation because of adequate production of metabolites and their translocation towards cob. The results of present experiment indicating positive response of various yield attributes to higher nitrogen fertilization accordance findings of several researchers (Chillar & Kumar, 2006; Bindhani et al., 2007; Gosavi & Bhagat, 2009; Prodhan, Bala, & Khoyumthem, 2007) opined that the higher green cob yield produced with application of higher nitrogen could be ascribed to its profound influence on vegetative and reproductive growth of the crop. The results of the present investigation are in accordance with findings of Raja (2001), Thakur and Sharma (1999) and Kumar (2009). Thakur et al. (1997) studied the response of baby corn to different levels of nitrogen and found that nitrogen fertilization had noticeable influence on crop growth and yield of baby corn. Application of 120 kg N ha^{-1} resulted in the maximum weight of baby corn without husk compared to other levels of N viz., 0, 20, 40, 60, 80 and 100 kg N ha^{-1} (Sahoo & Panda, 1997). These results corroborate the findings of Sunder Singh (2001) who observed comparable yields at 150 and 180 kg N ha^{-1}. Thakur et al. (1997) demonstrated that baby corn weight with and without husk was found increased significantly with successive increase in N levels up to 100 kg N ha^{-1}. Singh, Singh, Singh, Yadav, and Singh (2010) reported that significant increase in baby corn weight, cobs $plant^{-1}$, baby corn girth was observed with the application of 180 + 38.7 + 74.7 kg N+P+K ha^{-1} compared to 60 + 12.9 + 24.9 kg N + P + K ha^{-1}. Yield attributes increased with increased rates of N might be due to the fact that application of nitrogen to the maize plants maintained greenness of leaves for longer period which in turn helped in greater dry matter accumulation and this might have contributed much as a major source for the development of sink and thereby improved the yield attributes. Significantly highest fodder yield was recorded in Shuvra with 200 kg N ha^{-1} and the lowest was recorded in Khoibhutta with 0 kg N ha^{-1} indicating a faster growth under influence of higher level of nitrogen fertilization might have played a significant role in reducing competition for photosynthates and nutrients with other plants resulting in healthy plants.

5. Conclusion

The results revealed that both the long duration varieties Hybrid Baby Corn-271 and Shuvra produced comparable baby corn yield without husk at 160 and 200 kg N ha^{-1}. On the contrary, other two short duration varieties Khoibhutta and BARI Sweet Corn-1 produced comparable baby corn yield without husk at 120, 160 and 200 kg

ha^{-1}. Hence it may be suggested that to attain the maximum yield without husk and for cultivation of the following crop earlier Khoibhutta and BARI Sweet Corn-1 varieties may be grown with 120 kg N ha^{-1} in the similar areas having climatic and edaphic conditions of this experiment.

References

Ahmed, F. (1994). Maize Production Technology (*in Bengali*). International Fertilizer Development Center (IFDC)-Consultant of Ministry of Agriculture, Bangladesh.

Alwony, M. F., & Hasson, S. A. (1997). *Effect of different levels of dried blood and nitrogen on growth and yield of corn (Zea mays)*. International Wheat and Maize Improvement Centre, Mexico.

BARI-Bangladesh Agricultural Research Institute. (2008). *BARI Annual Research Report 2007-08*. Effect of season and population density on growth, fodder production and yield of baby corn at different locations. Agronomy Division, BARI, RARS, Hathazari, Chittagong, Bangladesh.

BARI-Bangladesh Agricultural Research Institute. (2004). *Production Technology of Baby Corn (in Bengali)*. Agronomy Division, BARI, Joydebpur, Gazipur, Bangladesh.

Bar-Zur, A., & Saadi, H. (1990). Prolific maize hybrids for baby corn. *Journal of Horticultural Science, 65*, 97-100.

Bindhani, A., Barik, K. C., Garnayak, L. M., & Mahapatra, P. K. (2007). Nitrogen management in Baby corn (*Zea mays*). *Indian Journal of Agronomy, 52*, 135-138.

Chillar, R. K., & Kumar, A. (2006). Growth and yield behaviour of sweet corn (*Zea mays* L. *saccharata*) under varying plant population and nitrogen level. In *Extended Summaries of Golden Jubilee National Symposium on conservation Agriculture and Environment* (pp. 277-278). Held during 26-28 October, 2006, Banaras Hindu University, Varanasi, India.

Das, S., Yadav, V. K., Jat, M. L., Asha, K., Sujay, R., Jyoti, K., ... Sekhar, J. C. (2008). Baby corn in India. DMR Technical Bulletin 2008 (pp. 1-45). Directorate of Maize Research, Pusa campus, New Delhi.

Gosavi, S. P., & Bhagat, S. B. (2009). Effect of nitrogen levels and spacing on yield attributes, yield and quality parameters of baby corn (*Zea mays*). *Annals Agricultural Research, 30*, 125-128.

Haque, M. M., Hamid, A., & Bhuiyan, N. I. (2001). Nutrient uptake and productivity as affected by nitrogen and potassium application levels in maize sweet potato intercropping system. *Korean Journal of Crop Science, 46*, 1-5.

Hossain, A., & Shahjahan, M. (2008). *Grain quality evaluation of the major varieties or cultivar of maize. Research Report for 2006-07* (pp. 1-6). Post Harvest and Technology Division, Bangladesh Agricultural Research Institute, Gazipur.

Kgasago, H. (2006). *Effect of planting dates and densities on yield and yield components of short and ultra-short growth period of maize (Zea mays* L.*)* (pp. 55-92). PhD Dissertation, University of Pretoria, South Africa.

Khaleque, M. A. (2005). *Study on growth and yield of modern wheat varieties in different management practices* (pp. 65-84). PhD Thesis. Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Kumar, A. (2009). Production potential and nitrogen-use efficiency of sweet corn (*Zea mays*) as influenced by different planting densities and nitrogen levels. *Indian Journal of Agricultural Science, 79*, 351-355.

Lee-Joung, K., Park, I. I., Chung, J., & Kim, J. (2005). Effect of planting densities and nitrogen levels on the growth characteristics, dry matter yield and nutritive value of corn for silage in alpine areas. *Journal of the Korean Society of Grassland Science, 25*, 239-244. http://dx.doi.org/10.5333/KGFS.2005.25.4.239

Mian, M. A. K., Ahmed, A., & Matin, A. (2002). Growth, yield and economics of hybrid maize as affected by rate and time of nitrogen application. *Bangladesh Journal of Agricultural Research, 27*, 41-46.

Modarres, A. M., Hamilton, R. I., Dwyer, L. M., Dijak, M., & Smith, D. L. (1998). Leafy reduce-stature maize for short season environments: Population density and planting pattern effect on hybrid performance. *Maydica, 43*, 227-234.

Pandey, A. K., Mani, V. P., Prakash, V., Singh, R. D., & Gupta, H. S. (2002). Effect of varieties and plant densities on yield, yield attributes and economics of baby corn (*Zea mays*). *Indian Journal of Agronomy, 47*, 221-226.

Pandey, A. K., Ved, P., Mani, V. P., & Singh, R. D. (2000). Effect of rate of nitrogen and time of application on yield and economics of baby corn (*Zea mays* L.). *Indian Journal of Agronomy, 45*, 338-343.

Prodhan, H. S., Bala, S., & Khoyumthem, P. (2007). Response to rate of nitrogen and effect of plant density on yield of baby corn. *Journal of Interacademicia, 11*, 265-269.

Raja, V. (2001). Effect of nitrogen and plant population on yield and quality of super sweet corn (*Zea mays*). *Indian Journal of Agronomy, 46*, 246-249.

Ramachandrappa, B. K., Nanjappa, I. I. V., & Shivakumar, I. I. K. (2004). Yield and quality of baby corn (*Zea mays* L.) as influenced by spacing and fertilization levels. *Acta Agronomica Hungarica, 52*, 237-243. http://dx.doi.org/10.1556/AAgr.52.2004.3.4

Rasheed, M., Ali, H., & Mahmood, T. (2004). Impact of nitrogen and sulfur application on growth and yield of maize crop. *Pakistan Journal of Research (Science), 15*, 153-157.

Sahoo, S. C., & Panda, M. M. (1997). Fertilizer requirement of baby corn (*Zea mays*) in wet and winter seasons. *Indian Journal of Agricultural Science, 67*, 397-398.

Sahoo, S. C., & Panda, M. M. (1999). Effect of level of nitrogen and plant population on yield of baby corn (*Zea mays*). *Indian Journal of Agricultural Science, 69*, 157-158.

Salahuddin, M., & Ivy, N. A. (2003). Cultivation of Maize. Production and Uses of Maize in Bangladesh (*In Bengali*) (pp. 29-35).

Singh, M. K., Singh, R. N., Singh, S. P., Yadav, M. K., & Singh, V. K. (2010). Integrated nutrient management for higher yield, quality and profitability of baby corn (*Zea mays*). *Indian Journal of Agronomy, 55*, 100-104.

Sorensen, I., Stone, P., & Rogers, B. (2000). Effect of sowing time on yield of a short and a long season maize hybrid. *Agronomy Journal of New Zealand, 30*, 63-66.

Sundar Singh, S. D. (2001). Effect of irrigation regimes and nitrogen levels on growth, yield and quality of baby corn. *Madras Agricultural Journal, 88*, 367-370.

Thakur, D. R., & Sharma, V. (1999). Effect of varying rates of nitrogen and its schedule of split application in baby corn (*Zea mays*). *Indian Journal of Agricultural Science, 69*, 93-95.

Thakur, D. R., Prakash, O. M., Kharwara, P. C., & Bhalla, S. K. (1997). Effect of nitrogen and plant spacing on growth, yield and economics of baby corn (Zea mays). *Indian Journal of Agronomy*, *42*(3), 479-483.

Tollenaar, M., Aguilera, A., & Nissanka, S. P. (1997). Grain yield is reduced more by weed interference in an old than in a new maize hybrid. *Agronomy Journal, 89*, 239-246. http://dx.doi.org/10.2134/agronj1997.00021962008900020014x

UNDP & FAO. (1988). Land Resources Appraisal of Bangladesh for Agricultural Development. *Report No. 2. Agro-Ecological Regions of Bangladesh* (pp. 212-221). United Nations Development Program and Food and Agriculture Organization. Bangladesh Agricultural Research Council, Farmgate, Dhaka.

Vafias, B. N., & Ipsilandis, C. G. (2005). Combining ability, gene action and yielding performance in maize. *Asian Journal of Plant Science, 4*, 50-55. http://dx.doi.org/10.3923/ajps.2005.50.55

Venekamps, J. H., Vries, F. W. T. P. de, & Koot, J. T. M. (1985). Influence of different levels of nitrogen on nitrogen nutrition and metabolism of maize plants, *Zea mays* L. Netherlands: *Agrobiological Research* (pp. 217-226).

Yellamanda Reddy, T., & Sankara Reddi, G. H. (2005). *Growth and Development of Crops* (p. 104). Principles of Agronomy. New Delhi: Kalyani publishers.

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Preparation and Control Efficiency of Seed Coating Agent by Antagonistic Actinomycetes Against Clubroot

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Abstract

In order to better use the antagonistic actinomycetes strain F22-29, the formula of the seed coating with the fermentation broth of F22-29 strain was screened and optimized by two orthogonal designed tests. The results showed that the best formula of seed coating agent was 1% sodium lignin sulfonate: 2% carboxy methylcellulose sodium: 2% dextrin. Compared with the root irrigation of the fermentation broth, the control efficiency of the seed coating against Rape *Plasmodiophora brassicae* was increased by 38.11%, and that of the seed coating against the Clubroot Disease in *Brassica napus* was increased by 63.16%. For Chinese cabbage coated seed, the control efficiency was increased by 15.78%.

Keywords: antagonistic actinomycetes, seed coating, clubroot, control effect

1. Introduction

The club-root disease of cruciferous crops is caused by the *Plasmodiophora brassicae* Woron. It can cause the decline of cruciferae crop yield. The concept of "seed coating" was firstly proposed by the Soviet Union in the 1960s. The seed coating has been developed rapidly in western countries, such as Germany and American in 1970s (Wu et al., 2003). ZBS biological seed coating with antagonistic bacteria is the first biological seed coating agent in China, which has been applied in the rice, wheat, corn, cotton and other crops (Shao, 2000). The BA-biological seed coating which is mixed with *Vivtorinox azotobacter*, *Bacterium anthracoides*, *Bacillus mucilaginosus*, and *Streptomyces jingyangensis*, by which the output of wheat, corn and other crops was increased obviously (Fang, 2004, 2005). The biological seed coating agent HND1 which has made with the bioactive metabolite of *Verticillium chlamydosporium* HDQ18 has good control effect on the soybeancyst nematode (Du, 2009). It shows that a seed coating treatment consisting of K-165 xanthan gum and talc is the most effective in prevention and treatment of the cotton black root rot (Chariklia et al., 2011). Recently research suggested that bacillus strains have a good effect on controling clubroot, such as *Bacillus subtilis* XF-1 and *Bacillus subtilis* Bs2004 (He et al., 2008; Liu, 2008). It was concluded by Jing Wang that antagonistic actinomycetes A316, A10 and antagonistic fungi T1 can strongly inhibit the germination of dormant spores, and do better in controlling rape clubroot in pot experiment and field experiment (Wang et al., 2011). Many research showed that the growth of crop can be promoted by the biological seed coating of antagonistic, by which the incidence rate of various diseases was reduced and the crop yield was increased. It's safety and environment-friendly. It plays the important role in plant disease control that can't be replaced by the chemical agent. Study on biological seed coating can effectively solve the problem of the preparation for antagonistic bacteria, which can treat plant diseases combined with prevention. Applications of biological seed coating also become a new highlights in the field of biological control of soil borne diseases (Guo et al., 2009). In this study, the fermentation broth of the strain F22-29 was used as active component, to screen out the suitable additives and determine the final formulation of seed coating agents, to provide the basis for product development.

2. Materials and Methods

2.1 Organism

Antagonistic actinomycetes strain F22-29 was isolated from Plant Pathology Laboratory of Sichuan Agricultural University, and was stored at zero to four degrees celsius. According to the study, the antagonistic actinomycetes

strain F22-29 was a mutant strain, it had a good control effect on clubroot in the field, the control efficiency was 59.99%, it was improved by 29.32% than the original strain (Lin, 2012).

2.2 Vegetable Varieties

We chose the three kinds of cruciferous vegetable seeds for experiment. Rape seeds N102-5 was developed by Department of Crop Breeding and Genetics in Sichuan Agricultural University. Chinese cabbage seeds Early maturing No. Five and *Brassica napus* seeds Xiyuan No. Four were purchased.

2.3 Mediums

Millet medium: glucose, sodium chloride, peptone, calcium carbonate.

Dispersant: sodium lignosulfate, sodium dodecyl benzene sulfonate, sodium dodecylsulfate, tween.

Membrane agent: carboxymethylcellulose sodium, sodium alginate, soluble starch, polyving akohol, chitosan.

Filling compound: dextrin, diatomite, light calcium carbonate, kaolin.

2.4 Screening of Auxiliary Agents

At first, the actinomycete fermentation liquid F22-29 was mixed with 3% filling compound, 2% dispersants, 1% membrane agent, and cultured at 25°C for 5 days. The control group was the untreated actinomycete fermentation liquid. In this experiment, the effects of various additiveson actinomycetes biological activity was determined by measuring the number of bacteria (living spores number), using the dilution method of plate counting (Guo, 2001).

2.5 Determination of Seed Coating Formulation

Selected the auxiliary agents which didn't affect the actinomycetes biological activity, used the orthogonal method $L_9(3^4)$ to determine the type and dosage of adjuvants, in order to determine the final formulation of seed coating. Seed germination rate was the main index of experiment, other physical traits of preparation were secondary screening index. Took 100 tablets of rape coated seed, were placed in the Perti dishes which with double wet filter paper in it, at 25°C for moisture culture germination, uncoated seeds as blank control. Repeated for 3 times (Lu, 2003).

2.6 Preparation and Coating

Bene tritum selected auxiliary agents and sifted them, then mixed with the actinomycete fermentation liquid F22-29 with different proportion, and it was the seed coating sample. The seed coating agent and rape seed mixed, placed in a Petri dish so that the surface of seed coating agent solidified membrane (Zhang, 1997).

2.7 Determination of Indexes

Determination of seed coating indexes was carried out in accordance with the national standards of the people's Republic of China GB/T 17768-1999 (State Bureau of technical supervision of the people's Republic of China, 1999).

2.8 Pot Experiments

In pot experiments, we used the soil which was made of sterilized soil and spore suspension, so the clubroot symptom development was by artificial inoculation. There were 3 proposals, coated seeds, root irrigation with actinomycete fermentation liquid F22-29, and uncoated seeds as control. Each treatment had 3 pots, 15 plants per pot, 3 vegetable seed varieties were 27 treatments. After 60 days, the disease symptoms were assessed using a scale consisting of four classes according toYoshikawa Hiroaki: 0 (no symptoms), 1 (1%~30% of roots had smaller clubs), 2 (31%~60% of main root and lateral roots had clubs), 3 (61%~100% of roots had bigger clubs, plant growth might be impaired)(Yoshikawa Hiroaki,1989).

$DI= (0n_0+ 1n_1 + 2n_2+ 3n_3) (100/3Nt)$

(n_0-n_3 is the number of plants in the indicated class and N_t is the total number of plants tested.)

Control Efficiency%= $[(DI_{control} - DI_{treatment})/DI_{control}]\times100$

Incidence%= The number of diseased plants /The total number of investigation×100

2.9 Data Analysis

Experimental data were analyzed using the LSD test ($P<0.05$) with significant difference analysis software DPS2006.

3. Results

3.1 Screening of Auxiliary Agents

The results showed that tween was a dispersant which had minimal impact on the biological activity of actinomycete F22-29, but sodium dodecyl benzene sulfonate had the biggest influence. For membrane agent, chitosan had minimal impact on the biological activity of actinomycete F22-29, polyving akohol greatly affects the biological activity. Light calcium carbonate was a filling compound which had minimal impact on the biological activity of actinomycete F22-29, but kaolin had the biggest influence (Table 1). Considering the physical properties of auxiliaries, we screened out the following auxiliary agents for further test: sodium lignosulfate, sodium dodecylsulfate, tween (Dispersant). carboxymethylcellulose sodium, soluble starch (Membrane agent). dextrin, diatomite, light calcium carbonate (Filling compound).

Table 1. Effects of Auxiliary on the biological activity of Actinomycete F22-29

Kinds of Auxiliary agents	Name of Auxiliary agents	Colony number (cfu/mL)
Dispersant	sodium lignosulfate	1.27×10^{6} c
	sodium dodecyl benzene sulfonate	1.10×10^{4} c
	sodium dodecylsulfate	1.15×10^{7} c
	tween	3.19×10^{7} c
Membrane agent	carboxymethylcellulose sodium	1.59×10^{8} abc
	sodium alginate	1.62×10^{8} abc
	soluble starch	6.92×10^{7} c
	polyving akohol	3.48×10^{6} c
	chitosan	1.82×10^{8} abc
Filling compound	dextrin	1.02×10^{8} bc
	diatomite	1.89×10^{8} abc
	light calcium carbonate	3.24×10^{8} ab
	kaolin	2.23×10^{5} c
CK	actinomycete strain F22-29	3.77×10^{8} a

Note: The different letters of the same series indicate the significant difference at 5% level ($P<0.05$), the same below.

3.2 Determination of Seed Coating Formulation

In the experiment, the first $L_9(3^4)$ orthogonal test was used to determine the species of dispersant, membrane agent and filling compound. Table 3 showed that the dispersing agent had the maximum range (R), so it greatly affects the seed germination rate, the Sodium lignosulfate which had the maximum average value of factor level was choosed (K-mean). In contrast, seed germination rate was smaller influenced by membrane agent and filling compound, and the carboxymethylcellulose sodium and dextrin were choosed for the next step test. The final seed coating formulation was sodium lignosulfate, carboxymethylcellulose sodium and dextrin.

Table 2. Arrangement of the first $L_9(3^4)$ orthogonal test

Level	Factor		
	Dispersant	Membrane agent	Filling compound
1	sodium lignosulfate	carboxymethylcellulose sodium	dextrin
2	sodium dodecylsulfate	sodium alginate	diatomite
3	tween	soluble starch	light calcium carbonate

Table 3. Results of the first $L_9(3^4)$ orthogonal test

Number	Factor			Relative germination rate (%)
	Dispersant	Membrane agent	Filling compound	
1	1	1	1	96.67 ab
2	1	2	2	95.33 abc
3	1	3	3	97.00 ab
4	2	1	2	93.33 abc
5	2	2	3	90.00 c
6	2	3	1	91.00 abc
7	3	1	3	95.67 abc
8	3	2	1	96.33 a
9	3	3	2	94.00 abc
K_{avg1}	96.33	95.22	94.67	
K_{avg2}	91.44	93.89	94.22	
K_{avg3}	95.33	94.00	94.22	
R	4.89	1.34	0.45	

3.3 Determination of Proportion

The second $L_9(3^4)$ orthogonal test was used to determine the auxiliary dosage. The results showed that dextrin had the maximum range(R), so the dosage of dextrin greatly affects the seed germination rate, the dosage of sodium lignosulfate and carboxymethylcellulose sodium had the smaller influence than dextrin. Considering the experimental results and the coating effect, we finalized the final seed coating dosage, it was 1% sodium lignosulfate: 2% carboxymethylcellulose sodium: 2% dextrin.

Table 4. Arrangement of the second $L_9(3^4)$ orthogonal test

Level	Factor		
	Sodium lignosulfate (%)	Carboxymethylcellulose sodium (%)	Dextrin (%)
1	2	3	3
2	1.5	2.5	2.5
3	1	2	2

Table 5. Results of the second $L_9(3^4)$ orthogonal test

Number	Factor			Relative germination rate (%)
	Sodium lignosulfate	Carboxymethylcellulose sodium	Dextrin	
1	1	1	1	66.33 d
2	1	2	2	82.67 abc
3	1	3	3	74.33 bcd
4	2	1	2	74.33 cd
5	2	2	3	67.00 d
6	2	3	1	64.00 d
7	3	1	3	75.00 bcd
8	3	2	1	61.33 d
9	3	3	2	83.67 ab
K_{avg1}	74.44	71.89	63.89	
K_{avg2}	68.44	70.33	80.22	
K_{avg3}	73.33	74.00	72.11	
R	6.00	3.67	16.34	

3.4 Determination of Indexes

PH value of seed coating agent was 9.06 by determined the main physical and chemical properties (Table 6). Studies showed that it was not conducive to clubroot when soil PH>7.2, therefore, the alkaline seed coating agent was helpful to reduce the harm of clubroot.

Table 6. The determination results of indexes

Item	PH	Effective composition content (%)	Suspension rate (%)	Abscission rate (%)	Low temperature stability	High temperature stability
Result	9.06	83.45	98.17	6.19	qualified	qualified

3.5 Pot Experiment

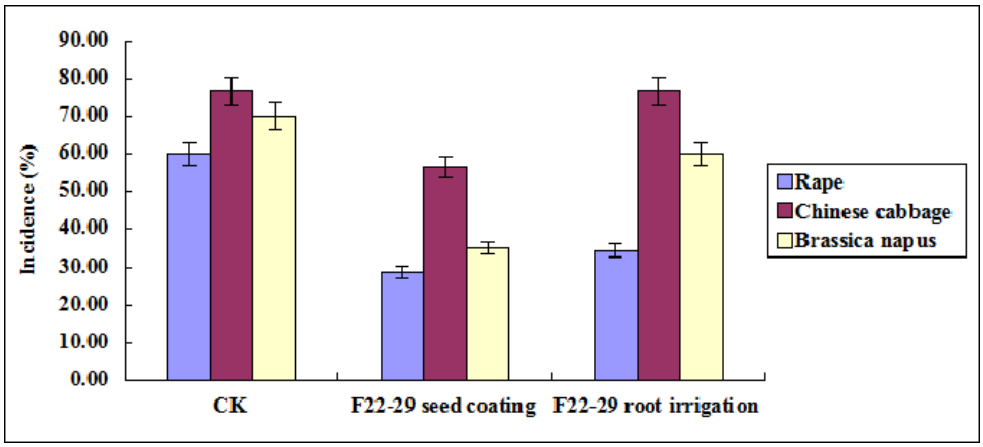

Figure 1. Incidence of pot experiment

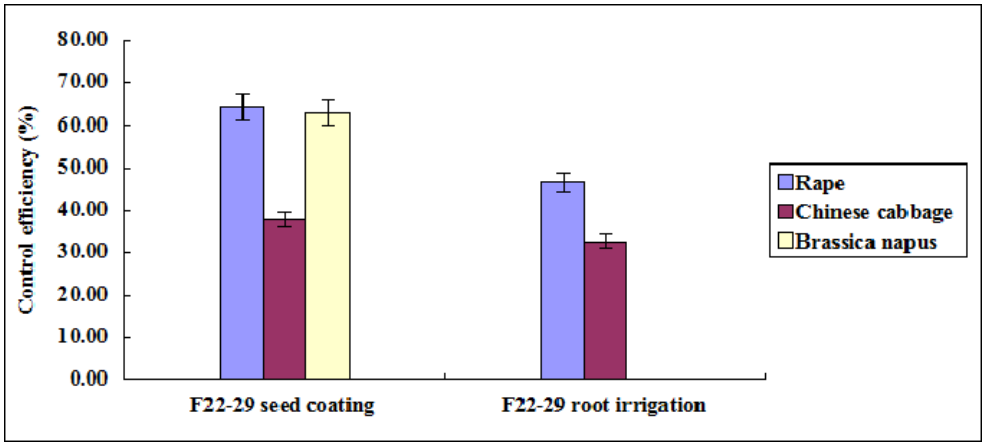

Figure 2. Control efficiency of pot experiment

In pot experiment, we prepared 3 kinds of coated seed (Rape, Chinese cabbage, *Brassica napus*). The results of pot experiment showed that the incidence of seed coating and root irrigation with actinomycete fermentation liquid F22-29 were both lower than the contorl which was only infected by pathogen. The incidence of antagonistic actinomycetes strain F22-29 seed coating and root irrigation against Rape clubroot were 28.57% and 34.29%, they were lower than the incidence of control which was 60.00%. The incidence of seed coating against Chinese cabbage clubroot were 56.67%, it was decreased by 26.09%

compared with the incidence of control. The incidence of seed coating and root irrigation with actinomycete fermentation liquid F22-29 against *Brassica napus* clubroot were 35.00% and 60.00%，they were reduced by 50.00% and 14.29% compared with the incidence of control (Figure 1). The control efficiency of seed coating with actinomycete fermentation liquid F22-29 was significantly better than the root irrigation with it. The control efficiency of seed coating against Rape clubroot was up to 64.44%, it was improved by 38.11% than control of root irrigation with actinomycete fermentation liquid F22-29. The control effect of seed coating against Chinese cabbage clubroot compared with actinomycete fermentation liquid F22-29 irrigation was improved up to 15.78%, *Brassica napus* seed coating agent had a particularly good control effect on *Brassica napus* clubroot, the control efficiency was up to 63.16% (Figure 2).

4. Discussion

In the study of the prevention and treatment of clubroot disease, we now focus on the separation of rhizosphere soil and microorganism of chemical agents in order to control clubroot. The application effect of chemical agents has a relationship with the time, concentration and times for application, etc., so that the control efficiency is often difficult to assess. There are many different kinds of microorganisms in biological world. So far a few of them have been studied, such as *Trichoderma* spp., *Streptomyces* and *Bacillus subtilis*. It's far from enough for the exploration of microorganisms resources (Li et al., 2013). Therefore, the development of biological actinomycetes seed coating agent not only uses microorganism resources to disease prevention and treatment, but also looks for a way to effective, convenient application method in field.

Resting sporangiums of crucifer clubroot disease spend summer and winter in soil and plant residue without compost manure. Resting sporangium has strong viability in soil, which could survive for 6~8 years in general. It could spread for short distance, by water, nematode in soil, insect activity and farming operation in the field, or by the seedlings with disease and carrying plants for long distance transmission. Resting sporangium in soil can germinate and infect root hair under proper conditions. Therefore, seedling stage is the key period for clubroot disease prevention. Though the method of actinomycetes fermentation liquid coating could a protective film was formed around the seed and soil around the seed was colonized by the actinomyces, which inhibited the germination of dormant spores in the soil to a certain extent, thereby reducing the infection of zoospores on root hairs and reducing the incidence of clubroot.

This study was based on the actinomycetes fermentation liquid F22-29 as effective component which could control crucifer clubroot, the final seed coating formulation and proportion was confirmed by auxiliary agents screening. Through the experiment, we determined the sodium lignosulfate, carboxymethylcellulose sodium, dextrin as dispersing agent, membrane agent and filling compound, as the proportion of 1%: 2%: 2%. The control effect of seed coating agent against Rape and *Brassica napus* clubroot reached more than 60%, the control effect by seed coating against Chinese cabbage clubroot compared with actinomycete fermentation liquid F22-29 was improved 15.78%. The control efficiency of seed coating with actinomycete fermentation liquid F22-29 was significantly better than the root irrigation with it, the results showed that there is also potential for exploiting the seed coating agent of actinomyce strain F22-29.

By the seed coating of antagonistic bacteria strain X203 and X189, Yan Zhang found that the control effect of seed coating treatment was higher than the root irrigation with ferment liquid, and the control effect of X203 coated seed on Chinese cabbage clubroot and Rape clubroot were higher than other treatments, up to 62.62% and 59.12% respectively (Zhang, 2010). It shows that the application of antagonistic strains of coating agent is an effective prevention method to control clubroot. Development of biocontrol actinomyces seed coating could cut the loss of manpower in the field. It is a more convenient and effective control method in prevention and treatment of clubroot.

In this experiment, the effective components of seed coating agent was only antagonistic, and other components were considered to adding into this agents such as elicitors, plant growth regulators, trace elements, in order to work at its best of seed coating agents. In addition, this experiment is a preliminary exploration on seed coating development with the active ingredient of the biocontrol actinomyces fermentation liquid, and the formulation and process of seed coating agents should be optimized in further.

a b

c

a: Chinese cabbage clubroot symptoms

b: *Brassica napus* clubroot symptoms

c: Rape clubroot symptoms

Figure 3. Clubroot symptoms of pot experiment

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References

Chariklia, S., Ioannis, A. S., & Iakovos, S. P. (2011). Evaluation of application methods and biocontrol efficacy of Paenibacillus alvei strain K-165, against the cotton black root rot pathogen Thielaviopsis basicola. *J. Biological Control., 58*, 68-73. http://dx.doi.org/10.1016/j.biocontrol.2011.04.002

Du, C. M., Li, H. Y., & Li, X. M. (2009). Control Effects of HND1 Biological Seed Coating on Soybean Cyst Nematode. *J. Soybean Science, 6*(28), 1126-1129.

Fang, X., Wang, Z. X., & Feng, J. (2004). A preliminary study on the effect of wheat BA- biological seed coating agent. *J. Journal of Microbiology, 2*(24), 60-61.

Fang, X., Wang, Z. X., & Feng, J. (2005). Effect of BA- biological seed coating agent on tomato. *J. Shandong Agricultural Sciences, 1*, 55-56.

Guo, N., Cheng, L. J., Duan, Y. X. (2009). Preparation and effects of biological seed coating on the soybean cyst nematode in soybean plots in Shenyang. *J. Plant protection, 35*(2), 158-162.

Guo, X. H., Xiao, C. G., & Zeng, Y. (2001). Biological characteristics of Plasmodiophora brassicae. *J. Journal of Southwest Agricultural University, 23*(1), 7-9.

He, Y. Q., Xiong, G. R., & Fan, C. M. (2008). Biological preparation capable of preventing and treating cruciferae club root and use there of. P.

Li, Z., Niu, Y. Z., & Wu, Y. C. (2013). Research progress of clubroot. *J. Journal of Southwest Agricultural University, 4*, 1733-1737.

Lin, S. (2012). *Screening and Mutation breeding of Antagonistic strains against Plasmodiophora brassicae*. D. Sichuan Agricultural University, Master.

Liu, Y., Ke, S. Y., & Huang, X. Q. (2008). Effect of Bacillus subtilis Strain Bs2004 on Promoting of Plant Health and Growth. *J. Chinese Journal of Biological Control, 24*(Suppl.), 46-49.

Lu, C. Y., & Wu, W. J. (2003). A preliminary study on the rape seed coating treatment effect. *J. Jiangsu Agricultural Sciences, 4*, 21-22.

Shao, B. F. (2000). *A microbial seed coating agent and its production method*.

State Bureau of technical supervision of the people's Republic of China. (1999). *GB/T 17768-1999. State Standard of the People's Republic of China*. S. Beijing: China standards Press.

Wang, J., Huang, Y., & Zhang, Y. (2011). Control of rapeseed clubroot by screened antagonistic microorganisms against Plasmodiophora brassicae. *J. Chinese Journal of Oil Crop Sciences, 33*(2), 169-174.

Wu, X. H., Liu, X. L., & Wang, H. M. (2003). Progressing on the Study of Seed Coating Formulations in China. *J. Pesticides, 5*, 1-5.

Yoshikawa, H., & Wang, S. (1989). Japan Cruciferae clubroot resistance breeding. *J. China Vegetables., 03*, 55-56.

Zhang, W. J. (1997). *The processing and application technologyof pesticides*. M. Beijing, China Agricultural University press.

Zhang, Y. (2010). *Screening of Antagonistic Strains Against Plasmodiophora Brassicae and Development of Seed Coating*. D. Sichuan Agricultural University, Master.

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Comparative Estimation of Technical Efficiency in Livestock-Oil Palm Integration in Johor, Malaysia: Evidence from Full and Partial Frontier Estimators

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Abstract

Two full frontiers (DEA and FDH) and two partial frontiers (order-alpha and order-m) were employed on the same data set for comparative estimation of technical efficiency in the goat-oil palm and cattle-oil palm integration systems. Data were collected from 255 livestock-oil palm integrated smallholder farms in Johor, Malaysia for the 2011 production season. Although the estimators differ in their assumptions but the technical efficiency estimates from the four distinct estimators based on the data set used appear to be similar both in magnitude and distribution as most farms produce either on the frontier or very close to the frontier. The small nature of the farms accounts for the negligible inefficiency recorded; as recommendation, larger farm size is indeed a policy tool that can guarantee frontier production to all farms.

Keywords: order-alpha, order-m, FFB, frontier, FDH

1. Introduction

Although oil palm crop originated from West Africa, its production has long crossed the shores of Africa. Substantial evidence abound not only to attest the production of oil palm outside the horizons of Africa but also to testify the long shift in its global index of production to the Asian continent. Global account for oil palm as a crop will be incomplete without mentioning the role Malaysia played and still playing in transforming the crop to a more economically viable status. Hardly is there any country in the world that invested so much on oil palm both in its up-stream and down-stream activities like Malaysia and hardly also is there a nation in the world that reaped so much economic benefit from oil palm like Malaysia. Malaysia surpassed Nigeria as the world leading producer nation in the 1970s up until Indonesia transcended Malaysia as the highest producer nation in 2007. Today, Malaysia is the second largest producer and highest exporter accounting for 44% of global exports (MPOC, 2013).

Considering the depletion in Malaysia's agricultural land owing to so much land devoted for the oil palm industry and the poor performance of the ruminant sector, there is the need for viable management strategies in the system. Strategies such as integration with livestock and further genetic modifications are avenues that guarantee FFB increase and livestock growth that can help Malaysia remain competitive in the future. Hence, this research focused on the estimation of production efficiency under both goat-oil palm and cattle-oil palm integration system with the view to dispel the aforementioned scenarios. Most efficiency studies on oil palm were estimated under sole production system and full frontier estimation techniques were mostly used. However, this research had ventured into integrated system and applied a combination of both full and partial frontiers to study the effect on the data set along methodological lines. Hence, this study used four approaches of estimating technical efficiency: two full frontiers (DEA and FDH) and two partial frontiers (order-alpha and order-m) on the same data set with a view to study if variations may exist in the efficiency scores they produce.

2. Methodology

2.1 Data Collection

Data collection for this study was cross sectional in nature, obtained from farmers integrating both goat-oil palm and cattle-oil palm in Batu Pahat, Johor Bahru, Kluang, Kota Tinggi, Kulaijaya, Ledang, Mersing, Muar, Pontian and Segamat districts of Johor State, Malaysia. Data collection basically covered production data for the year 2011 (January-December) and collection spanned between January and August, 2012. After outlier test and elimination, data on 255 sample size (plantations) were used for the analyses of this study. The study used a combination of FEAR 1.15 software developed by in 2010 on a 32-bit R version 2.14.0 and DEAP software developed by Coelli for its estimation. The FEAR estimates both the Farrell's and Sheppard's distance function; in this study, the input orientation of the Farrell's convention was used for all estimations; note the Farrell and Sheppard distance function are reciprocal of one another. The two full frontier estimators (DEA and FDH) and the two partial frontier estimators (order-m and order-α) applied in this research was to compare the application of four methodologies on the same data set with the view to understand whether or not exist any variability in the efficiency scores and why?

Figure 1. Map of the study area (Johor, Malaysia) according to districts considered during the research

Source: Malaysia books (2012).

2.2 Analytical Techniques: DEA Estimator

Following the existence of an underlying production, technical efficiency in DEA estimator can be estimated. If n represents samples of firm's observations that uses inputs k to produce outputs m, then, X_{ki} and Y_{mi} represents inputs and outputs vectors for the i[th] firm respectively. For firm using a firm utilizing X_{ki} to yield Y_{mi}, the input-oriented technical efficiency is based on CRS assumption as follows: In line with Abatania et al. (2012), technical efficiency can be empirically estimated as below:

$$TE_i(X_{ki}, Y_{mi}) = \min_{\theta,Z} \theta_i \,, (\theta: X_{ki}, Y_{mi})$$

$$subject\ to \begin{Bmatrix} y_{mi} \leq \sum_{i=1}^{I} Z_i y_{mi,}\, m = 1,2,\dots,m, \\ \sum_{i=1}^{I} Z_i x_{ki,} \leq \theta x_{ki,}\ k = 1,2,\dots,k, \\ Z_i \geq 0, i = 1,2,\dots,I, \end{Bmatrix}$$

Where: θ_i= technical efficiency of estimates calculated for each firm i, y_{mi} =quantity produced of output m by firm i , Z_i = denote intensity variable for firm i. A firm is adjudged technically efficient if $\theta = 1$, firms with values of $\theta < 1$ are considered technically inefficient. Note, being a CRS model in the above equation, it implies that all the firms operate at optimum level of production production (Mugera & Featherstone, 2008). However, in agricultural production rarely do we find all farms operating under CRS due to serial imperfections in agriculture. Therefore, additional constraints below are imposed to connote that farmers can as well produce

under variable returns to scale (VRS) and non-increasing returns to scale (NIRTS). Thus, $VRS\ constraint \rightarrow \sum_{i=1}^{I} Z_i = 1$and $NIRTS\ constraint \rightarrow \sum_{i=1}^{I} Z_i < 1$.

2.3 Estimation of Technical Efficiency

Amor and Muller (2010) defined technical efficiency in production as the ability of firms to produce maximum output given a set of inputs and technology while technical inefficiency relates to the failure to attain highest possible level of output given input and technology. Technical efficiency range between 0 and 1, a TE = 1 implies technically efficient production (on the frontier) while TE < 1 implies varying degrees of technical inefficiency (Vu, 2010).

$$TE_j = {}_{\theta_j^{CRS},\ x}^{Min}\theta_j^{CRS}$$

$$\theta_j^{CRS} X_i > X\lambda$$

$$\lambda \geq 0$$

Where: X = Input vector, Y = Output vector, θ_j^{CRS} = Technical efficiency of farm j under CRS.

2.4 Estimation of Scale Efficiency

Scale efficiency is estimated by taking the ratio of the two efficiencies measured above where scale efficiecy also lie between 0 and 1 (0≤SE≤1). SE = 1 implies efficient economy of scale, SE<1 implies that inputs are not scale efficient which can be a case of either increasing or decreasing returns to scale.

$$SE_j = \frac{\theta_j^{CRS}}{\theta_j^{VRS}} = \frac{TE_j}{EE_j}$$

Where: θ_j^{CRS}= Technical Efficiency under CRS and θ_j^{VRS}= Technical Efficiency under VRS.

However, Vu (2010) stated that the scale efficiency can also decompose farms with scale inefficiency into either increasing returns to scale (IRS) or decreasing returns to scale (DRS) simply by imposing a non-increasing returns to scale (NIRS) to the DEA by adding another convexity constraint ($\sum_{j=i}^{n} \lambda_j \leq 1$) to the to the first TE equation.

$$TE_j = \theta_j^{NIRS} = {}_{\theta_j^{CRS},\ x}^{Min}\theta_j^{CRS} + \sum_{j=i}^{n} \lambda_j$$

Where: $\sum_{j=i}^{n} \lambda_j \leq 1$, θ_j^{NIRS}= Technical Efficiency under non-increasing returns to scale and other variables as defined earlier.

The decision rules are: if $\theta_j^{NIRS} = \theta_j^{VRS}$ and SE_j<1, the farm is operating with decreasing returns to scale (DRS) otherwise increasing returns to scale (IRS) if $\theta_j^{NIRS} < \theta_j^{VRS}$

2.5 Free Disposal Hull (FDH) Estimator

The FDH estimator was introduced by Deprins et al. (1984), which is both a deterministic and non-parametric tool for measuring productive efficiency. It is deterministic due to its inability to accommodate stochastic properties, its non- parametric nature arise from its lack of functional form specification. Like the DEA estimator, the FDH is also very sensitive to outliers/ extreme observations, susceptible to dimensionality problems and highly sensitive to noise. However, the FDH and the DEA estimators differ substantially in that the DEA estimators assumes convex nature of production relationship; in the FDH such assumption is relaxed, thus no convexity is assumed.

2.6 Derivation of FDH Estimator

The derivation of FDH presented here is in line with De Borger et al. (1994). Suppose $y = y(y_1, y_2, \ldots, y_n)$ denote n non-negative outputs produced by utilizing numerous m non-negative inputs $X = X(X_1, X_2, \ldots, X_n)$ combination. Thus, the production possibility set Yrefers to the set of all combinations of inputs and outputs that are technically feasible, as shown below:

$$Y = \{(x, y) | x \in R_+^n, y \in R_+^n, (x, y) is\ feasible\ \}$$

Conveniently, the production technology can be modeled by an input correspondence$y \rightarrow L(y) \subseteq R_+^n$. For a specific vector of output y, the level set $L(y)$ represents the subject of all input vectors X that produce a minimum of the output vector y. Various production technologies can be defined by subjecting the level set $L(y)$ to various restrictions. While there may be some variations in the non-parametric estimators, regarding

imposition of restrictions or assumptions, but generally they are less restrictive or have very weak assumptions than the parametric approaches. FDH estimator can be defined by the axioms below:

$$0 \notin L(y)\ for\ y \geq 0, and\ L(0) = R_+^n \quad \text{Axiom 1}$$

Axiom 1 assumes that it is not possible to obtain semi positive output from a null input vector. Thus, no such thing as free production and that any non-negative input yields a minimum of zero level of output.

$$if \parallel y^l \parallel \rightarrow +\infty\ as\ l \rightarrow +\infty, then\ \bigcap_{l=1}^{+\infty} L(y^l)\ is\ empty \quad \text{Axiom 2}$$

Axiom 2 states that for any utilization of finite inputs, finite outputs are produced.

$$if\ x\ \in L(y) and\ x' \geq x, then\ x' \in L(y) \quad \text{Axiom 3}$$

Axiom 3 is called positive monotonicity or strong free disposability of inputs; implying that an increase in input *x* cannot lead to a decrease in output *y*.

$$L(y)\ is\ a\ closed\ correspondence \quad \text{Axiom 4}$$

The closedness axiom 4; states that an array of input vectors can each yield output bundle y and converge to x^*, then the same x^*can also yield output bundle *y*.

$$if\ y' \geq y, then\ L(y') \subseteq L(y) \quad \text{Axiom 5}$$

The last axiom (strong free disposability of output) provides for variable returns to scale and assumes any reduction in output bundles remain producible with the same quantity of input bundles. The specification of the FDH input correspondence is thus:

$$L(y)^{FDH} = \{x | x \in R_+^m, Z'N \geq y, Z'M \leq x, I_k'Z = |Z_i \in \{0,1\}\}$$

Where N represents kxn matrix of observed outputs, M represents kx1vector of intensity and I_k represents kx1 vector of ones. Hence, it is obvious that the axioms did not impose convexity assumption on the technology. Using the axioms, the specification of FDH output correspondence can be given as below:

$$P(x)^{FDH} = \{y | y \in R_+^n, Z'N \geq y, Z'M \leq x, I_k'Z = |, Z_i \in \{0,1\}\}$$

From the last two equations, the FDH graph correspondence can finally be defined with respect to either input or output correspondence as follows:

$$GR^{FDH} = \{(x,y) | x \in L(y)^{FDH}, x \in R_+^m, y \in R_+^n\}$$
$$= \{(x,y) | y \in P(x)^{FDH}, x \in R_+^m, y \in R_+^n\}$$

2.7 Order-alpha (α) Estimator

Order-alpha (α) is a generalization of the FDH estimator but in a different manner. While the FDH uses the concept of minimum input consumption among available peers for benchmarking, the order-α employs the (100-α)th percentile approach (Tauchmann, 2011).

$$\hat{\theta}_{\alpha i}^{oA} = \overset{P(100-\alpha)}{\underset{j \in B_i}{}} \left\{ \underset{k=1,\ldots,k}{max} \left\{ \frac{x_{kj}}{x_{ki}} \right\} \right\}$$

If α =100, both order-α and FDH gives the same output, while for values of α<100, some super-efficient firms may result and un-enveloped by the estimated production possibility frontier. The α is to order-α estimator what *m* is to order-m estimator; thus a decision (tuning) parameter that influence the output of the estimator.

2.8 Order-M Estimator

The order-m estimator is another non-convex and non-parametric estimator that is known for its important property of achieving root-n ($\sqrt{n}$) consistency that aid the estimator to circumvent the problem of dimensionality associated with the traditional non-parametric estimators such as the DEA. Using the order-m estimator to estimate $\mathcal{P}^{\partial t}$ will alter the root-n consistency property by losing it completely, to maintain the property, $\mathcal{P}_m^{\partial t}$ should be estimated instead. Order-m estimator provides robust estimates in relation with noise and outliers in the data set; for finite m, order-m estimates are more robust than DEA or FDH estimators and as m tend to infinity (∞), the order-m estimator converges to FDH estimator (Wheelock & Wilson, 2003).

2.9 Derivation for Order-M Estimator

Order-m is estimated based on expected maximum output frontiers; this helps to relax the convexity assumption and allow for noise (with zero expected value) in the output measures (Wheelock & Wilson, 2003). Remember, that the density $\mathfrak{f}^t(x,y)$ exerts bounded support on the production set $\mathcal{P}^t$. Then, the conditional distribution

function for the density $\mathfrak{f}^t(x,y) is\ \mathbb{F}^t_{y/x}(y_0|x_0) = \mathbb{P}_r(y \leq y_0 | x \leq x_0)$. Given level of inputs x_0 within the region of *x* and considering *miid* random variables $\{V_j\}^m_{j=1,} V_j \in \mathbb{R}^q_+$, drawn from the earlier stated conditional distribution $\mathbb{F}^t_{y/x}(\cdot\,|x_0)$, define the set as below:

$$\mathcal{A}^t_m(x_0) = \left\{ (x,y) \in \mathbb{R}^{p+q}_+ | x \leq x_0, \quad \bigcup_{j=1}^{m} y \leq \mathbb{V}_j \right\}$$

Where $\mathcal{A}^t_m(x_0)$ is random and depends on the specific draw of m vectors from the conditional distribution $\mathbb{F}^t_{y/x}(\cdot\,|x_0)$. To define the random distance function, see below:

$$\mathbb{D}\big(x,y|\mathcal{A}^t_m(x)\big) \equiv inf\{\theta > 0 | (x, y/\theta) \in \mathcal{A}^t_m(x)\}$$

For any value of $y \in \mathbb{R}^q_+$, provides the expected maximum output level of order-m for all values of *x* in order that:

$$\mathfrak{f}^t_x(x) = \mathfrak{f}^t(x,y) | \mathfrak{f}^t\big({}^{y}/_{x}\big) > 0 as y^{\partial t}_m(x) \equiv y | \mathbb{E}\big[\mathbb{D}\big(x,y|\mathcal{A}^t_m(x_0)\big)\big]$$

Thus, above is the output-oriented analog of input measure (Cazals et al., 2002). To form the order-m analog of $\mathcal{P}^t$, define as follows:

$$\mathcal{P}^t_m \equiv \big\{(x,y) | (x,y) \in \mathcal{P}^t, y \leq y^{\partial t}_m(x)\big\}$$

Above represents expected production set of order-m and finally, we denote of the compliment of $\mathcal{P}^t_m$ as $\mathcal{P}^{\partial t}_m$ and name it the order-m frontier.

To facilitate the understanding of order-m concept, consider (x,y) lying within $\mathcal{P}^t$. The projection of (x,y) onto the frontier $\mathcal{P}^{\partial t}$ is given as $\big(x, y|\mathbb{D}(x,y|\mathcal{P}^t)\big)$, given that the input bundles $x, \mathbb{D}(\mathcal{P}^t)^{-1}$ is the maximum feasible proportionate increase in output bundles, y. Other way, $y^{\partial t}_m(x)$ is the expected maximum output bundle (with equal output proportions as *y*) among *m* firms selected at random, on the condition that their inputs are equal to or less than *x*. Vividly, $y^{\partial t}_m(x) \leq y | \mathbb{D}(x,y|\mathcal{P}^t)$, and it can be shown that:

$$lim_{m\to\infty}\, y^{\partial t}_m(x) = y | \mathbb{D}(x,y|\mathcal{P}^t)\ and\ thus\ \ \mathcal{P}^t_m \to \mathcal{P}^t as\ \ m \to \infty.$$

Unlike the traditional non-parametric estimators that compares or benchmarks the output of a given firm to the maximum feasible output in the sample, the order-m estimator compares the firm's observed output bundles to what could be expected from any m randomly selected firms that utilize no more input bundles than the given firm.

2.10 Monte Carlo Technique for Order-M Estimator

Cazals et al. (2002) introduced a simple Monte Carlo technique; a simulation method for generating the order-m estimates of $\mathbb{E}\big[\mathbb{D}\big(x,y|\mathcal{A}^t_m(x)\big)\big]$ and hence $y^{\partial t}_m$. The random distance function for a specific draw $\{\mathbb{V}_j\}^m_{j=1}$ can be computed by:

$$\mathbb{D}\big(x,y|\mathcal{A}^t_m(x)\big) = \min_{j=1,\dots,m}\left[\max_{l=1,\dots,q}\left(\frac{yl}{v_j l}\right)\right]$$

Where yl and $v_j l$ denote the l^{th} elements of y and v_j implementing the Monte Carlo method requires drawing vitiates v_j from the empirical analog of the conditional distribution earlier stated $\mathbb{F}^t_{y/x}\big({}^{\cdot}/_{x_0}\big)$ as shown below:

$$\widehat{\mathbb{F}}^t_{y/x,n_t}(y_0|x_0) = \frac{\sum_{i=1}^{n_t} \mathbb{I}\big(x_i \leq x_0, y_i \leq y_0\big)}{\sum_{i=1}^{n_t} \mathbb{I}(x_i \leq x_0)}$$

Where $(x_i y_i) \in \varsigma^t_{n_t} \forall\ i = 1, \dots, n_t$. If $\big(x_0, y_0\big)$ is the point of concern, the steps are as follows:

(i) From the observations in $\varsigma^t_{n_t}$, drawn samples m times, independently and with replacement in order that $x_i \leq x_0$; drop the input vectors and denote the sample of the remaining output vectors by $\{\mathbb{V}_{kj}\}^m_{j=1}$.

(ii) Compute $\widehat{\mathbb{D}}_k\big(x_0, y_0 | \varsigma^t_{n_t}, m\big) = \min_{j=1,\dots,m}\left\{\min_{l=1,\dots,q}\left(\frac{vk_j l}{yol}\right)\right\}$

(iii) Where $vk_j l$ and yol denotes the l^{th} elements of vk_j and y_0.

(iv) Iterate steps (i)-(ii) *k* times to obtain $\left\{\widehat{\mathbb{D}}_k\big(x_0, y_0 | \varsigma^t_{n_t}, m\big)\right\}^k_{k=1}$

(v) Compute $\widehat{\mathbb{D}}_{m,n_t}\big(x_0, y_0\big) = \widehat{\mathbb{D}}\big(x_0, y_0 | \varsigma^t_{n_t}, m\big) = \mathbb{K}^{-1} \sum_{k=1}^{k} \widehat{\mathbb{D}}_k\big(x_0, y_0 | \varsigma^t_{n_t}, m\big)$ and estimator of $\mathbb{E}\big[\mathbb{D}\big(x,y|\mathcal{A}^t_m(x)\big)\big]$. Thus, an estimator $\hat{y}^{\partial t}_{m,n_t}$ of $y^{\partial t}_m$ can be estimated by replacing $\mathbb{E}\big[\mathbb{D}\big(x,y|\mathcal{A}^t_m(x)\big)\big]$ with $\widehat{\mathbb{D}}_{m,n_t}\big(x_0, y_0\big)$.

2.11 Definition of Input and Output Variables

In this study, estimation of efficiency was based on 7 inputs and 2 outputs. The input and output variables are as follows:

X_1 = Farm size (land) (Ha)

X_2 = Farm maintenance (RM/yr) (Source: maintenance of roads, paths and bridges and maintenance of farm building)

X_3 = Fertilizer (Kg)

X_4 = Capital (RM/yr) (Sources: land tax, fuel cost for machines, maintenance of machines, tools and equipment, depreciation, establishment cost)

X_5 = Family labor (Man-hour/yr)

X_6 = Hired labor (RM) (Sources: major hired labor operations; harvesting and weeding (land clearing))

X_7 =Other costs (RM) (Sources: salt, brown sugar, palm kernel cake (PKC) medicine, vaccine and supplements)

Y_1=Fresh Fruit Bunches (FFB) yield (MT/yr)

Y_1 =Livestock (Number of stock/yr)

3. Results and Discussion

3.1 Outlier Elimination and Descriptive Statistics of Data Used for the Analyses

Figure II and III show the box and whiskers plots for goat-oil palm and cattle-oil palm integrated farms respectively constructed after outlier elimination in the data set. Note, all the data lies within the box region, none lies outside the box or whiskers region. The outlier elimination process helps to improve the validity or accuracy or robustness of the efficiency estimates.

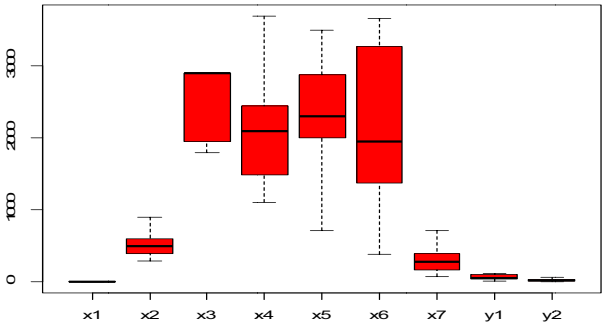

Figure II: Box and Whiskers plots for outlier detection and description of the statistical pattern of the data used for goat-oil palm integration

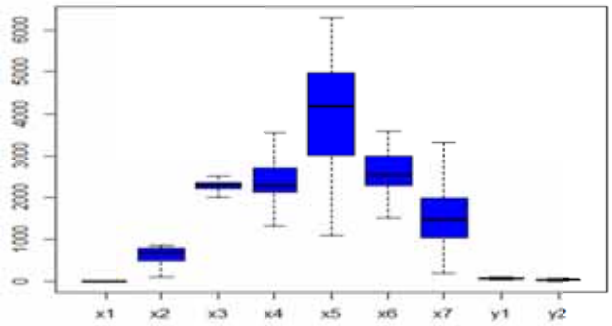

Figure III: Box and Whiskers plots for outlier detection and description of the statistical pattern of the data used for cattle-oil palm integration

Table 1 below also describes the statistical pattern or behavior of the data used for the efficiency analyses under goat-oil palm and cattle-oil palm integration respectively. In terms of farm size, the cattle-oil palm plantations maintain relatively larger farm size relative to the goat-oil palm plantations. The relatively larger farm size of the cattle-oil palm plantations perhaps explains its higher farm maintenance costs relative to goat-oil palm. The same reasoning of large farm size may be adduced for higher capital and hired labor under the cattle-oil palm relative to the goat-oil palm plantations.

Table 1. Descriptive Statistics of data used for both goat-oil palm and cattle-oil palm integration systems

Variable	Definition	Goat-Oil palm integration system				Cattle-Oil palm integration system			
		Minimum	Maximum	Mean	St.Dev.	Minimum	Maximum	Mean	St.Dev.
X1	Land (ha)	1.20	6.00	3.64	1.31	2.50	7.00	4.05	0.41
X2	Farm maintenance (RM/yr) (Sources: maintenance of roads, paths and bridges and maintenance of farm building)	290.00	900.00	510.30	168.21	120.00	850.00	624.70	172.14
X3	Fertilizer (Kg)	1800.00	2900.00	2480.00	480.06	2000.00	2500.00	2293.00	139.345
X4	Capital (RM/yr) (Sources: land tax, fuel cost for machines, maintenance of machines, tools and equipment, depreciation, establishment cost)	1106.00	3700.00	2034.00	649.05	1309.00	3563.00	2414.00	449.20
X5	Family labor (Man-hour/yr)	720.00	3500.00	2412.00	672.30	1080.00	6300.00	3954.00	1005.86
X6	Hired labor (RM) (Sources: major hired labor operations; harvesting and weeding (land clearing))	390.00	3660.00	2135.00	987.09	1500.00	3660.00	2658.00	560.06
X7	Other costs (RM) (Sources: salt, brown sugar, medicine, vaccine and supplements)	75.81	716.00	318.11	176.47	210.00	3312.00	1519.00	631.38
Y1	Fresh Fruit Bunches yield (MT/yr)	10.00	116.00	66.81	32.17	50.00	120.00	88.58	18.67
Y2	Livestock (Number of stock)	2.00	63.00	27.00	6.71	10.00	80.00	40.00	15.17

Source: Field survey (2012).

The farm size index and stocking rate of animals are important in explaining the variation in use of fertilizer, family labor and other costs. For instance, the fact that the cattle-oil palm plantations keep more animals relative to the goat-oil palm plantations in addition to the fact that the cattle deposit more dung than the goat as source of organic manure explains why the cattle-oil palm plantations apply lower levels of inorganic fertilizer in relation to the goat-oil palm plantations. Other source of variation in other costs in addition to farm size and stocking rate is the use of palm kernel cake (PKC) in the cattle-oil palm system while no evidence of it been use in the goat-oil palm system. The fact that 93% of the farmers under cattle-oil palm scheme are from FELDA as against 43% under goat-oil palm system may be the rationale behind higher yield of 88.58MT/yr in former relative to 66.81 MT/yr in the latter.

3.2 Results of Estimation of Technical Efficiency Based on DEA Estimator

Table 2 presents technical efficiency scores disaggregated according to variable returns to scale, constant returns to scale and scale efficiency assumptions under goat-oil palm integration. Overall, the technical efficiency estimates show that all the plantations operate between an efficiency range of 0.958 and 1.000 with a mean score of 0.997. On average all plantations produced at 99.7% efficiency which also translates to 0.3% inefficiency. The mean

efficiency estimate implies that under present production technology, goat-oil palm integrated plantations can on average potentially withdraw supply of input by 0.3% and still produce the same level of output bundle. This means that only 0.3% inefficiency level is present; suggesting very low prospects withdrawing the supply of inputs in order to enhance efficiency. The narrow efficiency range observed is an indication that there is no wide variation in yield among the plantations.

Table 2. Technical efficiency estimates disaggregated according to VRS, CRS and SE assumptions across goat-oil palm and cattle-oil palm integration based on DEA estimator

Efficiency range	TE-$_{VRS}$(PTE)	TE-$_{CRS}$(OTE)	SE
Goat-oil palm integration (N=65)			
<0.50	0	0	0
0.51-0.60	0	0	0
0.61-0.70	0	0	0
0.71-0.80	0	0	0
0.81-0.90	0	4 (6.15)	4(6.15)
0.91-0.99	10 (15.38)	26 (40.0)	25 (38.46)
1.00	55 (84.62)	35 (53.85)	36 (55.38)
Summary statistics			
Minimum	0.958	0.802	0.802
Maximum	1.000	1.000	1.000
Mean	0.997	0.977	0.979
Standard deviation	0.009	0.039	0.038
Cattle-oil palm integration (N=190)			
<0.50	0	0	0
0.51-0.60	0	0	0
0.61-0.70	0	0	0
0.71-0.80	0	0	0
0.81-0.90	0	0	0
0.91-0.99	0	0	0
1.00	190 (100.00)	190 (100.00)	190 (100.00)
Summary statistics			
Minimum	1.000	1.000	1.000
Maximum	1.000	1.000	1.000
Mean	1.000	1.000	1.000
Standard deviation	0.000	0.000	0.000

Source: Field Survey (2012).

In other words, the plantations seem to operate at relatively uniform yield. In comparison with the table for goat-oil palm integration, the cattle-oil palm integration shows all plantations as ostensibly efficient under all assumptions of VRS, CRS and SE and an average technical efficiency of 1.000 resulted. This means that the present production method and technology provides no room for input withdrawal (reduction) in order to produce the present level of output bundle; thus, suggesting no inefficiency at all. These very high technical efficiency levels estimated under both goat-oil palm and cattle-oil palm integrated systems in Table 2 is not surprising as many of these plantations had won productivity awards in the past and their many years of experience could be an added rationale for high

technical efficiency. Other reasons for the high technical efficiency could be the assumption surrounding the DEA estimator; the DEA is not robust to noise (De Witte & Marques, 2010), hence factors beyond farmers' control which cause inefficiency cannot be estimated using the DEA. Based on the foregoing limitations of the DEA, the study explored other efficiency estimators in an attempt to derive robust estimates.

The CRS assumption presents relatively lower estimates which range between 0.802 and 1.000 with a mean of 0.977; suggesting 97.7% efficiency level or 2.3% inefficiency level. These lower estimates under CRS relative to VRS assumption is in consonance with theory as the enveloping surface is tighter under CRS; thus, permitting lower efficiency estimates. Note also the percentage of plantations that attained 100% efficiency reduced from 55% under VRS down to 35% under CRS. The scale efficiency, like the CRS estimates also range between 0.802 and 1.000 with mean of 0.979. Thus, high levels of average scale efficiency, but comparing the scale efficiency scores with the pure technical efficiency (VRS estimates) in line with Padilla and Nuthall (2012), on average the SE (0.802) estimated here is lower than the PTE (0.997). In accordance with Padilla and Nuthall (2009), in the present study the lower value of SE against PTE implies that rather than managerial problems, scale of production or the small nature of farm size appear to be the major cause of inefficiency. Increase in scale of production is indeed an avenue to improving the efficiency in the goat-oil palm integrated system.

3.3 Results of TE Estimation Based on FDH, Order-α and Order-m Estimators

Table 3. Technical efficiency estimates based on FDH, order-alpha and order-m estimators for goat-oil palm and cattle-oil palm integration

Efficiency range	$TE_{FDH\text{-}Estimator}$	$TE_{ORDER\text{-}\alpha\text{-}Estimator}$	$TE_{ORDER\text{-}M\text{-}Estimator(NREP=2000)}$
Goat-oil palm integration (N=65)			
< 0.50	0	0	0
0.51-0.60	0	0	0
0.61-0.70	0	0	0
0.71-0.80	0	0	0
0.81-0.90	0	0	0
0.91-0.99	0	45 (69.23)	38 (58.46)
1.000	65 (100.00)	20 (30.77)	27 (41.54)
Summary			
Min	1.000	0.970	0.941
Max	1.000	1.000	1.000
Mean	1.000	0.998	0.990
Std. Dev.	0.000	0.004	0.016
Cattle-oil palm integration (N=190)			
< 0.50	0	0	0
0.51-0.60	0	0	0
0.61-0.70	0	0	0
0.71-0.80	0	0	0
0.81-0.90	0	0	10 (5.26)
0.91-0.99	0	170 (89.47)	142 (74.74)
1.000	190 (100.00)	20 (30.77)	38 (20.00)
Summary			
Min	1.000	0.910	0.853
Max	1.000	1.000	1.000
Mean	1.000	0.998	0.972
Std. Dev.	0.000	0.008	0.033

Source: Field Survey (2012).

Table 3 captures technical efficiency estimates based on three (FDH, order-α and order-m) estimators for goat-oil palm integrated plantations. The FDH estimator shows that all plantations are fully efficient (100%) showing no potentiality for inefficiency. This result is expected since unlike the DEA, the FDH estimator relaxes the convexity assumption and hence results to higher estimates relative to the DEA estimator. Similar result of 100% average efficiency was also estimated under the cattle-oil palm integrated plantations. Several studies such as De Borger et al. (1994) and De Witte and Marques (2010) that compared FDH and DEA estimators on the same data set reported higher FDH estimates as against the DEA estimates, purely due to the difference in convexity assumption.

In terms of order-α estimator, based on α-value = 0.95, under goat-oil palm integration shows estimates of 0.970, 1.000 and 0.998 as minimum, maximum and mean efficiency estimates respectively. This 99.8% level of average efficiency further implies only 0.2% inefficiency level to be adjusted. The result of mean efficiency under the goat-oil palm integrated plantation is consistent with that of cattle-oil palm integrated plantations; 0.910, 1.000 and 0.998 for minimum, maximum and mean scores were estimated. The order-m estimates allowing for 2000 bootstrap iteration for goat-oil palm system provided 0.941, 1.000 and 0.990 as estimates for minimum, maximum and average efficiency scores. Similarly, the order-m estimates under cattle-oil palm integration estimated in the table were 0.853, 1.000 and 0.972 as minimum, maximum and mean efficiency scores respectively. Comparing the results of TE scores under the four estimators (DEA, FDH, order-α and order-m), it can be observed that the mean scores did not vary much across the four estimators and under both goat and cattle integrated plantations. Results presented above all concur that under both goat and cattle integrated with oil palm, optimal production results and under both systems, farmers produce with less inefficiency level. Note that TE scores estimated under the partial estimators show lower values relative to those predicted by the full estimators. Thus, the full estimators show higher level of inefficiency relative to the partial estimators. This is not surprising considering the assumptions surrounding the partial estimators, which embeds some degree of bias associated with agricultural production that gives a proportion of factors beyond farmers control in its estimation aside the inefficiency itself. The foregoing attributes is not inherent in full frontier estimators, hence the relatively higher TE estimates.

4. Conclusions

The study revealed that the livestock-oil-palm integrated farms either operate close to the frontier or on the frontier; suggesting viable enterprise combination. The mean efficiency estimate implies that under present production technology, goat-oil palm integrated plantations can on average potentially withdraw supply of input by 0.3%, 0.0%, 0.2% and 1.0% under DEA, FDH, order-α and order-*m* estimators respectively and still produce the same level of output bundle. Similarly, the cattle-oil palm integrated plantations can on average potentially withdraw input supply by 0.0%, 0.0%, 0.2% and 2.8% under DEA, FDH, order-α and order-*m* estimators respectively and still produce the same level of output bundle. This in general connotes that livestock-oil palm integration is an optimal system of agricultural production and the farmers produce with minimal inefficiency level.

Based on the mean comparison in this study across the four estimators, it revealed a non-significant statistical difference in mean TE across the estimators used. However, relative to the nature of data used in this study, the FDH and the DEA estimators seem to show better result but in terms of capturing bias associated with agricultural production, the order-m and order-α are more suitable. The study also found small scale of farm operation as the main cause of inefficiency and suggested increase in farm size as medium for improving efficiency in the livestock-oil palm integration system.

Reference

Abatania, L. N., Hailu, A., & Mugera, A. W. (2012). *Analysis of farm household technical efficiency in Northern Ghana using bootstrap DEA*. A paper presented at the 56th annual conference of the Australian Agricultural and Research Economics Society. Fremantle W A, 7-10 February.

Amor, T. B., & Muller, C. (2010). Application of stochastic production frontier in the estimation of technical efficiency in irrigated agriculture in Tunisia. *Agricultural Journal, 5*(2), 50-56. Medwell pubishers. http://dx.doi.org/10.3923/aj.2010.50.56

Cazals, C., Florens, J. P., & Simar, L. (2002). Non-parametric frontier estimation: A robust approach. *Journal of econometrics, 106*, 1-25. http://dx.doi.org/10.1016/S0304-4076(01)00080-X

De witte, K., & Marques, R. C. (2010). *Influential observations in frontier models, a robust non-oriented approach to the water sector*. Ann Oper Res. http://dx.doi.org/10.1007/s10479-010-0754-6

DeBorger, B., Kerstens, K., Moesen, W., & Vanneste, J. (1994). A non-parametric free disposal hull (FDH) approach to technical efficiency: An illustration of radial and graph efficiency measures and some sensitivity results. *Swiss Journal of Economics and Statistics, 130*(4), 647-667.

Depriens, D., Simar, L., & Tulkens, H. (1984). Measuring labour efficiency in post offices. In M. Marchand (eds.), *The performance of public enterprises: Concepts and measurements* (pp. 243-267). Amsterdam: Elsevier.

MPOC. (2013). *Malaysian Palm Oil Council*. Annual Statistics, Annual Report, Kuala Lumpur Malaysia

Mugera, A. W. and Featherstone, A. M. (2008). Backyard hog production efficiency. Evidence from the Philippines. *Asian economic journal, 22*(3), 267-287. http://dx.doi.org/10.1111/j.1467-8381.2008.00278.x

Padilla-Fernandez, M. D., & Nuthall, P. L. (2009). Technical Efficiency in the production of sugarcane in central Negros are, Philippines: An application of data envelopment analysis. *Journal of International Society for Southeast Asian Agricultural Sciences (J.ISSAAS), 15*(1), 77-90.

Tauchmann, H. (2011). *Partial frontier efficiency analysis for stata*. Discussion Paper by Rheinisch-West f˙alisches Institutf˙ur Wirtschaftsforschung (RWI) Hohenzollernstraße 1-3, 45128 Essen, Germany.

Vu, L. H. (2010). *Efficiency of Rice Farming Households in Vietnam: A DEA with Bootstrap and Stochastic Frontier Application*. University of Minnesota, USA.

Wheelock and Wilson. (2003). *Robust non-parametric estimation of efficiency and technical change in US commercial Banking*. Working Paper by The Federal Reserve Bank of St Loius.

Wilson, W. P. (2010). *Asymptotic Properties of some non-parametric hyperbolic efficiency estimators*. Clemson computing and information technology group, Clemson University, USA.

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Bacillus subtilis LBF02 as Biocontrol Agent Against Leaf Spot Diseases Caused by *Cercospora lactucae-sativae* in Lettuce

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Abstract

More than 51 isolates of bacteria were obtained from leaves, crushed leaves and rhizosphere of lettuce plants. The bacteria isolates were purified and assayed against *Cercospora lactucae-sativae* on PDA plate by dual culture technique. Four isolates showed zone of inhibition against the pathogens. The antagonistic bacteria isolate LBF02 showed the highest percentage of growth inhibits against *C. lactucae-sativae* leaf spots with 80.82% inhibition, compared with the control. Based on morphological and biochemical tests, isolate LBF02 was identified as belonging to the *Bacillus subtilis* group. The LBF02 isolate was chosen for the formulation development. The formulation contained 40 ml of cell suspension, 89 g of rice flour, 1 ml of vegetable oil and 10 g of sucrose. The biocontrol of leaf spot diseases was tested by using a formulation applied in greenhouse experiments. The result showed that spraying 1 hour before or after the pathogen inoculation on lettuce plants was more effective in suppressing leaf spot disease than simply pathogen inoculation alone. Moreover, the antagonistic bacteria in formulation have the ability to survive for more than 6 months under storage at room temperature and survive for up to 15 days on lettuce leaves.

Keywords: biocontrol, *Cercospora lactucae-sativae*, *Bacillus subtilis*, lettuce

1. Introduction

Lettuce (*Lactuca sativa* L.) is the most popular leafy salad vegetable in Thailand. But, the vegetable is susceptible to leaf spot disease caused by *Cercospora lactucae-sativae*; this is disease that has important economic implications around the world and normally, this disease has been a major damage-causing one in Thailand (To-Anun et al., 2011). The effects of the pathogen are reduced yield and low qualities produce of the lettuce in greenhouses, hydroponics and fields (Hsieh & Goh, 1990). The fungal *Cercospora* sp. give rise to the leaf spot disease on numerous host plants in tropical regions and an increase in the disease usually occurs in the rainy season (Agrios, 2004). Crowded planting, high humidity and bad ventilation are conducive to the disease outbreak (Chupp, 1954; Koohakan et al., 2008). In the year 2004, due to an attack of just the *Cercospora longissima* comm losses of up to 68% were reported in the specific conditions (Gomes et al., 2004). All these antagonistic bacteria that have been isolated from the soil surrounding the plants and from the plant surfaces (Weller, 1988; Kim et al., 1997; Sindhu et al., 2002; Todorova & Kozhuharova, 2010) are active under the general mechanism of competitive exclusion or reduction of growth by other microorganisms, which is the interference process of the pathogens. Moreover, the antagonistic bacteria manufacture a diverse range of secondary metabolites due to enzymatic activity and therapeutics due to various mechanisms of secretion and are capable of catalyzing various biochemical reactions with novel enzymes of lytic enzymes, siderophores and antibiotics (Das et al., 2006). One of the bacteria biocontrol agents that have received much attention is the genus *Bacillus*. The *Bacillus* sp., because they produce active antagonistic metabolites, is abundant in soils and readily form endospores that survive under adverse environmental conditions (Silo-Suh et al., 1994). *Bacillus subtilis* showed inhibition against *Cercospora beticola* (Lindow & Brandl, 2003), *Cercospora beticola, Colletotrichum gloeosporioides* (Collins et al., 2003), *Pseudocercospora purpurea* (Eeden & Korsten, 2006) and *Rhizoctonia solani* Kühn (Kai et al., 2007). The antagonistic bacteria were chosen for formulation development such as for the formulation development of powder formulation and granule formulations of bacteria and used for controlling fungi growth (Chumthong et al., 2008; Kim et al., 2007; Lee et al., 2006). The formulations were applied by spraying on the plant leaves in the greenhouse experiments and fields.

2. Materials and Methods

2.1 Microorganisms and Screening of Antagonistic Bacteria

The fungus *C. lactucae-sativae* was isolated from lettuce by single spore isolation method (Choi et al., 1999) and maintained on V-8 juice agar (V8) medium. The antagonistic bacteria were isolated from lettuce leaf samples that were healthy. There were isolated by leaf wash technique using 10 g of leaf with 100 ml sterile distilled water. The bacteria isolated from crushed leaves, 3 g of plant leaf mixture was treated with 30 ml sterile distilled water. For the bacteria isolation from rhizosphere by soil dilution plate method, 1 g of soil rhizosphere was suspended in 10 ml of sterile distilled water and shaken at 180 rpm for 2 hours. Subsequently, they were serial diluted to 10^{-3} fractions and spread on plate under nutritive agar (NA) incubation at room temperature for 48 hours. The single colony of bacteria was selected and re-isolation was performed on NA until pure culture was obtained.

2.2 Dual Culture Inhibition Assays

Tests were performed on antagonistic bacteria isolates for inhibition effect against *C. lactucae-sativae* in potato dextrose agar (PDA). The antagonistic bacteria were touch single colony with paper dish at the defined medium. *C. lactucae-sativae* NH04 isolates were then inoculated on either side of the bacterial growth in four replicate plates incubated at room temperature for 30 days, assessed by measuring the size of the inhibition zone.

2.3 Inhibition Against Germination of Spores of Fungus

The antagonistic bacteria were cultured in 50 ml NGA broth and shaken at 160 rpm for 2, 4 and 6 days. The culture was separated by centrifugation at 5,000 rpm for 5 min at 4^{o}C. The cultures were then filtered using Whatman No. 1 filter paper. The supernatants of filtrated culture medium (FM), non-filtrated culture medium (NFM), NA and sterile distilled water were mixed with the spores of the fungus in the ratio 1:1 and spread on plate on water agar (WA). The spore was examined for germination at 3, 6, 9, 12 and 24 hrs, and the percentage of germination of the spores was counted.

2.4 Identification-Biochemical Studies

The test of the ability of the study bacteria LBF02 to isolate for physiological and biochemical characterization was carried out as described in *Bergey's Manual of Systematic Bacteriology* (Holt et al., 1984; Todorova & Kozhuharova, 2010; Zheng et al., 2007). The following sources of carbon were used: glucose, lactose, maltose, fructose, sucrose, mannitol, sodium citrate and urease. The remaining physical-biochemical conditions were as follows: growth at different temperatures, growth at different concentrations of NaCl, reduction of nitrates, Voges-Proskauer test, methyl red test, formation of indole, disintegration of casein, disintegration of gelatin, hydrolysis of starch and catalatic activity.

2.5 Bio-Product of Formulation

To generate formulations of the bacteria LBF02 isolate, 3 ml culture of bacterial cells were inoculated into 150 ml of nutrient glucose broth (NGB) and shaken at 150 rpm at 28°C for 5 days. The cells were harvested by centrifugation under 4°C at 5,000 rpm for 10 min and washed with 0.85% (w/v) NaCl and then centrifuged at 3,500 rpm for 5 min. The formulation contained 40 ml of cell suspension LBF02, 89 g of rice flour, 1 ml of vegetable oil and 10 g of sucrose, with the mixture completely dried at 45°C in a drying oven for 12 hours and subsequently ground in a blender to form a powder. The formulation without the bacteria was prepared in an identical way and referred to as "control" and maintained at room temperature. The viability tests consisted of spray formulation on plant leaves for 15 days, carried out after formulation and at 2-month intervals during storage at room temperature (28 ± 2^{o}C). Two plant leaves were suspended in 10 ml of sterile distilled water and shaken at 160 rpm for 30 minutes, after which the cfu was counted. The value (cfu/ml) of viable bacteria was taken as the average of three replications (three drops) per dilution.

2.6 Greenhouse Testing of Formulation for Control of C. lactucae-sativae

The formulation was performed under greenhouse condition. The lettuce plants were grown in pots to become 30-days-old. The *C. lactucae-sativae* inoculum was found to grow on the V8 medium for 7 days at room temperature. The spore suspension obtained was adjusted to 10^4 conidia using a haemacytometer. The formulations were prepared as 1 g mixed with 100 ml sterile distilled water before being sprayed on the lettuce plants. The products were applied by spraying 1 hour before or after the fungal pathogen treatment and sprayed 3 days before or after the fungal pathogen treatment on the lettuce plants. The plants were maintained under controlled growth for 5 days. The treatment had four replications, with the plants in each pot arranged in a Completely Randomized Design (CRD). The disease severity index of the leaf spot symptoms was recorded and graded on a 0 - 10 scale of Poonponkun et al. (2007), with the modification rating scale as follows: 0: plants did not

show any symptoms, 1: 1-10%, 2: 11-20%, 3: 21-30%, 4: 31-40%, 5: 41-50%, 6: 51-60%, 7: 61-70%, 8: 71-80%, 9: 81-90% plants showed evidence of spot symptoms and 10: 91-100% plants exhibited spot symptoms.

3. Results and Discussion

3.1 Microorganisms and Screening of Bacillus sp. Strains With an Antifungal

The fungus *C. lactucae-sativae* nine isolates were found on the leaf spot diseased lettuce. A total of 51 isolates were bacteria isolated from leaves, crushed leaves and rhizosphere of healthy lettuce. Four isolates of the antagonistic bacteria showed the highest percentage of inhibition of the pathogens. All of them were obtained from the crushed leaves as LBF02 and LBF03 isolates and from the rhizosphere as SRR02 and SRF08 isolates.

3.2 Dual Culture Inhibition Assays

The results of screening for antagonistic bacteria against *C. lactucae-sativae* produced 51 antagonistic bacteria isolates on PDA using the dual culture technique. Inhibition assays were conducted on the four isolates by forming zones of inhibition. These antagonistic bacteria isolates which were LBF02, LBF03, SRR02 and SRF08, showed the highest percentage of growth inhibition of *C. lactucae-sativae* with 80.82%, 79.30%, 75.12% and 73.67%, respectively. The LBF02 isolated exhibited the most pronounced antagonism against *C. lactucae-sativae* when compared with the control. Todorova & Kozhuharova (2010) report that previous studies of the antagonistic *Bacillus subtilis* strains TS 01 and ZR 02, which were isolated from soil, reveal that they showed the highest antifungal activity against *Alternaria solani*, *Botrytis cinerea*, *Monilia linhartiana*, *Phytophthora cryptogea* and *Rhizoctonia* sp. Favorable results were also found by Korsten & Jager (1995) who demonstrated the efficiency of *Bacillus subtilis* (isolate B246), *Bacillus cereus* (isolates B247 and B249) and *Bacillus licheniformis* (isolate B248) in hibiting effectively avocado post-harvest pathogens *Colletotrichum gloeosporioides, Phomopsis perseae, Drechslera setariae, Pestalotiopsis versicolor* and *Fusarium solani.*

3.3 Inhibition Against Germination of Spores of Fungus

The 100 conidia of *C. lactucae-sativae* were made to undergo germination in 3 hours in the sterilized distilled and NB culture methods. The results of the treatment of the spores which were treated using non-filtrated culture medium (NFM) and filtrated culture medium (FM) methods at 2, 4 and 6 days revealed that the NFM of 2, 4 and 6 days old culture demonstrated inhibition of spore germination of *C. lactucae-sativae* with 20, 23 and 28 spores, respectively. At 6 hours of cell treatment, the germ tubes were swelling and at 9 hours the spores of *C. lactucae-sativae* stopped germinating. The treatments of FM at 2, 4 and 6 days old culture demonstrated inhibition of spore germination, with 7, 8 and 10 spores, respectively (Figure 1). The spores of *C. lactucae-sativae* mixed FM experienced a reduction in the spore germination after 9 hours compared with the sterilized distilled water and the NB culture methods. Alderman & Beute (1986) reported about the conidia of *Cercospora arachidicola* atomized onto peanut leaves which began germinating after 2 hours; in that experiment, the maximum germination that is 82-85% occurred within 24-48 hours. Additionally, Folman (2004) reported using *Lysobacter enzymogenes* strain 3.1T8 as a potential biocontrol agent of *Pythium aphanidermatum* in cucumber. The antagonistic bacteria showed the activity, hemolytic activity and the production of a surface active compound, which decreased in the media of increasing strength, one or more low molecular compounds, caused rapid immobilization of zoospores of *P. aphanidermatum* and inhibited cyst germination. Moreover, Bacon, and Hinton (2006) reported the antagonistic bacteria as having the ability produce diverse range of enzymatic activities which can be sources of secondary metabolites and therapeutics by various secretion mechanisms and capable of catalyzing various biochemical reactions with novel enzymes of lytic enzymes, siderophores and antibiotics.

3.4 Identification-Biochemical Studies

Based on the biochemical and morphological tests according to *Bergey's Manual of Systematic Bacteriology* (Holt et al., 1984; Todorova & Kozhuharova, 2010; Zheng et al., 2007), the detailed physiological characteristics of LBF02 were investigated and compared with *Bacillus subtilis*, as shown in Table 1. The strain LBF02 consisted of spore-forming, Gram-positive rod shaped, bacteria colonies which were convex colonies with wrinkled surface, circular, white-cream, entire and opaque on nutrient agar. Catalatic reaction was positive, as well as liquefaction of gelatin, starch hydrolysis, nitrate reduction, Voges-Proskauer test, urease, methyl red test, hydrolysis of casein, as well as characters like oxidase, anaerobic growth, acid and no gas formation from different sugars. However, oxidase reaction was negative; so were the results of the tests for indole and H_2S production. These data indicated that strain LBF02 resembled a member of the Bacillus genus. Fritze (2004) reported about the conspicuous morphological feature of endospore formation that has lent itself from early on as an easily recognized property for taxonomic differentiation. The genus *Bacillus* groups were surely the largest and the most prominent. Based on

morphology, physiological tests (Todorova & Kozhuharova, 2010; Mishra et al., 2009), biolog and the 16S rDNA sequence, bacteria strain ZJB-063 was identified as *Bacillus subtilis* (Zheng et al., 2007).

3.5 Bio-Product of Formulation

The antagonistic bacteria of formulation survival tests were counted using the drop plate method on day 0, 1, 3, 5, 7, 10 and day 15 after spraying the formulation on the lettuce leaves. The results was that the numbers of bacteria on the lettuce leaves were seen to decline over time, with the bacteria quantities at 2.67×10^4, 1.33×10^4, 1.0×10^4, 1.0×10^4, 8.35×10^3, 3.35×10^3 and 2.83×10^3 cfu/ml respectively. The antagonistic bacteria in formulation was observed to survive more than 6 months under storage at room temperature and in the sprayed formulation on the leaves the antagonistic bacteria were seen to survives for up to 15 days on the lettuce leaves. Chumthong et al. (2008) reports that the result of the granule formulation of *B. megaterium* because of spraying with the formulation on leaf sheaths and leaf blades at the 7 days was that the number of bacteria on the surfaces of both the rice tissues was observed to be approximately 10^6 cfu/g of the plant.

3.6 Greenhouse Testing of Formulation for Control of C. lactucae-sativae

The tests conducted in the greenhouse experiments analyzed the effectiveness of formulation from isolate LBF02 on lettuce diseases, *C. lactucae-sativae* on lettuce that had grown up to 30-days. Upon evaluation of the damage according to the disease severity, when the data were analyzed statistically and compared, the mean of each treatment by CRD (completely randomized design) the confidence level was found to be 95 percent. The evaluations effective of nine treatments were tested the formulation on lettuce leaves. The formulation LBF02 was used by spraying it 1 hour before or after and by spraying 3 days before, the pathogen infection was found to have significantly reduced the infection indexes to 1.62, 1.87 and 2.12 respectively, whereas the control was found to be infested with 4.62. The inhibition is significantly at the 95 percent confidence level when compared with the control (Table 2 and Figure 2). Dhitikiattipong et al. (2011) reports that the result efficiency of powder formulation of antagonistic bacteria to control rice bakanae disease bacterized with BAK-131 and BAK-088 demonstrated bakanae incidences of 8.9% and 9.7%, respectively, which is comparable to the 8.2% of the mancozeb+carbendazim treatment and the 10.9% of the control treatment. In addition, Arunyanart et al. (2008) applied the powder formulation of the antagonistic bacteria *Bacillus subtilis* No. 33 and tested their effectiveness against the fungi *Curvularia lunata* (Wakk.) Boed, *Cercospora oryzae* Miyake, *Helminthosporium oryzae* Breda de Haan, *Fusarium semitectium* Bark & Rav, *Sarocladium oryzae* Sawada and *Trichoconis padwickii* Ganguly. Moreover, *Pseudomonas fluorescens* strain Pf1 was developed as powder formulation and applied as a seed treatment and foliar spray for the bacteria on the leaves. This has effectively controlled the *Pyricularia oryzae* disease and increased the grain yield (Vidhyasekaran et al., 1997).

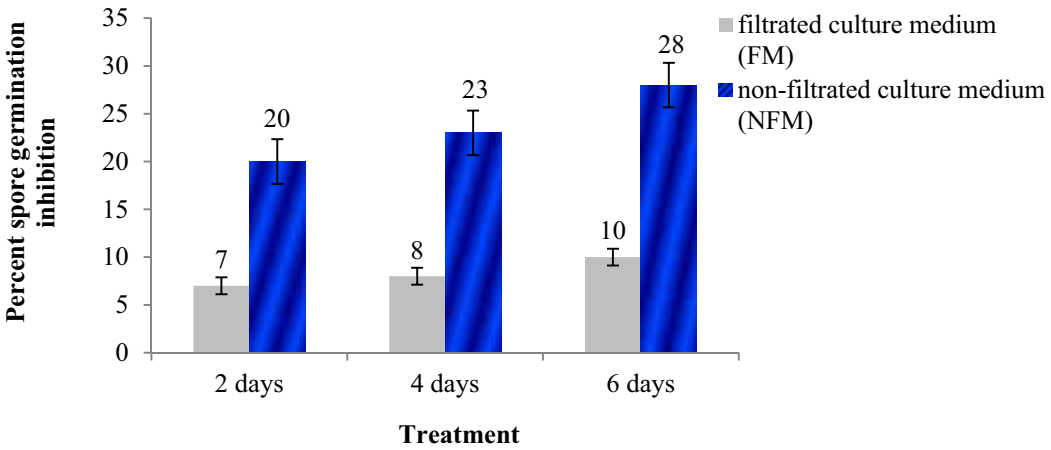

Figure1. The effect of 2 days, 4 days and 6 days old filtrated culture medium (FM), non-filtrated culture medium (NFM) treatment on the inhibition spores germination of *C. lactucae-sativae* on WA plates. The 100 percent of conidia *C. lactucae-sativae* germination took place under a condition of sterilized distilled water and NB culture treatment

Error bars represent the standard deviation of four replications.

Table1. Comparison of phenotypic characteristics for strain *B. subtilis* and LBF02 using conventional chemical tests

Characteristics	**Result**		**Characteristics**	**Result**	
	B. subtilis	**LBF02**		***B. subtilis***	**LBF02**
Gram staining	+	+	Methyl Red test	+	+
Cell shape	Rod	Rod	Indole production	-	-
Spores	+	+	Urease	+	+
Spore shape	Ellipsoid	Ellipsoid	Formation of H_2S	-	-
Spore position	Central	Central	Hydrolysis of casein	+	+
Mobility test	+	+	Utilization of citrate	+	+
Catalase	+	+	D-Glucose	+	+
Oxidase	+	+	Lactose	+	+
Liquefaction of gelatin	+	+	Maltose	+	+
Starch hydrolysis	+	+	Fructose	+	+
Nitrate reduction	+	+	Sucrose	+	+
VogesProskauer test	+	+	D-mannitol	+	+

"+" means positive and "**-**" means negative.

Table 2. Control of leaf spot disease on lettuce at five days after spray formulation treatments and pathogen inoculation in plants grown in pots under greenhouse conditions

Treatments*	**Severity of leaf spot****
1. Sprayed with sterile distilled water	0.00 d***
2. Sprayed with formulation without antagonistic bacteria	0.00 d
3. Sprayed with formulation of antagonistic bacteria	0.00 d
4. Sprayed with pathogen inoculation (*C. lactucae-sativae)*	4.62 ab
5. Sprayed with formulation without antagonistic bacteria and spray pathogen inoculation	4.12 b
6. Sprayed with formulation of antagonistic 1 hour before pathogen inoculation	1.62 c
7. Sprayed with formulation of antagonistic 1 hour after pathogen inoculation	1.87 c
8. Sprayed with formulation of antagonistic 3 days before pathogen inoculation	2.12 c
9. Sprayed with formulation of antagonistic 3 days after pathogen inoculation	5.37 a
CV (%)	32.20

* In treatments 1, 2 and 3, the lettuce plants were not inoculated with *C. lactucae-sativae*, while in treatments 4, 5, 6, 7, 8 and 9 the lettuce plants were inoculated with *C. lactucae-sativae*

** Severity index of leaf spot disease on the lettuce plants was defined as the percentage of diseased leaf area, where 0: plants did not show any symptoms, 1: 1-10%, 2: 11-20%, 3: 21-30%, 4: 31-40%, 5: 41-50%, 6: 51-60%, 7: 61-70%, 8: 71-80%, 9: 81-90% and 10: 91-100%.

*** Values within a column with different superscript are significant ($P < 0.05$).

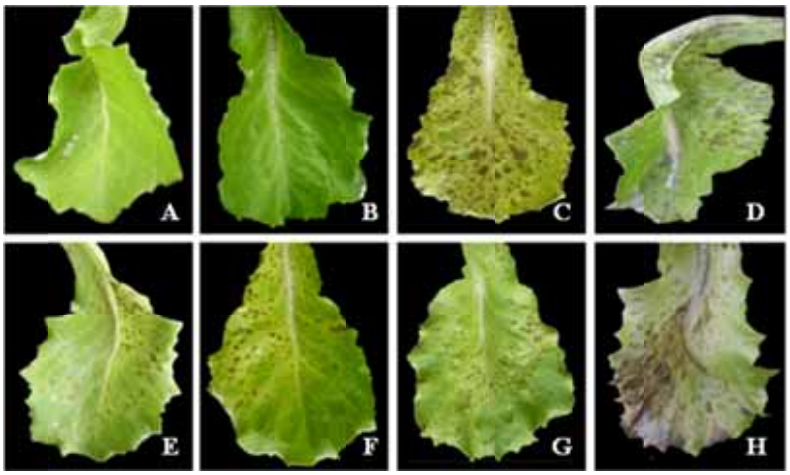

Figure 2. The comparison of efficiency upon the spraying of formulations after five days post inoculation for control of *C. lactucae-sativae* under greenhouse conditions. The following are the explanations: sprayed with formulation without the antagonistic bacteria (A), sprayed with the formulation of antagonistic bacteria (B), sprayed with the pathogen inoculation (*C. lactucae-sativae*) (C), sprayed with the formulation without antagonistic bacteria and spray pathogen inoculation (D), sprayed formulations were applied 1 hour prior to inoculation with *C. lactucae-sativae* (E), sprayed formulations were applied 1 hour after inoculation with *C. lactucae-sativae* (F), sprayed formulations were applied 3 days prior to inoculation with *C. lactucae-sativae* (G), sprayed formulations were applied 3 days after inoculation with *C. lactucae-sativae* (H)

4. Conclusion

The antagonistic bacteria LBF02 isolated showed the highest percentage of inhibition against *C. lactucae-sativae*. Non-filtrated culture medium (NFM) and filtrated culture medium (FM) treatment of LBF02 showed inhibition of the spore germination compared with the control. These data clearly indicate that the isolate LBF02 can be identified resembling a member of the *Bacillus subtilis* group. As for the greenhouse experiment, the results showed that using the formulation spray one hour before or after the pathogen inoculation on the lettuce plants was more effective in suppressing and managing leaf spot disease.

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References

Agrios, G. N. (2004). *Plant Pathology* (5th ed.). Elsevier Academic Press. London, 952.

Alderman, S. C., & Beute, M. K. (1986). Influence of temperature and moisture on Germination and germ tube elongation of *Cercospora arachidicola*. *Phytopathology, 76*(7), 715-719.

Arunyanart, P., Nilpanit, N., & Dhitikiattipon, R. (2008).Using Mixture Powder Formulation of Antagonistic Bacteria *Bacillus subtilis* and some Chemicals to Control Rice Seed Discoloration Disease. Bureau of rice Res. and Development; *Proceedings of rice and temperate cereal crops annual conference 2008 (2/2), Bangkok Thailand* (pp. 324-335).

Bacon, C. W., & Hinton, D. M. (2006) Bacterial endophytes: the endophytic niche, its occupants, and its utility. *In* S. S. Gnanamanickam (Ed.), *Plant-associated bacteria* (pp. 155-194). *The Netherlands: Springer*. http://dx.doi.org/10.1007/978-1-4020-4538-7_5

Choi, Y. W., Hyde, K. D., & Ho, W. H. (1999). Single spore isolation of fungi. *Fungal Diversity, 3*, 29-38.

Chupp, C. (1954). *A monograph of the fungus genus Cercospora.* Ithaca, New York. Published by the author, 667.

Chumthong, A., Kanjanamaneesathian, M., Pengnoo, A., & Wiwattanapatapee, R. (2008). Water-soluble granules containing *Bacillus megaterium* for biological control of rice sheath blight: formulation bacterial viability and efficacy testing. *World Journal of Microbiology and Biotechnology, 24*(11), 2499-2507. http://dx.doi.org/10.1007/s11274-008-9774-7

Collins, D. P., & Jacobsen, B. J. (2003). Optimizing a *Bacillus subtilis* isolate for biocontrol of sugar beet cercospora leaf spot. *Biological Control, 26*(2), 153-161. http://dx.doi.org/10.1016/S1049-9644(02)00132-9

Gomes, A. M., Michereff, S. J., & Mariano, R. L. (2004). Development and validation of a diagramatic key for Cercospora leaf spot of lettuce. *Summa Phytopathologica, 30*(1), 38-42.

Das, S., Lyla, P. S., & Khan, S. A. (2006) Marine microbial diversity and ecology: importance and future perspectives. *Current Sci., 90*(10), 1325-1335.

Dhitikiattipong, R., Srikoom, P., Rattanakarn, W., & Khemmuk, W. (2011). Efficacy of Powder Formulation of Antagonistic Bacteria to Control Rice Bakanae Disease in the Field. Bureau of Rice Research and Development; *Proceeding of 2nd Rice annual conference year 2011: Rice and national farmers' day*. Bangkok Thailand, 282-290.

Eeden, M. V., & Korsten, L. (2006).A predictive model for biological control of cercospora spot: Effect of nutrient availability. *South African Avocado Growers' Association Yearbook*, pp. 48-52.

Fritze, D. (2004). Taxonomy of the genus *Bacillus* and related genera: the aerobic endospore-forming bacteria. *Phytopathology, 94*(11), 1245-1248. http://dx.doi.org/10.1094/PHYTO.2004.94.11.1245.

Folman, L. B., De Klein, M. J. E. M., Postma, J., & Van Veen, J. A. (2004). Production of antifungal compounds by *Lysobacter enzymogenes* isolate 3.1T8 under different conditions in relation to its efficacy as biocontrol agent of *Pythium aphanidermatum* in cucumber. *Biological Control, 31*(2), 145-154. http://dx.doi.org/10.1016/j.biocontrol.2004.03.008

Holt, J. G. (1984). *Bergey's Manual of Systematic Bacteriology*. Vol. I and II.Williams Wilkins, Baltimore, USA, Vol. 1, 1-964, 2, 965-1599.

Hsieh, W. H., & Goh, T. K. (1990).*Cercospora and similar fungi from Taiwan*. Maw Chang Book Company.

Kai, M., Effmert, U., Berg, G., & Piechulla, B. (2007). Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani. Archives of Microbiology, 187*(5), 351-360. http://dx.doi.org/10.1007/s00203-006-0199-0

Kim, D. S., Weller, D. M., & Cook, R. J. (1997).Population dynamics of *Bacillus* sp. L324-92R12 and *Pseudomonas fluorescens* 2-79RN10 in the rhizosphere of wheat. *Phytopathology, 87*(5), 559-564. http://dx.doi.org/10.1094/PHYTO.1997.87.5.559

Kim, J. H., Lee, S. H., Kim, C. S., Lim, E. K., Choi, K. H., Kong, H. G., & Moon, B. J. (2006). Biological Control of Strawberry Gray Mold Caused by *Botrytis cinerea* Using *Bacillus licheniformis* N1 Formulation. *Journal of Microbiology and Biotechnology, 17*(3), 438-444.

Koohakan, P., Jeanaksorn, T., & Nuntagij, I. (2008). Major diseases of lettuce grown by commercial nutrient film technique in Thailand. *Science technology journal, 8*(2), 56-63.

Korsten, L. & De Jager, E. S. (1995). Mode of action of *Bacillus subtilis* for control of avocado postharvest pathogens. *South African Avocado Growers' Association Yearbook, 18*, 124-130.

Lee, J. P., Lee, S. W., Kim, C. S., Son, J. H., Song, J. H., Lee, K. Y., & Moon, B. J. (2006). Evaluation of formulations of *Bacillus licheniformis* for the biological control of tomato gray mold caused by *Botrytis cinerea*. *Biological control, 37*(3), 329-337. http://dx.doi.org/10.1016/j.biocontrol.2006.01.001

Lindow, S. E., & Brandl, M. T. (2003). Microbiology of the Phyllosphere. *Applied and environmental microbiology, 69*(4), 1875-1883. http://dx.doi.org/10.1128/AEM.69.4.1875-1883.2003

Mishra, B. S., Rath, R. R., Sahu, H. K., & Thatoi, H. N. (2009). Characterization and extracellular enzyme activity of predominant marine *Bacillus* spp. Isolated from sea water of Orissa Coast, India. *Malaysian Journal of Microbiology, 5*(2), 87-93.

Poonponkun, S., Bootta-noo, V., Atipunyakom, P., & LIkitaekarart, S. (2007). Efficacy of fungicides forcontrolling Colletotrichum of grapes plant diseases. *Research development of crop protection. Department of Agriculture*.

Silo-suh, L. A., Lethbridge, B. J., Raffel, S. J., He, H., Clardy, J., & Handesman, J. (1994). Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85 *and environmental microbiology, 60*(6), 2023-2030.

Sindhu, S. S., Gupta, S. K., Suneja, S., & Dadarwal, K. R. (2002). Enhancement of green gram nodulation and growth by *Bacillus* species. *Biologia mplantarum, 45*(1), 117-120. http://dx.doi.org/10.1023/A:1015117027863

To-Anun, C., Hidayat, I., & Meeboon, J. (2011). Genus *Cercospora* in Thailand: Taxonomy and Phylogeny (with a dichotomous key to species). *Plant Pathology & Quarantine, 1*(1), 11-87.

Todorova, S., & Kozhuharova, L. (2010). Characteristics and antimicrobial activity of *Bacillus subtilis* strains isolated from soil. *World Journal of Microbiology and Biotechnology, 26*(7), 1207-1216. http://dx.doi.org/10.1007/s11274-009-0290-1

Vidhyasekaran, P., Rabindran, R., Muthamilan, M., Nayar, K., Rajappan, K., Subramanian, N., & Vasumathi, K. (1997). Development of a powder formulation of *Pseudomonas fluorescens* forcontrol of rice blast. *Plant Pathology, 46*(3), 291-297. http://dx.doi.org/10.1046/j.1365-3059.1997.d01-27.x

Weller, D. M. (1988). Biological control of soil borne plant pathogens in the rhizosphere with bacteria. *Annual review of phytopathology, 26*(1), 379-407. http://dx.doi.org/10.1146/annurev.py.26.090188.002115

Zheng, Y. G., Chen, J., Liu, Z. Q., Wu, M. H., Xing, L. Y., & Shen, Y. C. (2008). Isolation, identification and characterization of *Bacillus subtilis* ZJB-063, a versatile nitrile-converting bacterium. *Applied Microbiology and Biotechnology, 77*(5), 985-993. http://dx.doi.org/10.1007/s00253-007-1236-x

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Comparison of Two Products of Direct-Fed Microbial Supplementation on the Nutrient Utilization and Ruminal Fermentation in Sheep

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Abstract

This experiment was undertaken to evaluate the potential impacts of supplementing two direct-fed microbial (DFM) products, namely Bactozyme and Ru-max, to the diet of 12 male Barki sheep (live body weight 46.6 ± 2.9 kg) on dry matter intake (DMI), apparent total tract digestibility of nutrients, nitrogen balance and rumen fermentation characteristics. The Bactozyme or Ru-max were supplemented at a rate of 1.0 g/head/day, mixed with the concentrate mixture. Animals were randomly allocated into 3 equal groups (n=4) and were subjected to the digestibility trails.

The results showed that the inclusion of either products of DFM had no positive impact on DMI, but non-significantly improved the apparent total tract digestibility of dry matter (DM), organic matter (OM) and crude protein (CP). However, the Bactozyme addition increased ($P<0.05$) the apparent total tract digestibility of neutral detergent fiber (NDF) and acid detergent fiber (ADF). The enhancement of the apparent total tract digestibility of cell wall was not significant for the two of DFM products and a non-significant improvement in cell wall digestion due to the Ru-max supplementation over the control group was found. The DFM products had positive impacts on the average of total digestible nutrients (TDN) and digestible crude protein (DCP) but non significant in comparison with the untreated animals. In addition, the nitrogen balance was improved ($P>0.05$) by 8 and 13% due to Ru-max and Bactozyme supplementation, respectively in comparison with the control group. The results also revealed that inclusion of DFM products had no impacts on rumen pH 3.0 and 6.0 h after feeding but Bactozyme reduced ($P<0.05$) the rumen pH 1.0 h after feeding compared to the control group. The inclusion of Bactozyme and Ru-max increased ($P>0.05$) the NH_3-N concentration in the rumen at 1.0 and 3.0 h after feeding but the Bactozyme decreased ($P<0.05$) the NH_3-N concentration and increased the volatile fatty acids (VFA) at 6.0 h after feeding compared to the control group. Overall, results indicated that the two DFM products had positive impacts on cell wall digestibility, which in turn improves metabolic energy supply and nutrients utilization in ruminants as well.

Keywords: feed additives, bactozyme, Ru-max, fermentation, nutrients digestibility

1. Introduction

Numerous studies have been conducted in an attempt to increase ruminant productivity by manipulating the rumen environment and to increase feed digestibility and nutrient utilization by the animals in order to supply sufficient nutrients to support a high level of milk production. One approach that has recently been widely investigated is the application of direct-fed microbial (DFM) preparations, in order to promote digestion and intestinal hygiene (Gourinier-Chateau et al., 1994), enhance animal performance and reduce usage of antibiotics (Jouany & Morgavi, 2007; Guedes et al., 2008; Wallace et al., 2008).

The definition of DFM is very broad and may include specific and nonspecific yeast, fungi, bacteria, cell fragments, and filtrates (Knowlton et al., 2002). The supplementation of DFM agents in dairy rations has become a generally accepted practice with the following stated benefits: increased ruminal digestion, dry matter intake (DMI), and milk production and reduced body temperature (Piva et al., 1993; Higginbotham et al., 1994; McGilliard & Stallings, 1998). *Enterococcus faecium* produces moderate amounts of lactic acid in the rumen. This could stimulate growth of lactic acid utilizer's microrganisms and stabilize ruminal pH (Nocek et al., 2002;

2003). Yeast and yeast products have been widely used in ruminant nutrition to manipulate rumen fermentation and improve animal performance (Bruno et al., 2009; Yalçin et al., 2011). In addition, other studies have shown substantial improvement of feed digestibility, rumen fermentation and animal performance due to fibrolytic enzymes supplementation (Bala et al., 2009; Gado et al., 2009; Holtshausen et al., 2011).

However, testing DFM supplementation produced variable and inconsistent results so far (cf. also Z. Mir & P. S. Mir, 1994). One main point to explain this is the diversity of DFM origin. Several biotic factors such as the strain of yeast, bacteria, fungi, enzymes and its viability, nature of the diet, animal type and its physiological status and level of performance may play considerable role in this regard. Still, direct comparisons among direct-fed microbial products have rarely been carried out, and a comprehensive analysis of variables indicative of the complex of influence of such products is mostly lacking. Therefore, the present study was conducted to compare the responses of Barki sheep to supplementation of two commercial DFM and determine their effect on feed intake, nutrients digestibility, nitrogen (N) utilization and rumen fermentation characteristics.

2. Materials and Methods

This experiment was conducted at the Milk Production Project, Animal Production Department, Faculty of Agriculture, Alexandria University, Egypt. All analyses were carried out at the Animal Nutrition Laboratory, Department of Animal Production, Faculty of Agriculture, Alexandria University, Egypt.

2.1 Animals and Management

Twelve adult male Barki sheep (live body weight, 46.6 ± 2.9 kg), a small Egyptian fat-tailed sheep breed, were randomly allocated to equal three groups (n=4). The control group received a basal diet, which composed concentrate mixture and Egyptian clover (*Alexandrium trifolum*) hay without supplement, the other two groups received the basal diet plus either 1.0 g of Ru-max/head/day or 1.0 g of Bactozyme /head/day according to the producer recommendations. The DFM product Ru-max® (Agri-King, Inc., Fulton, USA) is composed from cellulases, β-glucanases, amlyases, *Aspergillus oryzae, Enterococcus faecium* and *Saccharomyces Cerevisiae*. Bactozyme is a microbial feed additive (Dyno Vet. Company, the Egyptian - French Factory, Alexandria, Egypt) consisting of *Saccharomyces cerevisiae* ($20x10^{10}$ CFU), total live bacteria ($2x10^{10}$ CFU), *Lactobacillus acidophilus* ($2x10^{9}$ CFU), *Lactobacillus casei* ($0.4x10^{8}$ CFU), *Lactobacillus plantarum* ($1.6x10^{9}$ CFU), *Entrococcus faecium* ($4.0x10^{9}$ CFU), *Bacillus subtilis* ($6 x10^{9}$ CFU), *Bacillus licheniformis* ($6 x10^{9}$ CFU), phytase (2400 U), lipase (2400 U), xylanase (1200 U), cellulase (2400 U), pectinase 400 U, amylase (20000 U), protease (40000 U), β-gluconase (1000 U), fructo oligosaccharides (10 g), mannan oligosaccharides (10 g), calcium propionate (24 g), copper penta sulphate (10 g) and carrier up to 1 kg.

The animals were housed individually in metabolic crates under a protective roof and had free access to fresh water throughout the study. The basal diet consisted of clover hay and a concentrate mixture the composition of which is given in Table 2. The diet components were offered twice daily at 08:00 and 16:00 h in amounts of 750 and 750 g as fed/day foreach of clover hay and concentrate mixture, respectively. The experimental period lasted for 30 days, with the first 21 days being an adaptation period to the diet, followed by 7 days of sample collection (feces, urine, refusal feed). Individual intakes of clover hay and concentrate were recorded daily by weighing the feed offered and refused. During the collection period, also the complete output of feces was recorded by collection in buckets. Feces samples of 100 g/kg of total weight were collected and stored under 5˚C during the collection period. Directly afterwards, the samples collected during the 7 days were mixed. One kilogram of this mixture was dried at 60˚C for 72 h in a forced air oven, ground through a 1-mm screen and stored at room temperature until analysis. The remainder was kept in a freezer (–20˚C) for analysis of dry matter (DM) and total N. The urine was completely collected in plastic buckets containing 100 ml of H_2SO_4 (10%), and the amounts were recorded and samples (10%) were collected daily. These samples were stored in a freezer (–20˚C) during the collection period. Just after the collection period, the urine samples were pooled per animal and representative samples were frozen at –20˚C until further analysis.

The ruminal fluid was collected via the stomach tube at 1.0, 3.0 and 6.0 h after feeding consecutive for consecutive 2 days. The rumen pH was measured immediately after collection using pH meter. The rumen fluid was separated from the feed particles through four layers of gauze and stored at –20˚C for later analysis.

2.2 Sample Analyses

Chemical analyses were performed according to AOAC (2006). DM contents of feeds and refusals were determined by drying at 135˚C for 2 h, but of feces were dried at 105˚C overnight. OM was determined as the weight loss after ashing at 550˚C for 2 h. N content of feeds, feces and acidified urine was determined using the Kjeldahl method, and CP was calculated as 6.25 × N. Ether extract (EE) was analyzed according to AOAC (2006).

Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the procedures of Van Soest et al. (1991). No sodium sulfite and α-amylase were used in the procedure for NDF determination. Both NDF and ADF are expressed without residual ash. Concentrations of NH_3-N and total volatile fatty acids (VFA) in rumen fluid were determined by distillation using Markham apparatus according to the Preston (1995) and Warner (1964), respectively.

2.3 Statistical Analysis

Data were analyzed using the generalized linear model procedure (SAS, 2002). The following model was assumed: $Y_{ij}= \mu+ T_i + e_{ij}$ where: μ is the overall mean, T_i is the treatment type, e_{ij} is the random error term. Differences among means were tested using Duncan multiple range test (Steel & Torrie, 1980).

3. Results

The mean values of the proximate analysis on DM basis of the concentrate mixture and clover hay are presented in Table 1. The results of the proximate analysis showed that OM, CP and EE of concentrate mixture and clover hay were 895 vs. 890, 142 vs. 135 and 38.6 vs. 14 g/kg, respectively, while, NDF, ADF and hemicellulose content of the concentrate mixture were 398, 176 and 222, respectively and were 448, 282 and 166 g/kg for Egyptian clover, respectively.

Table 1. Ingredients and chemical composition of concentrate mixture and clover hay fed to Barki sheep

Ingredients,	g/kg DM	
Ground yellow corn	250	
Wheat bran	300	
Cotton seed meal	170	
Sunflower meal	245	
Limestone	20.0	
Sodium chloride	10.0	
Trace minerals*	5.0	
Items (g/kg DM)	Concentrate mixture	Clover hay
OM	895	890
CP	142	135
EE	38.6	014
NDF	398	448
ADF	176	282
Hemicellulose	222	166

*Mineral mixture contained (g/kg): Manganese Sulphate12.58, Zinc Sulphate 9.3, Copper Sulphate 3.2, Ferrous sulphate 16.67 Calcium iodate 0.081, Sodium selenite 0.4, Magnesium oxide 9.4, Cobalt sulphate 0.2, Sodium chloride Add to kg (M/s, Dyno vet company, Alexandria, Egypt).

Data of feed intake, nutrients apparent total tract digestibility and feeding values of rations without (control) or with Bactozyme and Ru-max are summarized in Table 2. The results showed that the inclusion of two products of DFM had no positive impact on DMI compared to the control group. The ratios of consumed clover hay and concentrate were 44.3:55.7; 44.0:56.0 and 43.3:56.7% for the control, Ru-maz and baztozyme groups, respectively. However, the supplementation of Bactozyme or Ru-max apparently improved the apparent total tract digestibility of DM, OM and CP but the improvement was not significant compared to the control. While the Bactozyme addition increased ($P<0.05$) the apparent total tract digestibility of NDF and ADF significantly, Ru-max only showed numerical increases compared to the control group. Although, DFM had positive responses on the mean values of TDN and DCP, no significant changes in comparison to the untreated animals were observed.

Table 2. Effects of Bactozyme and Ru-max supplementation on dry matter intake (DMI), nutrients apparent total tract digestibility and nutritive value in Braki sheep (Means ± SE)

	Control	Ru-Max	Bactozyme
DMI, g/head/d	1158.9±19.5	1154.4±19.5	1165.8±19.5
Apparent total tract digestibility, %			
Dry matter	66.9±1.3	68.1±1.3	69.9±1.3
Organic matter	67.3±1.3	68.4±1.3	70.6± 1.3
Crude protein	71.5±1.2	72.5±1.2	74.3±1.2
Ether extract	75.2±1.3^{b}	81.4±1.3^{a}	78.7±1.3^{ab}
Neutral detergent fiber	58.8±1.3^{b}	61.1±1.3^{ab}	63.2±1.3^{a}
Acid detergent fiber	40.6±2.3^{b}	45.4±2.0^{ab}	49.3±2.2^{a}
TDN	63.3±1.5	64.7±1.5	68.0±1.5
DCP	10.0±0.16	10.2±0.16	10.6±0.16

Different letters (a, b) in the same row indicate significant differences (P<0.05).

TDN: Total digestible nutrients; DCP: Digestible crude protein.

The effects of Bactozyme and Ru-max supplementation on N utilization are given in Table 3. Fecal and urinary N decreased (P>0.05) when Bactozyme and Ru-Max were supplemented compared to the control group. In addition, the N balance was apparently improved (P>0.05) by 8 and 13% due to Ru-max and Bactozyme supplementation, respectively in comparison to the control group.

Table 3. Effect of Bactozyme and Ru-max supplementation on nitrogen fractions of Barki sheep (Means ± SE)

	Control	Ru-Max	Bactozyme
N intake, g/d	25.8±0.53	25.7±0.53	25.9±0.53
Fecal N, g/d	7.4±0.67	6.9±0.67	6.1±0.67
Urinary N, g/d	9.2±1.47	7.9±1.47	8.7±1.47
Nitrogen balance, g/d	9.2±1.34	10.9±1.34	11.1±1.34

Mean values of rumen pH revealed that Bactozyme inclusion reduced (P< 0.05) rumen pH after 1.0 h of feeding but, pH at subsequent intervals such as 3.0 and 6.0 h after feeding were statistically comparable with control.

Table 4. Effect of Bactozyme and Ru-max supplementation on rumen pH after different times at morning feeding of Barki sheep

Groups	Rumen pH		
	1.0 h	3.0 h	6.0 h
Control	6.43±0.07^{a}	6.49±0.07	6.62±0.09
Ru-max	6.40±0.07^{ab}	6.47±0.07	6.67±0.09
Bactozyme	6.21±0.07^{b}	6.28±0.07	6.52±0.09

Different letters (a, b) in the same column indicate significant differences (P<0.05).

Bactozyme and Ru-max supplementation showed apparent increase (P> 0.05) in the NH_3-N at 1.0 and 3.0 h of post feeding. However, it was higher on Ru-max at 6.0 h of post feeding (P< 0.05) than Bactozyme and comparable to control. Bactozyme and Ru-max supplementation had no effects on VFA concentration either 1.0 or 3.0 h after feeding but VFA concentration was increased (P< 0.05) on both supplements compared to control.

Table 5. Effect of Bactozyme and Ru-max supplementation on NH_3-N concentration after different times at morning feeding of Barki sheep

Groups	NH_3-N (mg/dL) concentration		
	1.0 h	3.0 h	6.0 h
Control	15.5±2.0	19.4±1.88	19.9±1.7[ab]
Ru-max	18.7±2.1	21.4±2.4	20.8±1.5[a]
Bactozyme	19.4±2.1	20.1±2.1	16.3±1.8[b]

Different letters (a, b) in the same column indicate significant differences (P<0.05).

Table 6. Effect of two products of direct-fed microbial supplementation on VFA concentration after different times at morning feeding in Barki sheep

	VFA concentration (meq/dL)		
	1h	3h	6h
Control	10.51±0.69	8.73±0.80	7.25±0.41[b]
Ru-max	10.85±0.69	8.61±0.81	10.09±0.61[a]
Bactozyme	9.59±0.76	9.09±0.80	9.18±0.55[a]

Different letters (a, b) in the same row indicate significant differences (P<0.05).

4. Discussion

Cellulose and hemicellulose represent about 250-300 g/kg of most ruminant diets. These plant cell wall polymers are insoluble, structurally complex and not totally physically accessible, which explains why their degradation is sometimes limited. Moreover, the host enzymes are unable to hydrolyze these kinds of molecules. Improving the bioavailability of nutrients in a feedstuff by increasing the cell wall hydrolysis through the microbial supplement is a promising solution and, DFM preparations are reported to promote digestion and intestinal hygiene (Gourinier-Chateau et al., 1994), enhance animal performance and reduce usage of antibiotics (Jouany & Morgavi, 2007; Guedes et al., 2008; Wallace et al., 2008).

Ru-max and Bactozyme supplementation did not improve DMI and digestibility of DM, OM and CP but, digestibility of EE, NDF and ADF were improved significantly. Dawson (1992) also reported that the addition of DFM resulted in increased concentration of total anaerobic bacteria and the increase was associated with fibre digesting and lactic acid utilizing bacteria (Dawson 1992). The components that are used in DFM may be classified as lactic acid utilizing bacteria *Enterococcus*, (LUB), yeast products containing *Saccharomyces cerevisiae* and fungi *Aspergillus* oryzae, fibrolytic enzymes and cobalt carbonate. The LUB potentially moderatesrumen conditions and improve feed efficiency. Yeast DFM may reduce harmful oxygen, prevent excess lactate production, increase feed digestibility, and improve fermentation in the rumen. The DFM may also compete with and consequently inhibit the growth of pathogens, stimulate immune function, and modulate microbial balance in the gastrointestinal tract.

Many workers reported that the supplementation of DFM in ruminant rations has become a generally accepted practice due to increased ruminal digestion, DMI, performance and reduced body temperature (Piva et al., 1993; Higginbotham et al., 1994; McGilliard & Stallings, 1998). Nikkhah et al. (2004) and Raeth-Knight et al. (2007) have observed non-significant improvement of DMI by animals fed with yeast culture. Similalry, Bernard et al. (2010) and Arriola et al. (2011) reported that adding fibrolytic enzymes supplementation to dairy cow diet did not enhance DMI and no difference was found between cows supplemented with or without fibrolytic enzymes. DMI is often considered as a function of the initial rate of fiber digestion. An early stimulation of ruminal activity can be expected to have a major impact on the feed consumption and can provide a driving force for improved animal performance. Although significant improvement in the NDF digestibility was observed with Ru-max and Bactozyme supplementation, it could be presumed that they might be lagging in improving the initial rate of fiber digestion that could be translated into enhanced DMI. Supplementing lactating dairy cows with DFM products containing *Lactobacillus acidophilus* and *Propionibacterium freudenreichii* did not affect rumen fermentation in cows. On the other hand, Szasz, (2002), Ware and Zinn (2005) reported that Fibrozyme inclusion to the steers or heifer's diets increased total tract digestibility of NDF and ADF and increased DMI and average daily gain. Few

workers concluded improved feed intake, feed conversion rate, daily weight gain and total body weight in sheep, goat and cattle on administration of DFM (Torres-Rodriguez et al., 2007; Samli et al., 2007; Casey et al., 2007). Improvement from DFM supplementation could be anticipated due to positive effects on various digestive processes, especially cellulolysis, synthesis of microbial protein, stabilizers of ruminal pH and lactate, increased absorption of some nutrients and displayed a growth-promoting effect. The positive effect of the Bactozyme additive on NDF digestibility in this study might be related to stimulation of growth of cellulolytic bacteria. However, the effects of DFM on DMI appeared varying in this study probably due to variation in products, methods of applying, roughage: concentrate ratio (Yang et al., 2000; Bowman et al., 2002).

No increase in CP digestibility was observed in confirmation to Bassiouni et al. (2010) who supplemented fibrozyme to corn silage or rice straw. Mean values of DM and OM apparent total tract digestibility were not affected, while apparent total tract digestibility of NDF and ADF were improved ($P<0.05$) in treated groups compared to the control group. These results were in accordance with Beauchemin et al. (2003) and Arriola et al. (2011) who reported that exogenous enzymes improve apparent total tract digestibility of plant cell wall, and there is evidence for numerous potential modes of action suggesting their interdependence. Adding Bactozyme and Ru-max to a diet may increase the hydrolytic capacity of the rumen mainly due to increased bacterial attachment, stimulation of rumen microbial populations and synergistic effects with hydrolases of ruminal microorganisms. The net effect is increased enzymatic activity within the rumen, which enhances digestibility of the feed. Moreover, DFM supplementation to ruminant diets could also partly reduce digesta viscosity (Hristov et al., 2000) and alter ruminal fermentation (Gado et al., 2009; Arriola et al., 2011) and/or enhance attachment and colonization to the plant cell wall by ruminal microorganisms (Wang et al., 2001, Holtshausen et al., 2011) by synergism with enzymes or stimulate the rumen microbial numbers.

The TDN and DCP were not significantly changed between treatments as observed by Ismaiel et al. (2010). An apparent trend in improved N balance on study due to DFM supplementation was observed that was due to less of excretion of urinary nitrogen and fecal nitrogen in sheep fed bactozyme and Ru-max compared with control group, which in consistent with observations reported by El-Ashry et al. (2003); Ahmed and Salah (2006) and Ismaiel et al. (2010).

DFM feeding showed variable and inconsistent affect on altering rumen fermentation patterns. Some reports have demonstrated no effects of yeast culture supplementation on ruminal pH, rumen ammonia concentration, and VFA patterns *in vivo* (Wiedmeier et al., 1987) and *in vitro* (Newbold et al., 1996). Microbial conversion of peptides and amino acids to ammonia in the rumen is unfavourable to the host animal, because energy is required for microbial protein synthesis, and not all ammonia is incorporated into protein (Wallace et al., 1997). Consequently, if high levels of ammonia occur in the rumen, a large amount of N is excreted in urine and feces. For example, in animal production systems feeding high amounts of N, more than half of it is excreted in urine, mostly in the form of urea which is rapidly mineralised in NH_3/NH_4 + and then converted to nitrous oxide (N_2O), which has a global warming potential that is 296 fold that of carbon dioxide (CO_2) and more than 12 fold that of methane (Steinfeld et al., 2006). Because of the increasing concern of the role of livestock on climate change, nutritional strategies that aim at decreasing N loss in the rumen are of interest.

A decrease in NH_3 concentration is attributed to ruminal microbial proliferation, due to the increase of microbial use of available NH_3 for microbial N synthesis (Crocker et al., 1998). Some of the DFM e.g. galacto-oligosaccharides, are known to suppress ammonia producing bacteria, and stimulate the production of *Bifidobactrium,* which has the ability to assimilate ammonia as a N source (Deguchi et al., 1993). Beauchemin et al. (2000) found that enzyme supplementation decreased NH_3N value before and after feeding. This decreasing in NH_3N was likely caused by an increase in ruminal availability of slowly digestible carbohydrates due to enzyme supplementation.

Nutrients of forage cell walls are degraded to several metabolites, such as VFA, by ruminal bacteria, protozoa, and fungi. Results clearly indicated that no significant effect on VFA's concentration by Bactozyme or Ru-max supplementation at 1 or 3h after feeding but the VFA increased significantly at 6h after feeding compared to the control group. This result was associated with Kung et al. (2002); Sutton et al. (2003); Eun and Beauchemin (2005) who concluded that total VFA were not affected by enzyme supplementation, while Arriola et al. (2011) reported that fibrolytic enzymes supplementation increased ($P<0.03$) VFA concentration. However, the animal responses to DFM addition have been highly variable, apparently influenced by the composition of the diet and much remains to be elucidated about the dose- and diet-dependence of DFM effects (Chaucheyras-Durand et al., 2008). The variations among these studies may be due to differences in roughage: concentrate ratio, which may have effects on lactic acid concentration and rumen pH.

5. Conclusions

In conclusion, under the conditions of this study, both types of the investigated DFM (Ru-max and baztozyme) had no positive impacts on DMI, N utilization but improved cell wall digestibility as one of the key targets of the DFM products supplementation, which then would improve metabolic energy supply and nutrients utilization in ruminants as well.

References

Ahmed, B. M., & Salah, M. S. (2006). Effect of yeast culture as an additive to sheep feed on performance, digestibility, nitrogen balance and rumen fermentation. *J. Agri. Sci., 1*(14), 1-13. http://dx.doi.org/10.3168/jds.2010-3424

AOAC. (2006). *Official Methods of Analysis of AOAC International* (2000, 20th ed.). AOAC International, Arlington, VA, USA. http://dx.doi.org/10.1111/j.1740-0929.2009.00634.x

Arriola, K. G., Kim, S. C., Staples, C. R., & Adesogan, A. T. (2011). Effect of fibrolytic enzyme application to low and high concentrate diets on the performance of lactating dairy cattle. *J. Dairy Sci., 94*, 832-841.

Bala, P., Malik, R., & Srinivas, B. (2009). Effect of fortifying concentrate supplement with fibrolytic enzymes on nutrient utilization, milk yield and composition in lactating goats. *J. Anim. Sci., 80*, 265-272.

Bassiouni, M. I., Gaafar, H. M. A., Mohi El-Din, A. M. A., Metwally, A. M., & Elshora, M. A. H. (2010). Evaluation for rations supplemented with fibrolytic enzyme on dairy cows performance 3. Productive performance of lactating Friesian cows. *Livestock research for rural development, 22*(6).

Beauchemin, K. A., Colombatto, D., Morgavi, D. P., & Yang, W. Z. (2003). Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *J. Amin. Sci., 81* (E. Suppl. 2), E37-E47. http://dx.doi.org/10.3168/jds.S0022-0302(00)74914-9

Beauchemin, K. A., Rode, L. M., Maekawa, M., Morgavi, D. P., & Kampen, R. (2000). Evaluation of a nonstarchpolysaccharidase feed enzyme in dairy cow diets. *J. Dairy Sci., 83*, 543-553.

Bernard, J. K., Castro, J. J., Mullis, N. A., Adesogan , A. T., West, J. W., & Morantes, G. (2010). Effect of feeding alfalfa hay or Tifton 85 bermuda grass haylage with or without a cellulase enzyme on performance of Holstein cows. *J. Dairy Sci., 93*, 5280-5285. http://dx.doi.org/10.3168/jds.2010-3111

Bowman, G. R., Beauchemin, K. A., & Shelford, J. A. (2002). The proportion of the diet to which fibrolytic enzymes are added affects nutrient digestion by lactating dairy cows. *J. Dairy Sci., 85*, 3420-3429. http://dx.doi.org/10.3168/jds.S0022-0302(02)74430-5

Bruno, R. G. S., Rutigliano, H. M., Cerri, R. L., Robinson, P. H., & Santos, J. E. P. (2009). Effect of feeding *Saccharomyces cerevisiae* on performance of dairy cows during summer heat stress. *Anim. Feed Sci. Technol., 150*, 175-186. http://dx.doi.org/10.1016/j.anifeedsci.2008.09.001

Casey, P. G., Gardiner, G. E., Casey, G., Bradshaw, B., Lawlor, P. G., Lynch, P. B., … Hill, C. (2007). A 5-strain probiotic combination reduces pathogen shedding and alleviates disease signs in pigs challenged with *Salmonella enterica Serovar Typhimurium*. *Applied Environ. Microb., 73*, 1858-1863. http://dx.doi.org/10.1128/AEM.01840-06

Chaucheyras-Durand, F., Walker, N. D., & Bach, A. (2008). Effects of active dry yeasts on the rumen microbial ecosystem: Past, present and future. *Anim. Feed Sci. Technol., 145*, 5-26. http://dx.doi.org/10.1016/j.anifeedsci.2007.04.019

Crocker, L. M., DePeters, E. J., Fadel, J. G., Perez-Monti, H., Taylor, S. J., Wyckoff, J. A., & Zinn, R. A. (1998). Influence of processed corn grain in diets of dairy cows on digestion of nutrients and milk composition. *J. Dairy Sci., 81*, 2394-2407. http://dx.doi.org/10.3168/jds.S0022-0302(98)70131-6

Dawson, K. A. (1992). Current and future role of yeast culture in animal production: A review of research over the last six years. *Asia Pacific Lecture Tour on "Improving Nutrient Utilization while reducing pollution: New Dimensions Through Biotechnology"* (pp.15-37).

Deguchi, Y., Makino, A., Iwabuchi, A., Watanuki, M., & Yamashita, T. (1993). Selection of ammonia-assimilating bifidobacteria and their effect on ammonia levels in rat cecal contents and blood. *Microb. Ecol. Health Dis., 6*, 85-94. http://dx.doi.org/10.3109/08910609309141566

El-Ashry, M. A., Fayed, A. M., Youssef, K. M., Salem, F. A., & Aziz, H. A. (2003). Effect of feeding Flavomycin or yeast as feed supplement on lamb performance in Sinai.Egy. *J. Nutri Feeds., 6*, 1009-1022.

Eun, J. S., & Beauchemin, K. A. (2005). Effects of a proteolytic feed enzyme on intake, digestion, ruminal fermentation, and milk production. *J. Dairy Sci., 88*, 2140-2153. http://dx.doi.org/10.3168/jds.S0022-0302(05)72890-3

Gado, H. M., Salem, A. Z. M., Robinson, P. H., & Hassan, M. (2009). Influence of exogenous enzymes on nutrient digestibility, extent of ruminal fermentation as well as milk production and composition in dairy cows. *Anim. Feed Sci. Technol., 154*, 36-46. http://dx.doi.org/10.1016/j.anifeedsci.2009.07.006

Gourinier-Chateau, N., Larpent, J. P., Castellanos, M. I., & Larpent, J. L. (1994). *Probiotics in animal and human nutrition*. Technique et Documentation Lavoisier, Paris (France), Abstr.

Guedes, C. M., Gonçalves, D., Rodrigues, M. A. M., & Dias-da-Silva, A. (2008). Effects of a Saccharomyces cerevisiae yeast on ruminal fermentation and fibre degradation of maize silages in cows. *Anim. Feed Sci. Tech., 145*, 27-40. http://dx.doi.org/10.1016/j.anifeedsci.2007.06.037

Higginbotham, G. E, Coller, C. A., Aseltine, M. S., & Bath, D. L. (1994) Effect of yeast culture and Aspergillusoryzae extract on milk yield in a commercial dairy herd. *Journal of Dairy Science, 77*, 343-348. http://dx.doi.org/10.3168/jds.S0022-0302(94)76960-5

Holtshausen, L., Chung, Y. H., Gerardo-Cuervo, H., Oba, M., & Beauchemin, K. A. (2011). Improved milk production efficiency in early lactation dairy cattle with dietary addition of a developmental fibrolytic enzyme additive. *J. Dairy Sci., 94*, 899-907. http://dx.doi.org/10.3168/jds.2010-3573

Hristov, A. N., McAllister, T. A., & Cheng, K. J. (2000). Intraruminal supplementation with increasing levels of exogenous polysaccharides-degrading enzymes: Effects on nutrient digestion in cattle fed a barley grain diet. *J. Anim. Sci., 78*, 477-487.

Ismaiel, A. M., El-Far, A. H., & Abou-Ganema, I. I. (2010). Effect of Tonilisat and Roemin W2 Supplementations on the Performance of Lambs. *Intern. J. Biol. Life Sci., 6*(4).

Jouany J. P., & Morgavi, D. P. (2007). Use of 'natural' products as alternatives to antibiotic feed additives in ruminant production. *Animal, 1*, 1443-1466. http://dx.doi.org/10.1017/S1751731107000742

Knowlton, K. F., McKinney, J. M., & Cobb, C. (2002). Effect of a direct-fed fibrolytic enzyme formulation on nutrient intake, partitioning, and excretion in early and late lactation Holstein cows. *J. Dairy Sci., 85*, 3328-3335. http://dx.doi.org/10.3168/jds.S0022-0302(02)74421-4

Kung, L., Cohen, M. A., Rode, L. M., & Treacher, R. J. (2002). The effect of fibrolytic enzymes sprayed onto forages and fed in a total mixed ratio to lactating dairy cows. *J. Dairy Sci., 85*, 2396-2402. http://dx.doi.org/10.3168/jds.S0022-0302 (02)74321-X

McGilliard, M. L., & Stallings, C. C. (1998). Increase milk yield of commercial dairy herds fed a microbial and enzyme supplement. *J. Dairy Sci., 81*, 1353-1357. http://dx.doi.org/10.3168/jds.S0022-0302(98)75698-X

Mir, Z., & Mir, P. S. (1994). Effect of the addition of live yeast (*Saccharomyces cerevisiae*) on growth and carcass quality of steers fed high-forage or high-grain diets and on feed digestibility and in situ degradability. *J. Anim. Sci., 72*, 537- 545.

Newbold, C. J., Wallace, R. J., & McIntosh, F. M. (1996). Mode of action of the yeast Saccharomyces cerevisiae as a feed additive for ruminants. *Br. J. Nutr., 76*, 249-261. http://dx.doi.org/10.1079/BJN19960029

Nikkhah, A., Bonadaki, M. D., & Zali, A. (2004). Effects of feeding yeast (Saccharomyces cerevisiae) on productive performance of lactating Holstein dairy cow. *Ir. J. Agri. Sci., 35*, 53-60.

Nocek, J. E., Kautz, W. P., Leedle, J. A., & Allman, J. G. (2002). Ruminal supplementation of direct-fed microbials on diurnal pH variation and in situ digestion in dairy cattle. *J. Dairy Sci., 85*, 429-433. http://dx.doi.org/10.3168/jds.S0022-0302(02)74091-5

Nocek, J. E., Kautz, W. P., Leedle, J. A., & Block, E. (2003). Direct-fed microbial supplementation on the performance of dairy cattle during the transition period. *J. Dairy Sci., 86*, 331-335. http://dx.doi.org/10.3168/jds.S0022-0302(03)73610-8

Piva, G., Belladonna, S., Fusconi, G., & Siebaldi, F. (1993). Effects of yeast on dairy cows performance, ruminal fermentation, blood components, and milk manufacturing properties. *J. Dairy Sci., 76*, 2717-2722. http://dx.doi.org/10.3168/jds.S0022-0302(93)77608-0

Preston, T. R. (1995). *Biological and for research workers*. Rome: FAO, 1995 (Chap. 9, p.191).

Raeth-Knight, M. L., Linn, J. G., & Jung, H. (2007). Effect of direct-fed microbials on performance, diet digestibility, and rumen characteristics of Holstein dairy cows. *J. Dairy Sci., 90*, 1802-1809. http://dx.doi.org/10.3168/jds.2006-643

Samli, H. E., Senkoylu, N., Koc, F., Kanter, M., & Agma, A. (2007). Effects of enterococcus faecium and dried whey on broiler performance, gut histomorphology and intestinal microbiota. Arch. *Anim. Nutr., 61*, 42-49. http://dx.doi.org/10.1080/17450390601106655

SAS. (2002). *SAS users guide Statistical Analyses Systems Institute* (pp. 549-640). Cary, USA.

Steel, R. G. D., & Torrie, J. H. (1980). *Principles and Procedure of Statistics. A Biometrical Approach* (2nd ed., pp. 137-269). Mac Graw-Hill Com.

Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., Rosales, M., & de Haan, C. (2006). *Livestock's Long Shadow: Environmental Issues and Options*. Rome: Food and Agriculture Organization of the United Nations.

Sutton, J. D., Phipps, R. H., Beever, D. E., Humphries, D. J., Hartnell, G. F., Vicini, J. L., & Hard, D. L. (2003). Effect of method of application of a fibrolytic enzyme product on digestive processes and milk production in Holstein-Friesian cows. *J. Dairy Sci., 86*, 546-556. http://dx.doi.org/10.3168/jds.S0022-0302(03)73633-9

Szasz, J. L., McCalmant, T. M., Hunt, C. W., Grove, A. V., & Kennington, L. R. (2002). Effect of a fibrolytic enzyme preparation on intake and digestibility of bluegrass seed straw fed to beef cattle. *Proc. West. Sec. Am. Soc. Anim., Sci., 53*, 313.

Torres-Rodriguez, A., Donoghue, A. M., Donoghue, D. J., Barton, J. T., Tellez G., & Hargis, B. M. (2007). Performance and condemnation rate analysis of commercial Turkey flocks treated with a *Lactobacillus* sp.-based probiotic. *Poult. Sci., 86*, 444-446.

Van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for dietary fibre, neutral detergent fibre and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci., 74*, 3583-3597. http://dx.doi.org/10.3168/jds.S0022-0302(91)78551-2

Wallace, R. J., Colombatto, D., & Robinson, P. H. (2008). Enzymes, direct-fed microbials and plant extracts in ruminant nutrition. *Anim. Feed Sci. Tech. 145*, 1-4. http://dx.doi.org/10.1016/j.anifeedsci.2007.07.006

Wallace, R. J., Onodera, R., & Cotta, M. A. (1997). Metabolism of nitrogen-containing compounds. In P. N. Hobson & C. S. Stewart (Eds.), *The Rumen Microbial Ecosystem* (2nd ed., pp. 283-328). New York, NY: Blackie Academic and Professional. http://dx.doi.org/10.1007/978-94-009-1453-7_7

Wang, Y., McAllister, T. A., Rode, L. M., Beauchemin, K. A., Morgavi, D. P., Nsereko, V. L., … Yang, W. (2001). Effects of an exogenous enzyme preparation on microbial protein synthesis, enzyme activity and attachment to feed in the Rumen Simulation Technique (Rusitec). *Br. J. Nutr., 85*, 325-332. http://dx.doi.org/10.1079/BJN2000277

Ware, R. A., & Zinn, R .A. (2005). Influence of Maceration and fibrolytic enzyme on the feeding value of rice straw. *J. Anim. Vet. Adv., 4*, 387-392.

Warner, A. C. I. (1964). Production of volatile fatty acids in the rumen. Methods of measurements. *Nutr. Abs. Rev., 34*, 339.

Wiedmeier, R. D., Arambel, M. J., & Walters, J. L. (1987). Effect of yeast culture and *Aspergillus oryzae* fermentation extract on ruminal characteristics and nutrient digestibility. *J. Dairy Sci., 70*, 2063-2068. http://dx.doi.org/10.3168/jds.S0022-0302(87)80254-0

Yalçin, S., Yalçin, S., Can, P., Gurdal, A., Bag, C., & Eltan, O. (2011).The nutritive value of live yeast culture (*Saccharomyces cerevisiae*) and its effect on milk yield, milk composition and some blood parameters of dairy cows. *Asian-Aust. J. Anim. Sci., 24*, 1377-1385.

Yang, W. Z., Beauchemin, K. A., & Rode, L. M. (2000). A comparison of methods of adding fibrolytic enzymes to lactating cow diets. *J. Dairy Sci., 83*, 2512-2520. http://dx.doi.org/10.3168/jds.S0022-0302(00)75143-5

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Effects of Haulm Killing on Seed Potato Quality

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Abstract

In Northern Finland (65º40′N), haulm killing is used in seed potato production primarily to regulate tuber size. The most common haulm killing method is mechanical-chemical. We studied the effects of mechanical and mechanical-chemical haulm killing methods on seed potato quality, comparing to natural haulm senescence (control). The timing of haulm killing (when no more than 5% of the crop tubers were over 50 mm in size) and the time between destruction and harvest (21–26 days) were similar to practices followed in seed-potato production. Matilda was the cultivar used. In this study, haulm killing clearly increased plant disease pressure, as black scurf (*Rhizoctonia solani*) could be seen in tubers whose haulm had been destroyed. Black scurf was also observed in mechanical haulm killing. The yield from naturally senesced haulms had less black scurf than the other treatments. In addition, when haulm senescence occurred naturally, crop yield and starch content were highest compared to other treatments.

Keywords: seed potato, haulm killing, quality, black scurf

1. Introduction

Haulm killing can be used to advance harvesting, reduce foliage mass, obtain a suitable tuber size, strengthen tuber skins before harvesting and prevent plant pathogens from spreading amongst the foliage and crop (Struik & Wiersema, 1999). The aim of haulm killing in seed potato production is particularly to control soil-borne or seed-borne diseases, including viruses, black scurf (*Rhizoctonia solani*), late blight (*Phytophthora infestans)*, gangrene (*Phoma foveata)*, Verticillium wilt (*Verticillium dahliae)* and bacteria (Kempenaar & Struik, 2007).

The haulm can be destroyed using a variety of methods (steaming, flaming, electrocutting, vine pulling and mechanical or chemical methods or combinations of both). In the study of Misener and Everett (1981), different haulm killing methods were compared and haulm pulling to cut off the tubers from the roots was found to be most effective; 98–100% of the haulm dried and re-growth appeared in only 2–3%. Pulling immediately inhibits haulm starch synthesis, prevents phloem transport of photosynthesis products to the tubers (Tiessen et al., 2002) and prevents the supply of growth-stimulating hormones. Haulm killing would appear to be more effective whatever the method used if carried out once the foliage has already activated natural senescence (van Evert, van der Voet, van Valkengoed, Kooistra, & Kempenaar, 2012).

Effects of the timing of haulm killing in seed potato production on physiological condition have been studied in different production conditions and cultivars. In their studies, Brown, Beattie, and Laurence (2003) observed that the timing of haulm killing affected the physiological properties of seed potatoes, whereas Wurr, Fellows, Akehurst, Hambidge, and Lynn (2001) observed that the effects of the timing of haulm killing were not clera. According to Struik and Wiersema (1999), differences in disease resistance between cultivars must be taken into consideration in determining the timing of haulm killing. If the cultivar is not highly disease-resistant, it is recommended that the haulm is destroyed earlier than those of more disease-resistant cultivars. The presence of aphids must also be considered, along with the virus infection pressure in the production area. Early haulm killing decreases crop yield but, on the other hand, haulm killing carried out too late increases the risk of pathogens and potato diseases (Struik & Wiersema, 1999). The use of haulm killing or haulm killing chemicals is recommended when the foliage shows signs of natural senescence, because interrupting its growth while still immature causes crop starch content to remain low, and the physiological condition of tubers used to produce seed potatoes may remain unstable (Kempenaar & Struik, 2007).

The effects of the time between haulm killing and harvesting have become apparent primarily in the context of the physiological behaviour of seed potatoes (Wurr et al., 2001; Brown et al., 2003). The recommended time between haulm killing and harvest is 10–14 days. Skin set typically takes 10–14 days, depending on the cultivar and soil conditions (Halderson et al., 1988). If harvest is delayed in excess of the recommended time, there is a real risk of increased infection from plant diseases (Struik & Wiersema, 1999).

Under northern Europe production conditions, haulm killing is used primarily as a method of controlling tuber size, as tuber growth is relatively quick in long-day conditions (Temmerman et al., 2002). Haulm killing is often carried out on highly immature plants that may still be flowering, and the timing of haulm killing is not synchronized with foliage senescence or potato tuber maturation. When haulm killing has to be carried out at a relatively early stage, the time between destruction and harvest can be easily prolonged for as long as three or four weeks if weather conditions permit. In the present study we examined whether the methods and the chemicals used in the haulm killing practices of seed potato production affect seed-potato crop variables (crop yield), starch content and external quality).

2. Materials and Methods

2.1 Field Experiments

The soil type in the field plots was medium fine sand, with pH between 5.8–6.2. The plots were cultivated in 3-year crop rotation with barley as a rotation crop. The preparation of field plots was carried out according to normal cultivation practise: ploughing in autumn, seedbed preparation by S-tine harrow and horizontal rotary cultivator (Juko, Finland) in spring. The fertiliser used in the experiments was Potato Y1 (NPK 8–5–19 CCF) and the level of nitrogen fertiliser was 60 kg ha^{-1}. Field experiments were planted by semi-automatic planting machine (Kuppi-Juko, Finland) with 80 cm row-spacing and 28 cm planting density, between 19 May–6 June. Rimsulfuron (250 g kg^{-1}, 30+20 g ha^{-1} at 300 l H_2O, Titus® WSB, Berner) was used for weed control. The experimental squares were 3.2 m x 10.0 m in size, with four replications. The cultivar was the mid-to-late maturing Matilda, seed class E2.

2.2 Observations and Haulm Killing Treatments

After planting, we recorded the days to emergence, the developmental stages as defined by Hack et al. (1993) and observations of any foliage diseases. Temperature data were recorded regularly by the Finnish Meteorological Institute, Oulunsalo weather station (64.9 N, 25.4 E). The temperature calculations from the growing seasons were compared to the long-term averages (Table 1).

Table 1. Weather data representing average temperature sums (°C) and precipitation (mm) during the experiments. Data collected by the Finnish Meteorological Institute, Oulunsalo weather station (64.9 N, 25.4 E) April to September.

	Average temperature sums (°C)				Precipitation (mm)			
				20 years				20 years
	year 1	year 2	year 2	means	year 1	year 2	year 3	means
April	168	173	141	105	37	28	83	40
June	388	475	366	351	66	26	7	61
July	824	862	810	678	88	72	83	78
August	1068	1198	1086	928	66	51	31	64
September	1106	1303	1211	1034	67	59	14	54

The timing of haulm killing was determined by test harvesting during the growing season, and the haulm was crushed when no more than 5% of the crop tubers were over 50 mm in size. The target of yield tuber size was 35–55 mm. At the times of haulm killings, the foliage showed only a few signs of natural senescence. Mechanical haulm killing was carried out by crushing (Grimme, Germany) the foliage to 20–30 cm height. Reglone® (diquat dibromide, 200 g/l at 300 l/ha, Syngenta Crop Protection) or Spotlight Plus (carfentrazone-ethyl 60 g/l at 300 l/ha, Berner) were used for chemical haulm killing treatments two days after mechanical haulm killing (Table 2). As a control, the haulm was left to naturally senesce until harvest. The efficiency of chemical haulm killing was observed as browning of the green parts of the foliage on a 0–100% scale at 3, 7, 14 and 21 days after the chemical

was sprayed. The efficiency of mechanical crushing was assessed as a percentage of the haulm covered, with the condition following haulm crushing used as comparison. The re-growth of foliage was assessed on a scale of 0–100 (0=no growth, 100=growth in each plant).

Table 2. Haulm killing treatments carried out in the field experiments

	Treatment
Natural senescence	-
Mechanical	Haulm crushing
Mechanical-chemical 1	Crushing + Reglone 1.5 l/ha (diquat dibromide 200 g/l)
Mechanical-chemical 2	Crushing + Spotlight Plus 1.0 l/ha (carfentrazone-ethyl 60 g/l)

Table 3. Dates of planting, haulm killing, days after planting (DAP) and harvesting, days after haulm killing (DAH)

Year	Planting	Haulm killing		Harvesting
1.	6.6.	58 DAP	(3.8. mechanical) (8.8. chemical)	26 DAH (3.9.)
2.	21.5.	97 DAP	(26.8. mechanical) (28.8.chemical)	21 DAH (18.9.)
3.	19.5.	85 DAP	(12.8. mechanical) (14.8. chemical)	21 DAH (2.9.)

2.3 Yield and Quality Analyses

Harvesting (Underhaug 2100 harvester, Norway) was carried out 21–26 days after haulm killing (Table 3). Yields per plot were determined by weighing (Mettler, Germany, accuracy 0.02) and the tubers graded (SKALS RB814, Sweden) according to size (<35 mm, 35–55 mm, 55–70 mm and >70 mm). Starch content (specific gravity: 214.53 x (air weight/(air weight – water weight) – 217.76) and the external quality of the tubers was analysed by plot based on the 35–70 mm sized tubers. External quality was assessed visually by separating healthy and damaged tubers according to damage category. Damage was divided into the following categories: damage caused by disease (1 = healthy; 2–5 = damage types such as: scab, black scurf, soft rot, blackleg, other fungal or bacterial diseases; 6–10 = damage from physiological or other causes: turgor split, growth crack, misshape, greening, black spot, hollow heart, internal necrosis, other). The tubers in each damage category were weighed and the quantity of each damage type was calculated as a percentage of weight. As an additional definition of external quality, the quantity of black scurf was observed separately in each tuber as a percentage of tuber surface. In the analyses, the regulations concerning seed potato certification inspections were taken into consideration. These allow a maximum of 3% by weight of black scurf, skin spot and deep-scab (where the scab-covered area is more than one tenth of the tuber surface). In addition, the area covered by black scurf and common scab (where the area covered by common scab is over one third of the tuber surface) must not exceed 5% of the sample. Tubers below the certification limits were classified as healthy.

2.4 Statistical Analyses

The experimental model was fully randomized complete block. The statistical analyses were conducted using the Mixed Procedure from the SAS 9.2/SAS Enterprise Guide 4.3 (SAS Institute Inc., Cary, NC, USA) program, using a variance analysis model in balanced data from randomized complete block with means comparisons and contrasts.

3. Results

3.1 Observations and Haulm Killing Treatments

The foliage of the field experiments developed normally, emergence lasted 17–19 days after planting. *R. solani* stolon canker on potato stems was observed in 0–3.5% of all the plants. The *R. solani* infected plants were found to be scattered around the field experiment areas. Other foliage diseases were not observed. Mechanical-chemical

haulm killing was effective, destroying the haulm completely in two weeks (Figure 1). Of the chemicals, diquat dibromide (200 g/l) acted faster than carfentrazone ethyl (60 g/l), but a little re-growth (12%) was observed when diquat dibromide was used. Re-growth was also observed (24%) annually when only mechanical treatment was used.

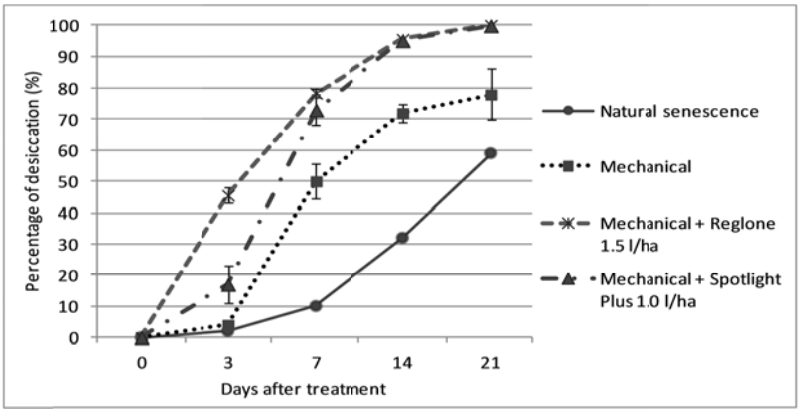

Figure 1. Potato leaf and stem desiccation visually rated at 3, 7, 14 and 21 days after treatments on a linear scale of 0–100, 0=no effect, 100=total kill of haulm

3.2 Yield and Quality Analyses

Naturally senesced haulm produced the largest yield (40.3 t/ha, p=<0.001 on average) and the tuber starch content (17.2%, p=<0.001) reached the level typical of the cultivar (Figure 2). On average, mechanical haulm killing affected yield and starch contents in the same way as mechanical-chemical haulm killing. At the annual level, the variance was such that during the first year, both the yield (31.4 t/ha, p=0.012) and starch content (14.8%, p=0.025) were higher in mechanical haulm killing than in mechanical-chemical haulm killing. During the next two years, there were no significant differences. The average mechanical-chemical yield level was 29.2 t/ha and starch content varied between 12.3–15.8%. The smallest <35 mm part, 7% of the total yield, was produced by the control (natural senescence). In mechanical haulm killing, <35 mm size was 17%, and 20% in mechanical-chemical haulm killing. The proportion of 35–55 mm was 71% in the control (natural senescence), 78% in mechanical haulm killing and 76% on average in mechanical-chemical haulm killing. Only naturally senesced haulm produced >70 mm tubers (Table 4).

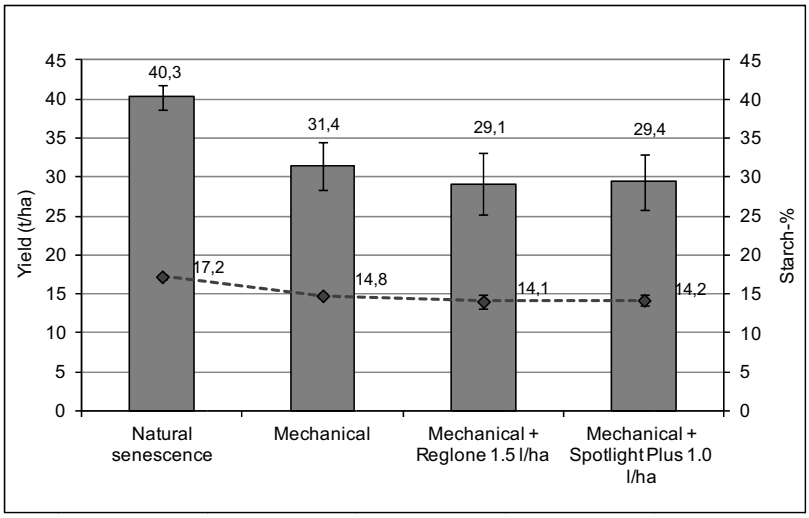

Figure 2. The values are the averages of three years. Bars represent yield and ♦ symbols starch content

There was abundant black scurf in the tubers (Figure 3). The incidence of black scurf varied between 0–60% by treatments and there was differences between the years. In the first two years, mechanical-chemical haulm killing increased the occurrence of black scurf (30% on average) to a statistically significant degree (p=>0.001) compared to other treatments. Mechanical haulm killing also increased the occurrence of black scurf (27% on average, p=0.028) compared to naturally senesced haulm. During the final year, there were no statistically significant differences between treatments with regard to the presence of black scurf. No other plant diseases were found except the presence of few mechanically damaged tubers (results not shown).

Table 4. Re-growth (%) after haulm killing and effects of haulm killing (cultivar Matilda) on seed potato yield (t/ha), tuber size distribution (%), starch content (%) and black scurf (*R. solani*) (%) in yield

	Re-growth (%)	Yield t/ha	<35	35-55	55-70	>70	Starch- %	Black scurf (%)
Year 1.								
No haulm killing	-	36.9***	4.2	75.7	15.0	5.1	17.4***	0.0
Mechanical	32	24.3**	17.9	79.8	2.3	0.0	14.2**	0.5
Mechanical+Regone 1.5 l/ha	18	20.1	19.5	78.3	2.2	0.0	12.3	36.8***
Mechanical+Spotlight 1.0l /ha	-	20.9	17.9	79.6	2.5	0.0	12.8	29.3***
Year 2.								
No haulm killing	-	41.0***	5.4	67.1	20.1	7.4	17.5***	10.9
Mechanical	29	34.3	13.2	78.4	8.4	0.0	14.7*	38.7***
Mechanical+Regone 1.5 l/ha	9	33.6	15.3	77.3	7.4	0.0	15.8	62.5***
Mechanical+Spotlight 1.0 l/ha	-	33.6	14.4	78.4	7.2	0.0	15.4	60.6***
Year 3.								
No haulm killing	-	43.0***	11.1	71.0	11.7	6.3	16.7**	13.0
Mechanical	10	35.5	20.5	77.9	1.6	0.0	15.5	12.4
Mechanical+Regone 1.5 l/ha	8	33.7	25.9	71.4	2.7	0.0	14.0	18.9
Mechanical+Spotlight 1.0 l/ha	-	33.5	22.9	77.0	0.1	0.0	14.5	16.2
Means								
No haulm killing	-	40.3***	7.1***	71.0	15.6***	6.3**	17.2***	7.9
Mechanical	24	31.4*	17.2	78.1	4.7	0.0	14.8*	17.2**
Mechanical+Regone 1.5 l/ha	12	29.1	20.2	75.7	4.1	0.0	14.1	39.4***
Mechanical+Spotlight 1.0 l/ha	-	29.4	20.3	77.1	2.5	0.0	14.2	35.3***

Statistically significant differences between haulm killing treatments at *P = 0.05, **P = 0.01 and ***P=0.001. There were no significant interactions between treatments and other variables measured.

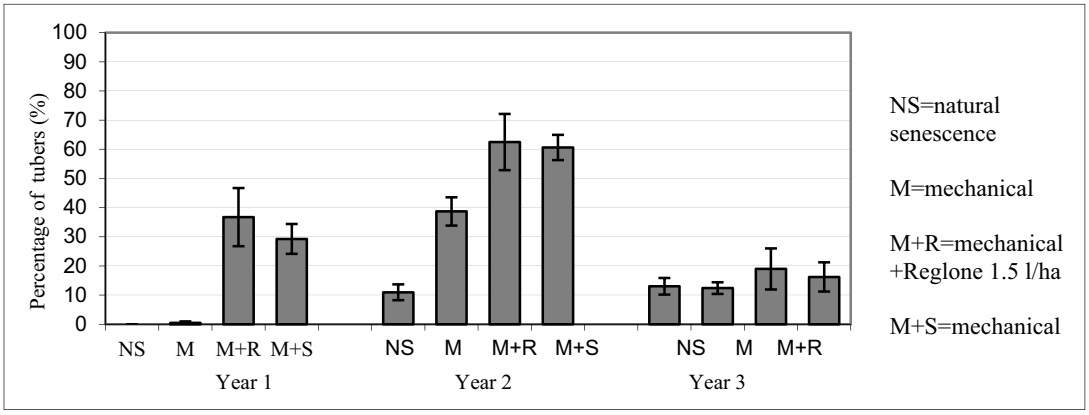

Figure 3. Percentage of tubers infected with black scurf (contamination area >10% of the tuber surface) among the yields by treatments based on analyses of external quality

4. Discussion

4.1 Haulm Killing Treatments

When haulm killing is used to regulate tuber size in short growing-season production conditions, in many cases, the haulm must be destroyed while still completely green and capable of photosynthesis. Earlier studies have found that in order to reach adequate efficiency, more chemicals are needed to destroy vital foliage than to destroy more senesced foliage (van Evert et al., 2012). To keep the use of haulm-killing chemicals at a reasonable level, additional tuber growth and photosynthesis can be efficiently interrupted by means of mechanical-chemical haulm killing.

The differences between the efficiency of the chemicals used for haulm killing are generally small. In earlier studies, as well as in our study, diquat was found to have a slightly more rapid effect than other chemicals (Ivany, 2001). Mechanical haulm killing also works well (Waterer, 2007). However, when applied to immature foliage, our study showed re-growth after crushing. Re-growth and reformation of photosynthetic capacity manifested as increased yield levels following mechanical haulm killing, compared to mechanical-chemical haulm killing under the first year. The results were contrary to the studies of Waterer (2007) in which the yield levels after mechanical haulm crushing were lower in comparison to yield levels after mechanical-chemical haulm crushing. Compared to other haulm killing methods, re-growth was least when following haulm pulling (Misener & Everett 1981; Halderson et al., 1988), because disconnecting foliage from the roots leads to instant cessation of phloem transport and interruption of growth (Tiessen et al., 2002). After haulm killing, previously synthesized metabolites may continue to increase in the still green stems and the remaining bottom leaves (Halderson et al., 1988) and growth-stimulating hormones have an opportunity to exert their effect on re-growth (Tiessen et al., 2002). Regardless of the method used, haulm killing would be most efficient if carried out at the time when the haulm has already started to senesce naturally (van Evert et al., 2012). Haulm vitality at the end of the growing season varies with weather conditions, production management and potato variety.

4.2 Yield and Quality

It is possible to regulate tuber size distribution by means of haulm killing; however this is achieved at the cost of yield and starch content. As a method of controlling tuber size mostly between 35–55 mm, haulm killing is easy to implement, and 70–80% of the tubers were distributed in the preferred size in our study. According to Struik and Wiersema (1999), the lower starch content and yield produced by haulm killing, as compared to the yields produced by natural senescence, are the natural result of haulm killing carried out at an early stage. When haulm killing is used in seed potato production primaly to achieve optimal tuber size distribution for commercial purposes, the physiological state of seed potatoes cannot be predicted. In addition to physiological state, the relatively low starch content of seed potatoes to which haulm killing is applied, may affect their vitality (Sabba et al., 2007) to produce new sprouts and roots during the next growing season.

The 10–14 day interval between haulm killing and harvest is considered adequate in terms of periderm maturity, depending on the cultivar (Waterer, 2007). Skin set typically takes 10–14 days, depending on the cultivar and soil conditions (Halderson et al., 1988). In northern seed potato production conditions, haulm killing is applied to immature plants and harvest is delayed as long as over 3 weeks after the haulm killing, weather conditions permitting. Tuber crops are known to be exposed to infection by black scurf when the haulm is destroyed while the root system is still in operation and the time between haulm killing and harvest is prolonged (Tsror, 2010). Black scurf caused by *R. solani* (Kühn AG-3) leads to substantial economic losses (Lootsma & Scholte, 1996), and in seed potato production, it may be an obstacle to certification. In our study, mechanical-chemical haulm killing in particular increased black scurf. The results are similar to those of Dijst (1988) where chemical haulm killing and cutting the stems, along with the prolonged period of time between haulm killing and harvest stimulated the formation of black scurf. According to Dijst (1988), water-insoluble components enable the formation of scleroses on the tuber surfaces, and the changes taking place in the relationship of inhibiting and stimulating factors after haulm killing could influence sclerosis formation. In addition, the colonisation by *R. solani* on the ground before harvest is a factor that significantly influences the occurrence of black scurf in tubers (Lootsma & Scholte, 1996). According to Lootsma and Scholte (1996), weather conditions are also significant in terms of the occurrence of *R. solani*, and wet and low temperatures favour its development. In this study, black scurf occurrence in tubers was the lowest after a dry growing season and the highest after cool and more moist conditions.

The results of our study showed that in seed potato production, the timing of haulm killing should not be determined only by tuber size even if the haulm killing methods in use work effectively. The best-selling part of the yield in terms of tuber size may be lost because of a plant disease. More research is needed to optimize haulm

killing and to suppress different soil-borne and seed-borne diseases. Even though haulm killing can decrease virus- or other seed- or soil-transmitted plant diseases in seed potato production, some haulm-killing methods or production conditions may, however, further the incidence of plant diseases (Kempenaar et al., 2008; Dijst, 1988). Production- and cultivar-specific demands must be taken into consideration (Ivany & Sanderson, 2001; Pavlista, 2001; Bethke & Busse, 2010) and sensors or other methods need to be developed to determine the timing or the amounts of chemicals used for haulm killing (van Evert et al., 2012). Our study provides significant information regarding haulm killing in the northern conditions (latitudes 65º40′N), specifically to the as well as seed potato production of the cultivar Matilda. In this case haulm killing in seed potato production is questionable since total yield and starch content was lower and black scurf infection higher compared to the control.

References

Bethke, P. C., & Busse J. S. (2010). Vine-Kill treatment and harvest date have persistent effects on tuber physiology. *American Journal of Potato Research, 87*, 299-309. http//dx.doi.org/10.1007/s12230-010-9137-4

Brown, P. H., Beattie, B., & Laurence, R. (2003). Intergenerational effects on seed potato physiological aging. *Acta Horticulture, 619*, 241-249.

Dijst, G. (1988). Effect of periderm and water-soluble exudates of potato tubers on black scurf formation before and after haulm destruction. *Netherlands Journal of Plant Pathology, 94*, 247-266. http//dx.doi.org/10.1007/BF01977316

Hack, H., Gall, H., Klemke, T., Klose, R., Meier, U., Stauss, R., & Witzenberger, A. (1993). The BBCH-scale for phonological growth stages of potato (*Solanum tuberosum* L.). *Proceedings of the 12th Annual Congress of the European Association for Potato Research, Paris,* 153-154.

Halderson, J. L., Haderlie, L. C., & Skrobacki, A. (1988). Mechanical vine killing of potatoes. *American Potato Journal, 65*, 415-423. http//dx.doi.org/10.1007/BF02852962

Ivany, J., & Sanderson, J. (2001). Response of potato (Solanum tuberosum) cultivars to glufosinate-ammonium and diquat used as desiccants. Weed Technology*, 15*, 341–345.

Kempenaar, C., & Struik, P. C. (2007) Haulm killing. *Potato Research, 50*, 341-345. http//dx.doi.org/10.1007/s11540-008-9082-5.

Lootsma, M., & Scholte, K. (1996). Effects of soil disinfection and potato harvesting methods on stem infection by *Rhizoctonia solani* Kühn in the following year. *Potato Research, 39*, 15-22. http//dx.doi.org/10.1007/BF02358202

Misener, G. C., & Everett, C. F. (1981). Vine pulling as a means of top killing potatoes. *American Potato Journal, 58*, 103-109. http//dx.doi.org/10.1007/BF02854379

Pavlista, A. (2001). Hydrothol as a vine desiccant of Atlantic Potatoes. *Journal of Vegetable Crop Production pitää olla kursiivi, 7*, 59-68. http://dx.doi.org/10.1300/J068v07n01_08

Sabba, R. P., Bussan, A. J., Michaelis, B. A., Hughes, R., Drilias, M. J., & Glynn, M. T. (2007). Effect of planting and vine-kill timing on sugars, specific gravity and skin set in processing potato cultivars. *American Journal of Potato Research, 84*, 205-215. http://dx.doi.org/10.1007/BF02986270

Struik, P. C., & Wiersema, S. G. (1999). *Seed Potato Technology*. The Netherlands: Wageningen Pers.

Temmerman, L., Wolf, J., Colls, J., Bindi, M., Fangmeier, A., Finnan, J., Ojanperä, K., & Pleijel, H. (2002). Effect of climatic conditions on tuber yield (*Solanum tuberosum* L.) in the European "CHIP" experiments. *European Journal of Agronomy, 17*, 234-355. http://dx.doi.org/10.1016/S1161-0301(02)00064-3

Tiessen, A., Hendriks, J. H., M., Stitt, M., Branscheid, A., Gibon, Y., Farré, E. M., & Geigenberg, P. (2002). Starch synthesis in potato tubers is regulated by post-translational redox modification of ADP-glucose pyrophosphorylase: A novel regulatory mechanism linking starch synthesis to the sucrose supply. *The Plant Cell, 14*, 2191-2213. http://dx.doi.org/10.1105/tpc.003640

Tsror, L. (2010). Biology, epidemiology and management of *Rhizoctonia solani* on potato. *Journal of Phytopathology, 158*, 649-658. http://dx.doi.org/10.1111/j.1439-0434.2010.01671.x

van Evert, F. K., van der Voet, P., van Valkengoed, E., Kooistra, L., & Kempenaar, C. (2012). Satellite-based herbicide rate recommendation for potato haulm killing. *European Journal of Agronomy, 43*, 49-57. http://dx.doi.org/10.1016/j.eja.2012.05.004

Waterer, D. (2007). Vine desiccation characteristics and influence of time and method of top kill on yields and quality of four cultivars of potato (*Solanum tuberosum* L.). *Canadian Journal of Plant Science, 87,* 129-135. http://dx.doi.org/10.4141/P06-074

Wurr, D. C. E., Fellows, J. R., Akehurst, J. M., Hambidge, A. J., & Lynn., J. R. (2001). The effect of cultural and environmental factors on potato seed tuber morphology and subsequent sprout and stem development. *Journal of Agricultural Science, Cambridge, 136*, 55-63. http://dx.doi.org/10.1017/S0021859600008431

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Heavy Metal Accumulation in Roadside Soils and Grasses of Dhaka City, Bangladesh

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Abstract

Heavy metals are important environmental pollutants and their toxicity in human, plants and animals has been received much more attention. A study was conducted to investigate the heavy metal pollution of roadside soil and grasses of Dhanka city, Bangladesh. The highest levels of metal concentrations were found in the samples from heavy traffic congestion. The results revealed that Cu, Pb, Zn, Cr and Cd levels in the roadside soils of Dhaka city were low compared to several cities of the world. In grass samples, the highest concentration of heavy metals were found in the order- Zn > Cu > Cr > Pb > Cd. The maximum concentration of Cu was found in the sample collected from Kalabagan and mean value was 85.20μg g^{-1}, which was higher than critical toxic level for most plants. Similarly for Zn, a critical toxic level for plants is 100 μg g^{-1}, which exceeded by the mean value obtained from the present study. The highest concentration of Pb and Cd were found in the samples collected from Soinik Club and Bijoy Saroni, respectively. The study revealed that the contamination factor for Pb, Zn and Cd were several times higher compared to Cu and Cr, which indicates that Pb, Zn and Cd were the major pollutants in the roadside soils. Finally, the I_{geo} calculations of the roadside soils of Dhaka city also revealed moderate pollution level in soils by Pb, Zn and Cd from anthropogenic sources in the study area.

Keywords: environmental pollution, pollutants, pollution load index, pollutions level, Dhaka city, soil quality

1. Introduction

Environmental pollution has increasing in tremendous rate after global industrialization that has negative impacts on human health and ecosystem services (Onder et al., 2007). The contribution of cars and road transports to the global emission of atmospheric pollutants is regularly increasing (Viard et al., 2004). The road transports also induce the contamination of nearer soils by a pollutant transfer via the atmospheric fallouts (Viard et al., 2004; Nabuloa et al., 2006) or road runoff (Mitsch & Gosselink, 1993; Nabuloa et al., 2006). Dhaka is the busiest city of Bangladesh and peak period total travel demand in Dhaka City is 1.3 million vehicle Km^{-1} and 59.5 thousand vehicle $hours^{-1}$ (Habib, 2002). Therefore, emission from these transport vehicles results in significant heavy metal accumulation in roadside soils of Dhaka city.

Nowadays the toxic effects of heavy metals are burning issues and have been studied by many researchers (Flora, 2002; Yang et al., 2002; Nordberg, 2003; Massadeh et al., 2006). Entrance of heavy metals may occur in human and animal food chain as a result of their uptake by edible plants grown in contaminated soil (Bakirdere & Yaman, 2008). The toxic and hazardous effects of some heavy metals on human health are very significant and may cause many fatal diseases. Lead (Pb) is one of the heavy metal that is responsible for anemia, neurological disorder, hyperactivity and changes in blood enzymes in human body (Flora, 2002; Mortula & Rahman, 2002). Cadmium (Cd) and Zn are important toxic metals and longtime exposure of which may causes renal, pulmonary, hepatic, skeletal, reproductive and many other carcinogenic effects (Roa et al., 2001; Alam et al., 2003; Arora et al., 2008; Bhuiyan et al., 2011).

It is widely recognized that the principal reasons of heavy metals (Pb, Cu and Cd) derived from traffic congestion, long-range transport and household heating (Grigalaviciene et al., 2005; Viard et al., 2004). The spreading of contaminants is influenced by meteorological parameters such as rainfall, wind, and traffic intensity (Bakirdere & Yaman, 2008). The same meteorological conditions affect the concentration of same contaminants in the

roadside soil (Viard et al., 2004). The traffic density determine the lead level in soil and vegetation (Olajire & Ayodele, 1997; Othman et al., 1997; Carlosena et al., 1998; Swaileh et al., 2001; Viard et al., 2004; Grigalaviciene et al., 2005; Hjortenkrans et al., 2006).

Soil samples and vegetation is the most economic and reasonable ways for assessing heavy metal status in the atmosphere (Onder et al., 2007). Scots pines (Yilmaz & Zengin, 2003), acacia (Aksoy et al., 2000a), grass (Fatoki, 2003), other plants (Aksoy et al., 2000b), and other organisms such as fish (Rashed, 2001) have also been used for monitoring. In order to asses contamination by metals in the vicinity of a highway, several studies have been carried out dealing with the different compartments: study of global deposits, roadside soil and vegetation (Viard et al., 2004). Information on accumulation of heavy metal on roadside soil of this city due to highway traffic and vehicles is very limited (Aktaruzzaman et al., 2013). But this could be the new threat for agriculture. Determination of heavy metal accumulation in roadside soil may be an index of the environmental pollution of Dhaka city. Keeping this view in mind, the research was conducted to know the heavy metal accumulation of roadside soil and grasses of Dhaka city, Bangladesh as well as to access the geochemical deposition of heavy metal along with the pollution level of densely populated Dhaka city.

2. Materials and Methods

2.1 Study Location and Sampling Procedures

Table 1. Details about soil and plant samples collected from Dhaka city, Bangladesh

Locations	Sample ID	Type of sample	Distance from road (m)	Sampling time in 2011	Dry weight of grass (g)
South	1a	Soil	1	April-July-August	
Jatrabari	b	Soil	3		
Saydabad	2a	Soil	1	April-May-July	
Matijhil	3a	Soil &Grasses	1	May-July-August	9.43
Khilgaon	4a	Soil	1	April-June-August	
	b	Soil	10		
Shahbag	5a	Soil	1	April-May-October	
	b	Soil	15		
Azimpur	6a	Soil & Grasses	1	May-July-September	8.72
Sonargaon	7a	Soil & Grasses	3	April-June-August	6.30
hotel	b	Soil	20		
Kalabagan	8a	Soil & Grasses	2	June-July-October	7.58
	b	Soil	20		
Mogbazar	9a	Soil	1	May-July-September	
	b	Soil	8		
Satrastarmor	10a	Soil	1	May-June-August	
Bijoysaroni	11a	Soil	1	April-June-September	
	b	Soil & Grasses	8		9.15
Mohakhali	12a	Soil	1	May-August-October	
Agargaon	13a	Soil & Grasses	1		9.31
	b	Soil	15		
Mirpur-10	14a	Soil & Grasses	1	May, June, August	9.60
	b	Soil	15		
Gulshan-1	15a	Soil and Grasses	2	April-July-September	7.56
	b	Soil	15		
Soinik club	16a	Soil & Grasses	1	May-July-August	8.55
	b	Soil	5		
Banani	17a	Soil & Grasses	1	June-August-October	11.09
	b	Soil	20		
Airport	18a	Soil & Grasses	1	May-June-August	8.22
	b	Soil	8		
Azampur	19a	Soil	2	June-July-August	
Abdullapur	20a	Soil	1	May-June-September	
	b	Soil	5		

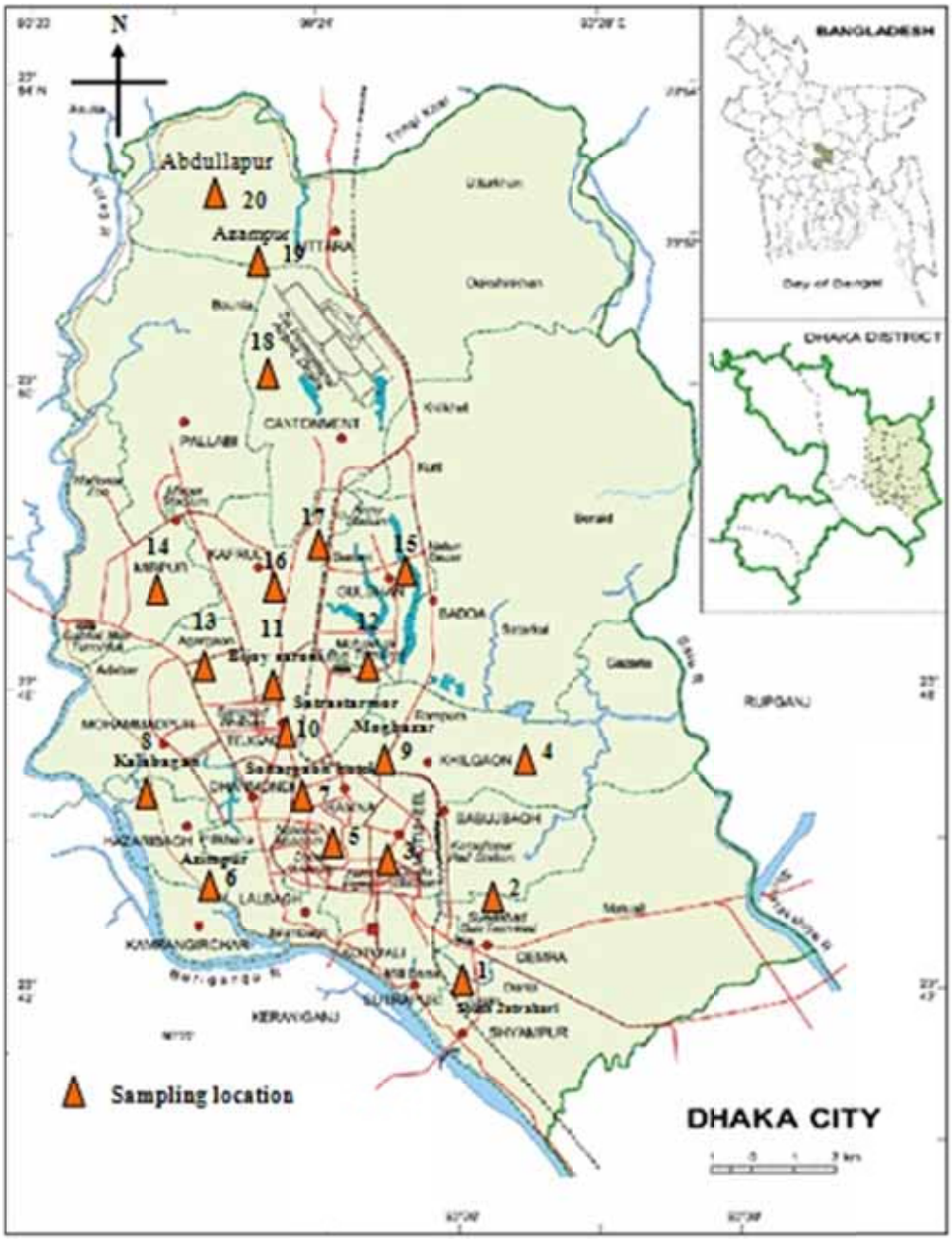

Figure 1. Sampling site at Dhaka city of Bangladesh

The study area is located at Dhaka city of Bangladesh which recently heavily polluted due to traffic congestion and remarkable metallic contamination caused by automobile emission in the roadside ecosystem. Total lengths of the surveyed roads were fifty kilometers from South Jatrabari to Abdullahpur, Dhaka. Completely randomized design was followed to collect the sample from the study location. Twenty sampling location were selected for collecting soil samples and eleven location for grass samples randomly by using the table of random number and samples were collected three times from the same place. Three different times sampling were done from 20 locations so that the variations of the heavy metal concentration independent of sampling period and not affected by the meteorological conditions (temperature, rainfall and wind). Sample collection started from South Jatrabari and moved towards Abdullapur. The soil and plant samples were collected to assess the pollution level during summer season (April to October), 2011 and wind flow during this time were north south direction. Usually, 350 – 500 g of each surface composite soil sample was collected at a depth of 0-20 cm from 20 locations of Dhaka city (Table 1, Figure 1). The soil samples were put into the individual polythene bag with definite marking and tagging. The soil

samples were air dried, cleaned by removing roots, plant fragments and stones. Then the samples were ground and subsequently sieved by using a 2 mm stainless steel sieve. The chemical analysis was carried out at the Laboratory of Department of Agricultural Chemistry, Bangladesh Agriculture University (BAU), Mymensingh, Bangladesh. The concentrations of heavy metals in grass and soil samples were determined at Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur.

For analysis of heavy metal content, each soil sample was homogenized and dried at 105°C. After that, in a beaker about 1.0 g of soil was taken and mixed with 3 ml of HNO_3/H_2O_2 (2/1). This mixture was shaken slowly and kept in a hot plate for drying. After cooling, about 2 ml of 0.75 mol l^{-1} nitric acid were added to the remainder and centrifuged. The clear digests were analyzed by using direct STAT-FAAS for Pb and Cd and FAAS for Cu as the procedure stated by Bakirdere and Yaman, 2008. Control analysis were carried out by using the same procedure.

For the analysis of vegetations, grass sample [common name: *Fulkhori* (*Ageratum conyzodes*) of Asteraceae family] were collected from 11 different locations in Dhaka roadsides shown in Table 1 & Figure 1. The collected samples were cleaned and washed with distilled water and kept in oven for drying at 110°C. About 5.0 g of dried sample were taken in 250 ml pyrex beaker and added 0.6 ml of concentrated sulphuric acid to each sample that helps to reduce the volatilization loss in ashing stage. After that the beaker was positioned into ashing furnace and kept for 4-5 hrs at 500°C until ashing was completed by turning the color of whistish. The process was repeated to get the white ash. After that, 3.0 ml of conc. HNO_3/H_2O_2 mixture (2/1) was put into the ashed sample and kept in hot plate for drying with continuous stirring. Then, approximately 2.0 ml of 1.0 mol l^{-1} HNO_3 was added to the left over residues sample and diluted to get required volume. Cu contents were examined by FAAS whereas Pb and Cd contents of clear digests were analyzed by using STAT-FAAS (Bakirdere & Yaman, 2008). Blank analyses were also carried out by using the same procedure.

2.2 Determination of Heavy Metals

The determination of different heavy metals in soil and grass sample was done by using an atomic absorption spectrophotometer (AAS) (Varian Spectra AA55B, Australia). Mono element hollow cathode lamp was employed for the determination of each heavy metal of interest. At first the AAS was calibrated followed by the manufacturer's recommendation. Then the extract was diluted (if required) and/or run directly in AAS for the determination of heavy metal in the sample. A standard line was prepared by plotting the absorbance reading on Y-axis versus the concentration of each standard solution of metal on X-axis. Then, the concentration of metal was calculated in the soil samples of interest by plotting the AAS reading on the standard line. The statistical analyses of the analytical results obtained from roadside soil and grasses samples were performed as outlined by Gomez and Gomez (1984).

2.3 Index of Geoaccumulation (I_{geo})

A geoaccumulation indexing (I_{geo}) approach was used to quantify the degree of anthropogenic contamination, and to compare the different metals in soils and sediments (Muller, 1969). This quantitative check of metal pollution in soils and sediments was proposed in the form of an equation defined as the index of geoaccumulation, as follows:

$$I_{geo} = \log_2 (C_n / 1.5 \times B_n)$$

Where, C_n is measured concentration of trace metal in the sediment, and B_n is the geochemical background for the same element which is either directly measured in pre-civilization sediments of the area or taken from the literature (average shale value described by Turekian & Wedepohl, 1961). The factor 1.5 is introduced to include possible variations of the background values that are due to lithologic variations.

2.4 Pollution Load Index

Pollution load index (PLI) is a multi-metal approach, which has been introduced by Tomlinson et al. (1980) for an overall assessment of soil and sediment quality with respect to trace metal concentrations. According to Tomlinson et al. (1980), PLI describes the quality of a site or an estuary in terms of easily understood by the non-specialist and which can be used to compare the pollution status of different sites and estuaries. The PLI for a single site is the *n*th root of *n* number of multiplied together contamination factor (CF) values. The CF and PLI for a single site can be obtained as follows:

$$CF = C_{\text{Metal concentration}} / C_{\text{Background concentration of the same metal,}}$$

$$\text{PLI for a site} = n\text{th } \sqrt{CF_1 \times CF_2 \ldots \times CF_n}$$

While computing the contamination factor (CF) for pollution load index (PLI) of soils of the studied region, average shale value for each heavy metal described by Turekian and Wedepohl (1961) were considered as

background concentration values. The concept of a baseline is a fundamental issue to the formation of a PLI (Tomlinson et al., 1980; Angula, 1966).

2.5 Statistical Analysis

Soils and plant samples were analyzed separately. The data represent means calculated from three replicated sample point for each. The statistical analyses of the analytical results obtained from roadside soil and grasses samples were performed as outlined by Gomez and Gomez (1984). Analysis of variance was performed using the Proc Mixed procedure of Statistical Analysis System (SAS Inst., 1999).

3. Results and Discussion

3.1 Heavy Metal Status in Roadside Soils

Total Cu, Zn, Pb, Cd and Cr concentrations of soils collected from different locations of Dhaka metropolitan city are presented in Table 2. The results of variance analysis showed that there were significant differences for soil sample collected in different locations (Table 3). The highest total Cu concentration of soils was 53.56 μg g^{-1}, which recorded at Sonargaon hotel (at a distance of 20 m from road), while the lowest content was 1.28 μg g^{-1} found at Banani (close to road). The mean value was 35.74 μg g^{-1} which indicates that distribution of Cu in roadside soils of Dhaka metropolitan area was generally lower than the world-wide values (100-300 μg g^{-1}; Fergusson, 1991).

Total Zinc concentrations in both close to road and a distance from the road samples varied with a range from 33.21 to 352.35 μg g^{-1} while the mean value was 136.73 μg g^{-1}. Chamon et al. (2009) reported that the soil collected from Tejgaon industrial area contained 685 μg g^{-1} Zn. This study recorded the highest concentration of Zn in soils collected from Agargaon and the lowest concentration was found at Mirpur-10 samples. The highest Pb concentration of soils was 104.62 μg g^{-1}, which was recorded at Bijoy Saroni (close to road), while the lowest content was 1.53 μg g^{-1} found at Mirpur-10 (at a distance from road). The mean value of Pb was 38.92 μg g^{-1} which is low in compare to others city in the world. The highest Cd concentration of soils was 0.672 μg g^{-1} which has been recorded at Bijoy Sarani (close to road), while the lowest content was 0.064 μg g^{-1} found at Sainik Club (at a distance from road). The mean value was 0.336 μg g^{-1} (Table 2), which also indicates that distribution of Cd in roadside soils of Dhaka metropolitan area were generally lower compared with several cities in the world (Table 4). Total Cr concentrations in both close to road and a distance from the road samples varied with a range from trace to 70.96 μg g^{-1} while the mean value was 21.74 μg g^{-1} (Table 2). On the contrary, Chamon et al. (2009) reported that the soil collected from Tejgaon industrial area contained 173 μg g^{-1} Cr.

Table 2. Total Cu, Zn, Pb, Cd and Cr concentrations in roadside soils collected from twenty selected locations of Dhaka city, Bangladesh

Location	Sample ID	Heavy metal concentration (μg g^{-1})				
		Cu	Zn	Pb	Cd	Cr
South	1a	48.06cd	115.83j	36.32lm	0.34fgh	68.62a
Jatrabari	b	19.49k	81.81no	16.85q	0.10l	0.00n
Saydabad	2a	22.61k	169.16f	38.24kl	0.32f-i	28.28f
Matijhil	3a	28.12j	115.16j	20.14p	0.50bcd	20.87hi
Khilgaon	4a	19.65k	49.41r	11.77r	0.24ij	21.24hi
	b	53.29ab	279.45b	65.19e	0.53bc	70.96a
Shahbag	5a	42.68efg	129.57hi	46.00i	0.46cde	15.74jk
	b	18.64k	88.29mn	53.93h	0.34fgh	18.25ij
Azimpur	6a	38.57ghi	163.96f	15.03q	0.14ki	13.24kl
Sonargaon	7a	50.09abc	166.05f	92.60b	0.58b	28.14f
hotel	b	53.56a	130.41h	52.47h	0.24ij	27.89f
Kalabagan	8a	41.17e-h	84.24mno	42.68j	0.38efg	0.00n
	b	6.51l	71.28p	19.46p	0.24ij	0.00n
Mogbazar	9a	23.39k	103.68l	21.50p	0.19jk	25.92fg
	b	52.92abc	76.14op	11.26r	0.10l	0.00n

Satrastarmor	10a	43.73def	105.46kl	25.84o	0.29hi	0.23n
Bijoy saroni	11a	53.13ab	183.87e	104.62a	0.67a	4.50m
	b	37.06hi	161.19f	53.49h	0.43de	41.48bc
Mohakhali	12a	28.36j	183.87e	27.48o	0.19jk	31.88e
Agargaon	13a	53.29ab	352.35a	86.56c	0.53bc	40.89c
	b	51.90abc	249.48c	76.91d	0.53bc	36.74d
Mirpur-10	14a	36.79hi	167.67f	37.08l	0.38efg	44.58b
	B	44.59de	33.21s	1.53t	0.53bc	0.00n
Gulshan-1	15a	35.07i	120.83ij	59.59f	0.27hij	23.58gh
	b	50.02abc	241.20c	34.17mn	0.19jk	13.85kl
Soinik club	16a	10.48l	92.50m	21.86p	0.30ghi	10.90l
	b	39.37f-i	225.75d	14.61q	0.06l	1.89mn
Banani	17a	1.28m	34.83s	7.12s	0.10l	0.00n
	b	39.30d-i	120.69ij	56.48g	0.43de	25.27fg
Airport	18a	48.38bcd	114.21jk	40.45k	0.34fgh	34.81de
	b	52.65abc	146.61g	56.35g	0.43de	36.04d
Azampur	19a	30.46j	120.30ij	33.69n	0.40ef	25.68fg
abdullahpur	20a	22.21k	90.72mn	26.33o	0.24ij	27.90f
	b	18.64k	58.32q	15.84q	0.14kl	0.00n
Range		1.28-53.56	33.21-352.35	1.53-104.62	0.06-0.67	0.00-70.96
Mean		35.750	136.103	38.924	0.328	21.745
Std. dev.		15.07	71.04	25.39	0.16	18.73
SE		2.58	12.18	4.35	0.03	3.21
LSD		4.33	8.87	2.23	0.073	3.11

Table 3. Summary of variance analysis for heavy metals measured from soil samples collected from different traffic congested areas of Dhaka city, Bangladesh

Heavy metals	Sources of variation	Sum of Squares	df	Mean Square	F	P value	Sig.
	Between Groups	22480.52	33	681.228	96.405	0.01	**
Cu	Within Groups	480.507	68	7.066			
	Total	22961.02	101				
	Between Groups	499555.5	33	15138.05	510.42	0.01	**
Zn	Within Groups	2016.746	68	29.658			
	Total	501572.3	101				
Pb	Between Groups	63803.87	33	1933.45	1034.04	0.01	**
	Within Groups	127.147	68	1.87			
	Total	63931.01	101				
Cd	Between Groups	2.475	33	0.075	41.811	0.01	**
	Within Groups	0.122	68	0.002			
	Total	2.597	101				
Cr	Between Groups	34720.15	33	1052.126	288.581	0.01	**
	Within Groups	247.919	68	3.646			
	Total	34968.07	101				

Table 4. Comparison of mean concentration of metals (μg g^{-1}) in roadside dust of Dhaka city with other cities of the world

City	Cu	Zn	Pb	Cd	Cr	Digestion
Amman[a]	177	358	236	1.7	-	$HCL+HNO_3$
Bahrain[b]	-	151.8	697.2	72	144.4	$HCL+HNO_3$
Birmingham[c]	466.9	534	48	1.6	-	$HCLO_4+HNO_3+H_2SO_4$
London[d]	87	174	232.7	1.4	-	$HCL+HNO_3$
Manchester[e]	113	653	265	-	-	HNO_3
Istanbul[f]	38.1	156	99.3	-	-	$HCL+HNO_3$
Israel[g]	60.4	82.2	87.4	0.27	42.4	$HCL+HNO_3$
Greece[h]	42.7	137.8	359.4	0.2	193.2	$HCLO_4+HNO_3+H_2SO_4$
(DhakaCity)	**35.75**	**136.103**	**38.924**	**0.328**	**21.745**	**$HCL+HNO_3$**

[a]Al-Khashman (2007); [b]Akhter and Madany (1993); [c]Charlesworth et al. (2003); [d]Akbar et al. (2006); [e]Robertson et al. (2003);[f]Guney et al. (2010); [g]Swailehet al. (2004); [h]Christoforidis and Stamatis (2009).

3.2 Heavy Metal Concentration in Roadside Grass

In case of grass (*Ageratum conyzodes*) samples, the highest heavy metal concentration values were found in the following order- Zn > Cu > Cr > Pb > Cd (Tables 5 & 6) of areas located near to industrial side found higher Zn content compare to others location and highely Zn polluted region is Agargaon, Dhaka. Analysis of variance in Table 6 showed that significance variation for each of the heavy metal concentration between groups due to the sample collected from different locations.

Table 5. Total Cu, Zn, Pb, Cd and Cr concentrations in roadside grasses collected from eleven selected locations of Dhaka city, Bangladesh

Locations	**Sample ID**	**Heavy metal concentration (μg g^{-1})**				
		Cu	**Zn**	**Pb**	**Cd**	**Cr**
Matijhil	3a	66.30g	86.70h	2.64e	0.42def	15.72e
Azimpur	6a	75.60b-e	108.90d	3.42d	0.72ab	29.46c
Sonarga Hotel	7a	73.80de	101.40e	2.40ef	0.60bc	31.62c
Kalabagan	8a	85.20a	119.10b	3.18d	0.48cde	35.34b
Bijoy sarani	11b	77.21bcd	120.20ab	2.50ef	0.41def	30.79c
Agargaon	13a	78.60bc	122.10a	2.16fg	0.30f	35.82bb
Mirpur-10	14b	75.30cde	116.70c	5.40ab	0.54cd	41.76q
Gulshan-1	15a	80.10b	77.70i	5.16b	0.36ef	36.06b
Soinik club	16b	74.70cde	97.20f	5.70a	0.72ab	36.30b
Banani	17a	67.20fg	90.30g	1.80f	0.42def	23.34d
Airport	18b	71.40ef	96.30f	3.96c	0.78a	38.58qb
Range		66.30- 65.20	77.70- 122.10	1.80-5.70	0.30-0.78	15.72- 41.76
Mean		75.037	103.327	3.484	0.523	32.254
Std. dev.		5.47	15.13	1.38	0.16	7.39
SE		1.65	4.56	0.42	0.05	2.23
LSD		2.45	1.28	0.24	0.09	1.84

Table 6. Summary of variance analysis for heavy metals measured from grass samples collected from different traffic congested areas of Dhaka city, Bangladesh

Heavy metals	Sources of variation	Sum of Squares	df	Mean Square	F	P value	Sig.
Cu	Between Groups	898.046	10	89.805	14.355	0.01	**
	Within Groups	137.628	22	6.256			
	Total	1035.675	32				
Zn	Between Groups	6868.025	10	686.803	403.786	0.01	**
	Within Groups	37.42	22	1.701			
	Total	6905.445	32				
Pb	Between Groups	57.475	10	5.747	92.376	0.01	**
	Within Groups	1.369	22	0.062			
	Total	58.844	32				
Cd	Between Groups	0.786	10	0.079	9.268	0.01	**
	Within Groups	0.187	22	0.008			
	Total	0.973	32				
Cr	Between Groups	1639.986	10	163.999	46.45	0.01	**
	Within Groups	77.674	22	3.531			
	Total	1717.661	32				

3.3 Assessment of Pollution Level

The geoaccumulation index (I_{geo}) introduced by Muller (1969) was also used to assess heavy metal pollution in roadside soils of Dhaka metropolitan city. Table 5 shows the geoaccumuation index, which includes seven grades.

Table 7. Measure of metal pollution in soil sample collected from Dhaka city by using geoaccumulation index

Index of Geo- accumulation	I_{geo} Class	Designation of soil or sediment quality
10 – 5	6	Extremely polluted
4 – 5	5	Strongly/ extremely polluted
3 – 4	4	Strongly polluted
2 – 3	3	Moderately/ strongly polluted
1 – 2	2	Moderately polluted
0 – 1	1	Uncontaminated/ moderately polluted
0	0	Unpolluted

The calculated I_{geo} for heavy metals of roadside soils of Dhaka city, and their corresponding contamination intensity are illustrated in Figures 2 & 3.

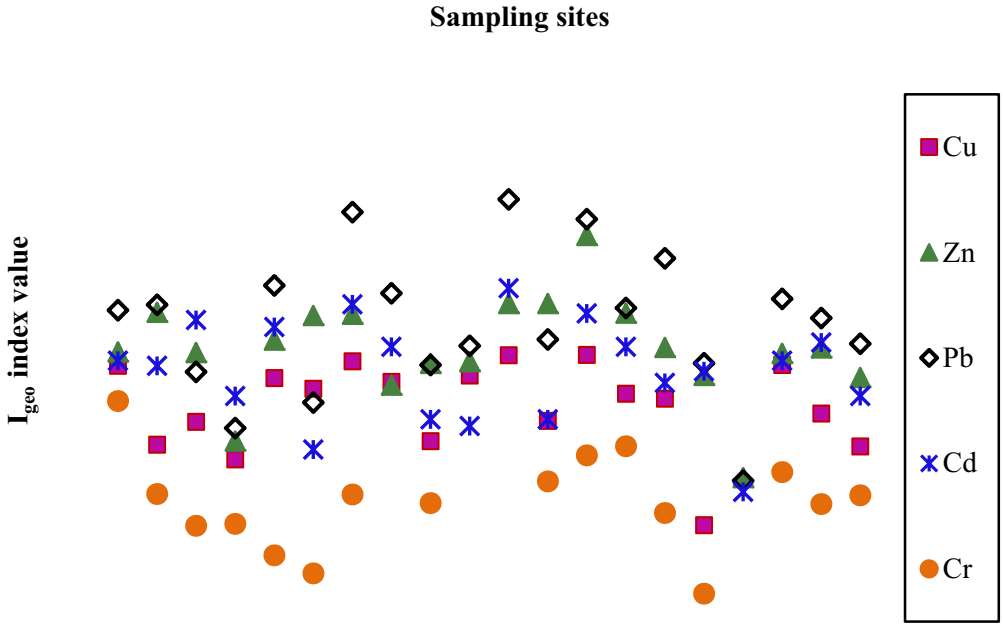

Figure 2. Geoaccumulation index (I_{geo}) of heavy metals in different roadside soils (very close of 1-3 m to the road) of Dhaka metropolitan city

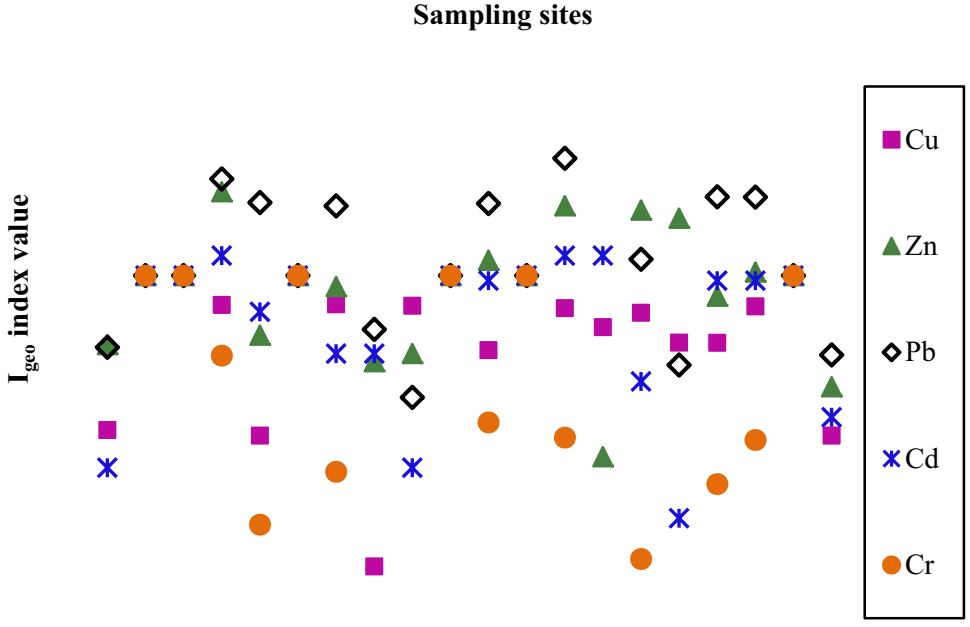

Figure 3. Geoaccumulation index (I_{geo}) of heavy metals in different roadside soils (at a distance of 5-20 m from the road) of Dhaka metropolitan city

3.4 Pollution Load Index (PLI)

The PLI values range from 0.08-1.72 for soil samples collected from 20 locations of very close to the road of Dhaka metropolitan city. On the other hand, the PLI values range from 0.16-1.74 for soil samples collected from 14 locations at a distance from the roadside of Dhaka metropolitan city (Table 8). While computing the contamination factor (CF) for pollution load index (PLI) of soils of the studied region, average shale value for each

heavy metal described by Turekian and Wedepohl (1961) were considered as background concentration values. The concept of a baseline is a fundamental issue to the formation of a PLI (Tomlinson et al., 1980). The index provides a simple, comparative means for assessing a site or estuarine quality: a value of zero indicates perfection, a value of one that only baseline levels of pollutants are present, and values above one would indicate progressive deterioration of the site and estuarine quality (Tomlinson et al., 1980).

Table 8. The contamination factor (CF) for each heavy metal at each sampling site of Dhaka metropolitan city.

Sampling sites (very close: 1-3 m from the road)	Contamination factor					PLI
	Cu	Zn	Pb	Cd	Cr	
1	1.07	1.22	1.82	1.12	0.76	1.15
2	0.50	1.78	1.91	1.07	0.31	0.89
3	0.62	1.21	1.01	1.65	0.23	0.78
4	0.44	0.52	0.59	0.80	0.24	0.48
5	0.95	1.36	2.30	1.55	0.17	0.96
6	0.86	1.73	0.75	0.48	0.15	0.60
7	1.11	1.75	4.63	1.92	0.31	1.40
8	0.91	0.89	2.13	1.28	0.00	0.34
9	0.52	1.09	1.07	0.64	0.29	0.65
10	0.97	1.11	1.29	0.60	0.00	0.29
11	1.18	1.94	5.23	2.24	0.05	1.06
12	0.63	1.94	1.37	0.64	0.35	0.82
13	1.18	3.71	4.33	1.76	0.45	1.72
14	0.82	1.76	1.85	1.28	0.50	1.11
15	0.78	1.27	2.98	0.91	0.26	0.93
16	0.23	0.97	1.09	1.01	0.12	0.50
17	0.03	0.37	0.36	0.32	0.00	0.08
18	1.08	1.20	2.02	1.12	0.39	1.03
19	0.68	1.27	1.68	1.33	0.29	0.89
20	0.49	0.95	1.32	0.80	0.31	0.69
Sampling sites at a distance of 5-20 m from the road						
1	0.43	0.86	0.84	0.32	0.00	0.19
4	1.18	2.94	3.26	1.76	0.79	1.74
5	0.41	0.93	2.70	1.12	0.20	0.75
7	1.19	1.37	2.62	0.80	0.31	1.01
8	0.14	0.75	0.97	0.80	0.00	0.18
9	1.18	0.80	0.56	0.32	0.00	0.21
11	0.82	1.70	2.67	1.44	0.46	1.20
13	1.15	2.63	3.85	1.76	0.41	1.53
14	0.99	0.35	0.08	1.76	0.00	0.16
15	1.11	2.54	1.71	0.64	0.15	0.86
16	0.87	2.38	0.73	0.21	0.02	0.37
17	0.87	1.27	2.82	1.44	0.28	1.05
18	1.17	1.54	2.82	1.44	0.40	1.24
20	0.41	0.61	0.79	0.48	0.00	0.18

4. Discussions

Road site soil gave higher Cu concentration might be the reason for industrial pollution near to sampling location. The results supports the finding of Chamon et al. (2009) and reported that the soil collected from Tejgaon industrial area contained 99.7 μg g^{-1} Cu. The source of Cu in the roadside soils was indicated by research as being due to corrosion of metallic parts of cars derived from engine wear, thrust bearing, brushing and bearing metals (Al-Khashman, 2004, 2007; Das & Nolting, 1993). It is apparent from Table 4 that Zn levels in roadside soils of Dhaka city were also low compared to several cities of the world. According to Elik (2003), higher concentration of Zn and Cd in heavy traffic zones indicates that fragmentation of car tires is a likely source of these metals (Yaylali-Abanuz, 2011; Mondol et al., 2011; Sorme & Lagerkvist, 2002; Kabata-Pendias, 2000). Other possible sources of Zn in relation to automobile traffic are: wearing of brake lining, losses of oil and cooling liquid and wearing of road paved surface (Saeedi et al., 2009).

The highest Pb values have been detected in the present study at soils close to road collected from Bijoy Saroni carrying heavy traffic (Al-Khashman, 2007; Fergusson, 1991). Lead pollution in the environmental samples including soil, dust, sediments and natural water comes from combustion of gasoline that contains tetraethyl lead as an anti-knocking agent (Rahman et al., 2012; Tuzen, 2003). The Pb is converted to PbO and PbO_2 which is then transformed into volatile $PbCl_2$, $PbBr_2$ and PbBrCl by the addition of dichloro- or dibromomethane to the gasoline. Therefore, Pb compounds emit into the atmosphere from vehicle exhaust gases using Pb added petroleum products (Saeedi et al., 2009).

Higher concentration of Cd in heavy traffic zones indicates that fragmentation of car tires is a likely source of these metals (Elik, 2003). Furthermore, Ni and Cd could also be traced to electroplating, and cadmium to tyres. The Cd is released as a combustion product in the accumulators of motor vehicles or in carburetors (Al-Khashman, 2007). Cadmium is now most commonly encountered in Cd-Ni battery production, although it continues to be used in paints as well as in plastic production where it is an effective stabilizing agent (Kabadayi & Cesur, 2010; Mohiuddin et al., 2010). However, the range of the median value of Cd in roadside soils and dust samples world-wide has been examined as being 0.5-4.0 μg g^{-1} (Fergusson & Kim, 1991). Similarly to Cd, the highest concentration of Cr was found in soils collected from Khilgaon and the lowest concentration was recorded at South Jatrabari samples. According to Al-Khashman (2007), the Cr in roadside soil and dust is associated with the chrome plating of some motor vehicle parts (Ahmed et al., 2012). The level of Cr in the investigated area was generally lower than those determined in other cities in the world (Table 4).

In the plant sample, the clearest effect of Zn excess is reduction of root growth for the low tolerant plant a critical toxic level of Zn for plants is 100 100 μg g^{-1}, which is exceeded by the mean value obtained from the present study (Rauna et al., 1988). Zinc has limited mobility in the plant and it can be accumulated in the root system when Zn has been added to the soil (Onder et al., 2007; Ahmed & Gani, 2010). The highest concentration of Cu was found in the sample collected from Kalabagan and mean value was 85.20 μg g^{-1}, which is above than critical toxic level for most plant. Robson and Reuter (1981) explained that there are different tolerance ranges for plants but a critical toxic level of Cu is in the range of 20 to 30 μg g^{-1} for most plants. The highest concentration of Pb and Cd was found in the sample collected from Soinik Club and Bijoy Saroni, respectively. In city areas, Pb comes from industrial and domestic wastewater and air pollution resulting from vehicle exhaust output and incineration of fossil fuels into the environment (Coskun et al., 2006). The main source of environmental Cd pollution is the ferrous steel industry. In addition, vehicle wheels, mineral oils and usage of waste mud may introduce Cd into the soil and this increases Cd levels of the plants (Sharma et al., 2006; Loska et al., 2004).

The I_{geo} values for Cu and Cr exhibited zero class, indicating unpolluted soil quality. The values for Cd, among the 20 locations of very close to the road and 14 locations at a distance from the road, 5 and 3 sites, respectively exhibited I_{geo} class 0-1, indicating uncontaminated/ moderately polluted soil quality by Cd. Similarly, in case of Zn, among the locations 7 sites of very close to the road and 6 sites at a distance from the road showed positive values ($0 < I_{geo} < 2$), that means I_{geo} class 1-2, indicating moderately polluted soil quality. Considering Pb, among the 20 locations of very close to the road, 55% sampling sites exhibited I_{geo} class 1-2, indicating moderately polluted soil quality by Pb. On the other hand, among the 14 locations at a distance from the roadside, 8 sites showed positive values ($0 < I_{geo} < 2$), that means I_{geo} class 1-2, indicating also moderately polluted soil quality. Finally, the I_{geo} calculations of the roadside soils of Dhaka metropolitan city revealed moderate pollution level in soils by Pb, Zn and Cd from anthropogenic sources in the study area.

PLI values out of 34 locations, total 12 sites had the value >1.0 indicates pollution load in the respective sites. On the other hand, it is evident from Table 6 that the contamination factor for both Zn, Pb and Cd were several times

higher compared to Cu and Cr, which indicates that Zn, Pb and Cd were the major pollutants in the roadside soils of Dhaka metropolitan city giving rise to PLI values for the study area.

5. Conclusion

This study recorded the highest concentration of total Cu (53.56 μg g^{-1}), Zn (352.35 μg g^{-1}), Pb (104.62 μg g^{-1}), Cr (70.96 μg g^{-1}) and Cd (0.672 μg g^{-1}) in soil samples collected at Sonargaon hotel (at a distance from road), Agargaon, Bijoy Saroni (close to road), Khilgaon and Bijoy Sarani (close to road), respectively. The I_{geo} calculations of the roadside soils of Dhaka metropolitan city revealed moderate pollution level in soils by Pb, Zn and Cd from anthropogenic sources in the study area. The higher PLI values of these pollutants indicate that the air and soil quality heavily deteriorates and which may have adverse effects on mankind and earth ecosystem.

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References

Ahmed, G., Miah, M. A., Anawar, H. M., Chowdhury, D. A., & Ahmad, J. U. (2012). Influence of multi-industrial activities on trace metal contamination: An approach towards surface water body in the vicinity of Dhaka Export Processing Zone (DEPZ). *Environmental Monitoring Assessment, 184*(7), 4181-4190. http://dx.doi.org/10.1007/s10661-011-2254-9

Ahmed, J. U., & Gani, M. A. (2010). Heavy metal contaminations in water, soil and vegetation of the industrial areas in dhaka, Bangladesh. *Environmental Monitoring Assessment, 166*, 347-357. http://dx.doi.org/10.1007/s10661-009-1006-6

Akbar, K. F., Hale, W. H., Headley, A. D., & Athar, M. (2006). Heavy metal contamination of roadside soils of Northern England. *Soil Water Res, 1*(4), 158-163. Retrieved from http://www.cazv.cz/userfiles/File/SWR%201_158-163.pdf

Akhter, M. S., & Madany, I. M. (1993). Heavy metals in street and house dust in Bahrain. *Water, Air, Soil and Pollution, 66*, 111-9. http://dx.doi.org/10.1007/BF00477063

Aksoy, A., Ahin, U., & Duman, F. (2000a). *Robinia pseudo-acacia* L. as a possible biomonitor of heavy metal pollution in Kayseri. *Tropical Journal of Botany, 24*, 279-284. Retrieved from http://journals.tubitak.gov.tr/botany/issues/bot-00-24-5/bot-24-5-3-9910-4.pdf

Aksoy, A., Çelik , A., & Öztürk, M. (2000b). Plants as possible indicators of heavy metal pollution in Turkey, *Chemia Inzyneria Ekologiczna, 11*, 1152-1161.

Aktaruzzaman, M., Fakhruddin, A. N. M., Chowdhury, M. A. Z., Fardous, Z., & Alam, M. K. (2013). Accumulation of heavy metals in soil and their transfer to leafy vegetables in the region of Dhaka Aricha Highways, Savar, Bangladesh. *Pakistan Journal of Biological Science, 16*(7), 332-338. http://dx.doi.org/10.3923/pjbs.2013.332.338

Alam, M. G. M., Snow, E. T., & Tanaka, A. (2003). Arsenic and heavy metal concentration of vegetables grown in Samta village, Bangladesh. *Science of Total Environment, 111*, 811-815. http://dx.doi.org/10.1016/S0048-9697(02)00651-4

Al-Khashman, O. A. (2004). Heavy metal distribution in dust, street dust and soil from the work place in Karak Industrial Estate, Jordan. *Atmospheric Environment, 38*, 6803-6812. http://dx.doi.org/10.1016/j.atmosenv.2004.09.011

Al-Khashman, O. A. (2007). The investigation of metal concentrations in street dust samples in Aqaba city, Jordan. Environ. *Geochemistry & Health, 29*, 197-207. http://dx.doi.org/10.1007/s10653-006-9065-x

Angula, E. (1966). The Tomlinson Pollution Index applied to heavy metal, Mussel-Watch data: A useful index to assess coastal pollution. *Science of Total Environment, 187*, 19-56. http://dx.doi.org/10.1016/0048-9697(96)05128-5

Arora, M., Kiran, B., Rani, A., Rani, S., Kaur, B., & Mittal, M. (2008). Heavy metal accumulation in vegetables irrigated with water from different sources. *Food Chemistry, 111*, 811-815. http://dx.doi.org/10.1016/j.foodchem.2008.04.049

Bakirdere, S., & Yaman, M. (2008). Determination of lead, cadmium and copper in roadside soil and plants in Elazig, Turkey. *Environmental Monitoring Assessment, 136*, 401-410. http://dx.doi.org/10.1007/s10661-007-9695-1

Bhuiyan, M. A. H., Suruvi, N. I., Dampare, S. B., Islam, M. A., Quraishi, S. B., Ganyaglo, S., & Suzuki, S. (2011). Investigation of the possible sources of heavy metal contamination in lagoon and canal water in the tannery industrial area in Dhaka, Bangladesh. *Environmental Monitoring Assessment, 175*, 633-649. http://dx.doi.org/10.1007/s10661-010-1557-6

Carlosena, A., Andrade, J. M., & Prada, D. (1999). Searching for heavy metals grouping roadside soils as a function of motorized traffic influence. *Talanta, 47*, 753-767. http://dx.doi.org/10.1016/S0039-9140(98)00117-9

Chamon, A. S., Mondol, M. N., & Ullah, S. M. (2009). Amelioration of heavy metals from contaminated soils of Hazaribagh and Tejgaon areas from Bangladesh using red mud. *Bangladesh Journal of Science and Industrial Research, 44*(4), 479-484. http://dx.doi.org/10.3329/bjsir.v44i4.4602

Charlesworth, S., Everett, M., McCarthy, R., Ordonez, A., & de Miguel, E. (2003). A comparative study of heavy metal concentration and distribution in deposited street dusts in a large and a small urban area: Birmingham and Coventry, West Midlands, UK. *Environmental International, 29*, 563-573. http://dx.doi.org/10.1016/S0160-4120(03)00015-1

Christoforidis, A., & Stamatis, N. (2009). Heavy metal contamination in street dust and roadside soil along the major national road in Kavala's region, Greece. *Geoderma, 151*(3/4), 257-263. http://dx.doi.org/10.1016/j.geoderma.2009.04.016

Coşkun, M., Steinnes, E., Frontasyeva, V. F., Sjobakk, T. E., & Demnika, S. (2006). Heavy metal pollution of surface soil in the Thrace region, Turkey. *Environmental Monitoring Assessment, 119*, 545-556. http://dx.doi.org/10.1007/s10661-005-9042-3

Das, J. D., & Nolting, R. F. (1993). *Distribution of trace metals in sediments and pore waters in the N.W. Mediterranean Sea*. NIOZ, EROS-200 Project, 10.

Elik, A. (2003). Heavy metal accumulation in street dust samples in Sivas. *Communications Soil Science and Plant Analogy, 34*, 145-156. http://dx.doi.org/10.1081/CSS-120017422

Fatoki, O. S. (2003). Lead, Cadmium and Zinc Accumulation A long some Selected Major Roads of Eastern Cape. *International Journal of Environmetal Studies, 60*, 199-204. http://dx.doi.org/10.1080/00207230304734

Fergusson, J. E. (1991). *The Heavy Elements: Chemistry, Environment Impact and Health Effects*. Oxford: Pergamon Press, London.

Fergusson, J. E., & Kim, N. D. (1991). Trace elements in street and house dusts: sources and speciation. *Science of Total Environment, 100*, 125-150. http://dx.doi.org/10.1016/0048-9697(91)90376-P

Gomez, A. K., & Gomez, A. A. (1984). *Statistical procedures for agricultural research* (2nd ed., pp. 207-215). New York: John Wiley and Sons.

Grigalaviciene, I., Rutkoviene, V. & Marozas, V., (2005). The accumulation of heavy metals Pb, Cu and Cd at roadside forest soil. *Polish Journal of Environmental Studies, 14*, 109-115. Retrieved from http://www.6csnfn.pjoes.com/pdf/14.1/109-115.pdf

Guney, M., Onay, T. T., & Copty, N. K. (2010). Impact of overland traffic on heavy metal levels in highway dust and soils of Istanbul, Turkey. *Environmental Monitoring Assessment,* 164, 101-110. http://dx.doi.org/10.1007/s10661-009-0878-9

Habib, K. M. N. (2002). *Evaluation of planning options to alleviate traffic congestion and resulting air pollution in Dhaka City*. M. Sc. Thesis, Department of Civil Engineering, BUET, Dhaka.

Hjortenkrans, D., Bergback, B., & Haggerud, A. (2006). New metal emission patterns in road traffic environments. *Environmental Monitoring Assessment, 117*, 85-98. http://dx.doi.org/10.1007/s10661-009-0878-9

Kabadayi, F., & Cesur, H. (2010). Determination of Cu, Pb, Zn, Ni, Co, Cd and Mn in road dusts of Samsun City. *Environmental Monitoring Assessment, 168*, 241-253. http://dx.doi.org/10.1007/s10661-009-1108-1

Kabata-Pendias, A. (2000). *Trace Element in Soils and Plants*. Boca Raton, FL, USA: CRC Press. http://dx.doi.org/10.1201/9781420039900

Loska, K., Wiechula, D., & Korus, I. (2004). Metal contamination of farming soils affected by industry. *Environmental International, 30*, 159-165. http://dx.doi.org/10.1016/S0160-4120(03)00157-0

Massadeh, A., Al-Sharif, L., Dalale'h, R., & Hassanet, M. (2006). Analysis of lead levels in local Jordanian and imported sheep meat and organs using atomic absorption spectrometry. *Environmental Monitoring Assessment, 115*, 87-93. http://dx.doi.org/10.1007/s10661-006-6497-9

Mitsch, W. J., & Gosselink, J. G. (1993). *Wetlands* (2nd ed.). Van Nostrand-Reinhold, USA.

Mohiuddin, K. M., Zakir, H. M., Otomo, K., Sharmin, S., & Shikazono, N. (2010). Geochemical distribution of trace metal pollutants in water and sediments of downstream of an urban river. *International Journal of Environmental Science & Technology, 7*(1), 17-28. Retrieved from http://www.sid.ir/en/VEWSSID/J_pdf/92220100103.pdf

Mondol, M. N., Chamon, A. S., Faiz, B., & Elahi, S. F. (2011). Seasonal variation of heavy metal concentrations in Water and plant samples around Tejgaon industrial Area of Bangladesh. *Bangladesh Journal of Academic Science, 35*, 19-41. http://dx.doi.org/10.3329/jbas.v35i1.7968

Mortula, M. M., & Rahman, M. S. (2002). Study on waste disposal at DEPZ. Bangladesh Environmental Protection Agency, *(BAPA). 2*, 807-817.

Muller, G. (1969). Index of Geoaccumulation in sediments of the Rhine River. *Geojournal, 2*(3), 108-118.

Nabuloa, G., Oryem-Origa, H., & Diamond, M. (2006). Assessment of lead, cadmium, and zinc contamination of roadside soils, surface films, and vegetables in Kampala City, Uganda. *Environmental Research, 101*, 42-52. http://dx.doi.org/10.1016/j.envres.2005.12.016

Nordberg, G. (2003). Cadmium and human health: A perspective based on recent studies in China. *The Journal of Trace Elements in Experimental Medicine, 16*, 307-319. http://dx.doi.org/10.1002/jtra.10039

Olajire, A. A., & Ayodele, E. T. (1997). Contamination of roadside soil and grass with heavy metals. *Environmental International, 23*, 91-101. http://dx.doi.org/10.1016/S0160-4120(96)00080-3

Onder, S., Dursun, S., Gezgin, S., & Demirbas, A. (2007). Determination of Heavy Metal Pollution in Grass and Soil of City Centre Green Areas (Konya, Turkey). *Polish Journal of Environmental Studies, 16*(1), 145-154. Retrieved from http://www.6csnfn.pjoes.com/pdf/16.1/145-154.pdf

Othman, I., AlOudat, M., & AlMasri, M. S. (1997). Lead levels in roadside soils and vegetation of Damascus city. *Sci. Total Environment, 207*, 43-48. http://dx.doi.org/10.1016/S0048-9697(97)00243-X

Rahman, S. H., Khanam, D., Adyel, T. M., Islam, M. S., Ahsan, M. A., & Akbor, M. A. (2012). Assessment of Heavy Metal Contamination of Agricultural Soil around Dhaka Export Processing Zone (DEPZ), Bangladesh: Implication of Seasonal Variation and Indices. *Applied Science, 2*, 584-601. http://dx.doi.org/10.3390/app2030584

Rashed, M. N. (2001). Monitoring of environmental heavy metals in fish from Nasser Lake. *Environmental International, 27*, 27-33. http://dx.doi.org/10.1016/S0160-4120(01)00050-2

Rauna, A., Poschenrieder, C. H., & Arcelo, J. (1988). Growth and biomass partitioning in Zn-toxic bush beans. *Journal of Plant Nutrition, 11*, 577-588. http://dx.doi.org/10.1080/01904168809363824

Roa, M. S., Gopalkrishnan, R., & Venkatesh, B. R. (2001). Medical geology-An emerging field in environmental science. In *National Symposium on Role of Earth Sciences, Integrated and RelatedSocietal Issues* (pp. 213-222). Lucknow, India, 2-4 November 2001, *GSI Spl. Pub.* No. 65.

Robertson, D. J., Taylor, K. G., & Hoon, S. R. (2003). Geochemical and mineral magnetic characterization of urban sediment particulates, Manchester, UK. *Applied Geochemistry, 18*, 269-282. http://dx.doi.org/10.1016/S0883-2927(02)00125-7

Robson, A. D., & Reuter, D. J. (1981). Diagnosis of Copper Deficiency and Toxicity. In J. F. Loneragen, A. D. Robson, & R. D. Graham (Eds.), *Copper in Soils and Plants* (pp. 287-312). London: Academic Press.

Saeedi, M., Hosseinzadeh, M., Jamshidi, A., & Pajooheshfar, S. P. (2009). Assessment of heavy metals contamination and leaching characteristics in highway side soils, Iran. *Environmental Monitoring Assessment, 151*(1/4), 231-241. http://dx.doi.org/10.1007/s10661-008-0264-z

SAS Institute. (1999). SAS for windows SAS Inst., Cary, N.C.

Schwar, M. J. R., Moorcroft, J. S., Laxen, D. P. H., Thompson, M., & Armorgie, C. (1988). Baseline metal in dust concentrations in Greater London. *Science of Total Environment, 68*, 25-43. http://dx.doi.org/10.1016/0048-9697(88)90359-2

Sharma, R. K., Agrawal, M., & Marshall, F. (2006). Heavy metal contamination in vegetables grown in wastewater irrigated areas of Varanasi, India. *Bulletin of Environvironmental Contamination and Toxicology, 77*, 312-318. http://dx.doi.org/10.1007/s00128-006-1065-0

Singh, A. K., Hasnain, S. I., & Banerjee, B. K. (2003). Geochemical partitioning of heavy metals in sediments of Bamodar river-a tributary of the lower Garga, India. *Environemtal Geology, 39*, 90-98. http://dx.doi.org/10.1007/s002540050439

Sorme, L., & Lagerkvist, R. (2002). Sources of trace metals in urban wastewater in Stockholm. *Science of the Total Environment, 298*(1-3), 131-145. http://dx.doi.org/10.1016/S0048-9697(02)00197-3

Swaileh, K. M., Hussein, R. M., & Abu-Elhaj, S. (2004). Assessment of heavy metal contamination in roadside surface soil and vegetation from the West bank. *Environmental Contamination and Toxicology, 47*, 23-30. http://dx.doi.org/10.1007/s00244-003-3045-2

Swaileh, K. M., Rabay'a, N., Salim, R., Ezzughayyar, A., & Rabbo, A. A. (2001). Concentrations of heavy metals in roadside soils, plants, and landsnails from the West Bank, Palestine. *Journal of Environmental Science and Health Part A: Toxic/Hazardous substances and Environmental Engineering, 36*(5), 765-778. http://dx.doi.org/10.1081/ESE-100103759

Tomlinson, D. C., Wilson, J. G., Harris, C. R., & Jeffrey, D. W. (1980). Problems in the assessment of heavy-metal levels in estuaries and the formation of a pollution index. *Helgoland Marine Research, 33*, 566-575. http://dx.doi.org/10.1007/BF02414780

Turekian, K. K., & Wedepohl, K. H. (1961). Distribution of the elements in some major units of the earth's crust. *Bulletin: The Geological Society of America, 72*, 175-192. http://dx.doi.org/10.1130/0016-7606(1961)72[175:doteis]2.0.co;2

Tuzen, M. (2003). Determination of heavy metals in soil, mushroom and plant samples by atomic absorption spectrometry. *Microchemistry Journal, 74*, 289-297. http://dx.doi.org/10.1016/S0026-265X(03)00035-3

Viard, W., Pihan, F., Promeyrat, S., & Pihan, J. C. (2004). Integrated assessment of heavy metal (Pb, Zn, Cd) highway pollution: Bioaccumulation in soil, Graminaceae and land snails. *Chemosphere, 55*, 1349-1359. http://dx.doi.org/10.1016/j.chemosphere.2004.01.003

Yang, X. E., Long, X. X., Ni, W. Z., Ye, Z. Q., He, Z. L., & Stoffella, P. J. (2002). Assessing copper thresholds for phytotoxicity and potential dietary toxicity in selected vegetable crops. *Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes, 37*(6), 625-635. http://dx.doi.org/10.1081/PFC-120015443

Yaylali-Abanuz, G. (2011). Heavy metal contamination of surface soil around Gebze industrial area, Turkey. *Microchemical Journal, 2*(99), 82-92. http://dx.doi.org/10.1016/j.microc.2011.04.004

Yilmaz, S., & Zengin , M. (2003). Monitoring environmental pollution in Erzurum by chemical analysis of Scots pine (*Pinus sylvestris* L.) needles. *Environment International, 1097*, 1-7. http://dx.doi.org/10.1016/S0160-4120(03)00097-7

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Hybrid of Artificial Neural Network-Genetic Algorithm for Prediction of Reference Evapotranspiration (ET_0) in Arid and Semiarid Regions

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Abstract

Evapotranspiration is a principal requirement in designing any irrigation project, especially in arid and semiarid regions. Precise prediction of Evapotranspiration would reduce the squandering of huge quantities of water. Feedforward Backpropagation Neural Network (FFBPNN) model is employed in this study to evaluate the performance of Artificial Neural Networks (ANNs) in comparison with Empirical FAO Penman-Monteith (P-M) Equation in predicting reference evapotranspiration (ET_0); later, a hybrid model of ANN-Genetic Algorithm (GA) is proposed for the same evaluation function. Daily averages of maximum air temperature (T_{max}), minimum air temperature (T_{min}), relative humidity (R_h), radiation hours (R), and wind speed (U_2) from Mosul station (Nineveh, Iraq) are used as inputs to the ANN simulation model to predict ET_0 values obtained using P-M Equation. The main performance evaluation functions for both models are the Mean Square Errors (MSE) and the Correlation Coefficient (R^2). Both models yield promising results, but the hybrid model shows a higher efficiency in prediction of Evapotranspiration and could be recommended for modeling ET_0 in arid and semiarid regions.

Keywords: evapotranspiration, FAO Penman-Monteith Equation, artificial neural network, genetic algorithm

1. Introduction

Increasing demand for water and scarcity of water supply are growing concerns in both arid and semiarid regions of the world. Iraq, which consists of both arid and semiarid climates, suffers from a low rainfall rate of about 160 mm per year and tremendous amount of water loss from evaporation and transpiration (Evapotranspiration). The condition is further aggravated by other climate events such as drought and salinity (Kerr, 1998). Native date palm, cotton, barley, and wheat are common products in Iraq that depend on irrigation because of scarcity of rain water. An understanding of the precise prediction of Evapotranspiration would allow for optimization of water use in irrigation projects.

The specific concept of Evapotranspiration (ET) is influenced by alteration of weather parameters, crop types, stage of growth, and other environmental conditions. The precise determination of ET produced the need for another comprehensive concept, namely, Reference Evapotranspiration (ET_0), which can be defined as "the rate of Evapotranspiration from an extensive surface of 8 to 15 cm tall, green grass cover of uniform height, actively growing, completely shading the ground and not short of water" (Doorenbos & Pruitt, 1977). The extensive surface resembles the reference surface indicated by FAO experts as the "A hypothetical reference crop with an assumed crop height of 0.12 m, a fixed surface resistance of 70 s m^{-1} and an albedo of 0.23" (Allen et al., 1998). Methods for predicting evapotranspiration are either Direct or Indirect methods. Direct Methods, such as Lysimeters (Wright, 1988), or field experiments, are mostly for scientific researches. Indirect Methods are usually empirical and depend on weather parameters such as solar radiation; air temperature; air humidity; and wind speed, measured or estimated at meteorological stations. Many empirical methods have been studied,

issued, and applied in the prediction of ET, but the Food and Agriculture Organization (FAO) of the United Nations assumes that the combination of energy balance/aerodynamic equations provides the most accurate results for prediction of ET_0, and it adopted the FAO Penman-Monteith (P-M) Equation as the only standard equation for estimation of ET_0 (Allen et al., 1998).

Artificial Intelligence (AI) applications distinguished itself in many scientific fields during the last decades; however, researchers in hydrology, among many other fields, are still attracted to apply Artificial Neural Networks (ANNs) in modeling stream flow, rainfall, suspended sediment and ET. Kumar et al. (2002) found that application of Multiple Layers Perception (MLP) of ANN, with backpropagation algorithm; gives an accurate estimation of ET_0. In 2005, Trajkovic approved the possibility of achieving reliable results of ET_0 on the basis of temperature data only; FAO P-M Equation is used in comparison with another three temperature-based empirical equations and Radial-Based Function (RBF) Network. The RBF model better predicted FAO P-M ET_0 than the other calibrated empirical methods. Kisi (2006) investigated the possibility of modeling ET_0 using the technique of Generalized Regression Neural Network. The results of the intelligent model were successful. In 2007, Kisi and Öztürk investigated the accuracy of Adaptive Neurofuzzy Inference System Models (ANFIS) in modeling ET_0. Values of ET_0 were obtained using FAO P-M Equation with four years records of daily climate parameters of Pomona Station and Santa Monika Station, operated by the California Irrigation Management Information System (CIMIS), the results were compared with ANFIS and ANN, and also in comparison with Hargreaves and Ritchie empirical methods. The comparative results proved the superiority of ANFIS with inputs of T, U_2, RH and R in modeling daily ET_0 over the ANN and empirical methods. Kisi (2008) examined the accuracy of three ANN techniques, namely, the Generalized Regression (GR), MLP and Radial Basis Neural Networks (RBNNs) in a model of P-M Evapotranspiration. Results proved that both MLP and RBNN techniques could be successfully used in modeling ET_0. Landeras et al. (2008) implemented Seven ANN techniques with different inputs and compared the results to ten empirical and semi-empirical ET_0 equations calibrated to FAO P-M equation using meteorological data as inputs. The comparisons criteria are the statistical error techniques; using PM56 daily ET_0 values as a reference. ANN techniques have obtained better results than the calibrated equations. El-Baroudy et al. (2010) compared the Evolutionary Polynomial Regression (EPR) to ANN and Genetic Programming (GP). The EPR model provided a performance comparable to that of GP and ANN models. Tabari and Talaee (2012) indicated that the main obstacle in application of FAO P-M Equation is the wide range of meteorological data essential as an input for calculation of ET_0, and because of the nonlinearity of ET phenomenon. They used a multi-layer neural network (MLNN) with variable inputs of data sets for modeling of ET_0 in the semiarid region in Hamedan, the model with all required climate parameters used as inputs performed best among other MLP models. Khoshhal and Mokarram (2012) evaluated different structures of MLP for estimation of ET_0, using meteorological data of Eghlid station in Iran for the period 2000-2010 as inputs and P-M ET_0, obtained using the same meteorological data, as output. The performance of 10 ANN models with different inputs were evaluated, the functions used for evaluation is root mean square error (RMSE), mean absolute error (MAE), and determination coefficient (R^2). The model with T_{min}, T_{max}, R_h, R, U_2 is more accurate in predicting ET_0 than other models.

This study investigates the performance of ANN and a hybrid of Artificial Neural Network-Genetic Algorithm (ANN-GA) techniques in predicting ET_0 in comparison with values obtained using P-M Equation with historical data from Mosul meteorological stations in Iraq. Evaluation of the proposed Artificial Intelligence techniques will be carried out against estimations done using the empirical method.

2. Study Area

The study area, named Mosul, is located at the center of Nineveh Governorate, northern Iraq, between latitude 36° 22' 00" N, longitude 43° 07' 00" E, and altitude 222.6 m above sea level, with an overall area of 37,323 km^2 (Figure 1) (Statistical report, 2009). The weather data used in this study are obtained from the main meteorological station in Mosul (Global Station Code 608), which includes the daily averages of: maximum air temperature (T_{max}), minimum air temperature (T_{min}), relative humidity (R_h), radiation hours (R), and wind speed (U_2) from 1980–2005.

Figure 1. Study area in Iraq

3. Techniques in Calculating Reference Evapotranspiration (ET_0)

3.1 Penman-Monteith Empirical Equation (P-M Equation)

The precise determination of ET_0 is a fundamental requirement in planning and scheduling any irrigation project. The empirical method in estimating ET_0 is a practical application if used with the proper crop coefficient (Kc) to find the crop Evapotranspiration (Etc) (i.e., Etc = ET_0 × Kc) (Yoder et al., 2005).

ET_0 is measured in millimeters per unit time where the time could be extended from seconds to minutes, hours, or even the overall growing season. Allen et al. in 1998 issued FAO paper 56 with the latest form of the FAO P-M Equation adopted by the organization as the only present method for calculation of ET_0. The climatic parameters are the only factors affecting ET_0, thus, ET_0 could be computed using records of weather data for a specific location, at any time intervals such as daily, ten days, or monthly. The equation formula shows all weather data that control energy exchange in the evaporation and transpiration process.

$$ETo = \frac{0.408\Delta(R_n - G) + \gamma \frac{900}{T+273} u_2 (e_s - e_a)}{\Delta + \gamma (1 + 0.34 u_2)} \quad (1)$$

ET_0 = Reference Evapotranspiration (mm/day), Δ = slope of vapor pressure curve (kPa $°C^{-1}$), Rn = net radiation (MJ $m^{-2}day^{-1}$), G = density of soil heat flux (MJ $m^{-2}day^{-1}$), Υ = psychometric constant (kPa $°C^{-1}$), T = average daily air temperature (°C), U_2 = wind speed at height 2 m (m s^{-1}), (e_s-e_a) = deficit in saturation vapor pressure.

The equation does not take into consideration the crop characteristics; Kc combines all the physical and physiological differences between crops for calculation of ETc.

The altitude and latitude data of study location should be specified. These data are essential for computation of radiation, daylight hours (N), or to adjust some weather parameters for the local average value of atmospheric pressure

Standard weather data, including (T_{max}), (T_{min}), (R_h), (R), and wind speed (U_2), over a period of 26 years were collected and organized in daily averages of monthly intervals form, then utilized in P-M equation for estimation of ET_0.

3.2 Feedforward Backpropagation Neural Networks

ANN architecture is a simulation of information processing that occurs in the biological brain. It starts with receiving, learning, adapting, recognizing the pattern, and then performing a desired function (Target) by trial of different weights of the information elements in a computation model. A typical ANN model contains an input layer that receives the input data. The hidden layers, with number of nodes that would satisfy the problem requirements, would recognize patterns and organize these data through multiple trial processes to predict the output (El-Baroudy et al., 2010). In this study, the Supervised Feedforward Backpropagation Neural Network (FFBPNN) is used for prediction of ET_0. In the FFBPNN, neurons are connected forward where each layer of the neural network connects to the next layer. In our work, the network model has an input layer, one hidden

layer, and an output layer, using one hidden layer to represent the nonlinear relationship of ET_0 is sufficient (Kumar et al., 2002). The number of actual input variables is seven (7), representing weather parameters, altitude, and latitude values. The weather parameters used as inputs are the historical records obtained from the main meteorological station in Mosul for the period 1980–2005. The total available data is divided into two main categories; 80% of the data, records of 240 monthly averages of each of the weather parameters, is for training the model; the other 20% of data records, representing 72 monthly averages of each of the weather parameters, is used in testing the performance of the model. In the training period, the input data are designed to be classified again into three subclasses, training; testing; and valuating. The backpropagation algorithm is then employed to activate the ten (10) neurons in the hidden layer, determined using trial-and-error methods with application of 5, 7, 9, 10 and 15 neurons), for weighing and training of information elements to achieve the desired target as shown in Figure 2 (Badde et al., 2013). Backpropagation is a form of supervised training in ANN, whereby the network has to be provided with both sample inputs and anticipated outputs during training. In this work, all input and output variables were normalized before training. The function Min and Max (premnmx, postmnmx, tramnmx) were used to standardize the data within a range of [-1, 1] (Malek, 2008). The model was implemented using MATLAB.

$$XN = (X - MinX) / (MaxX - MinX) * 2 - 1 \quad (2)$$

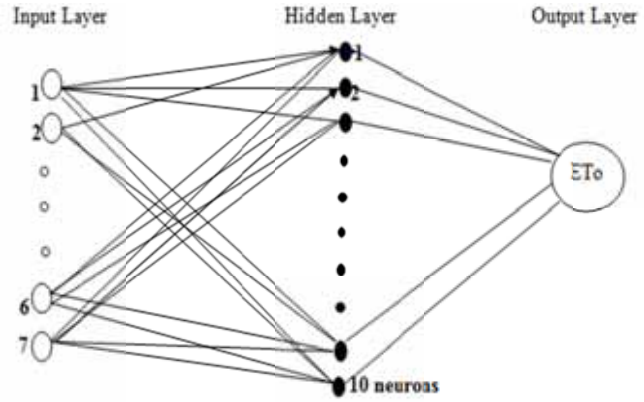

Figure 2. Feedforward backpropagation neural network

After training and testing performance of the simulation model, the FFBPNN model is implemented also to prepare for making prediction, and then to predict ET_0 for any time interval depending on new input data that have not been used in the training or testing periods.

The same ANN model was tested also for inputs to include weather data only, excluding altitude and latitude data; which are constant parameters for the identified study location.

3.3 Genetic Algorithm

Genetic Algorithm (GA) is a method of probabilistic optimization based on evolution theory. Evolution from one generation to another is composed of three main stages: Selection of strings according to their fitness, crossover of strings, and random mutation for selected strings to compute new generation (Azzini, 2007). The records of weather data used in FFBPNN model are used in this study, in the same pattern, in a hybrid model of Binary GA with ANN (GA-ANN). The GA technique is used in this model to improve the performance of ANN by changing the number of hidden layers to three (3) instead of only one (1) layer used in the ANN prediction method. The standard performance function is the Mean Square Errors (MSE), which is used to minimize the connection weights depending on cost of elements and accuracy.

$$MSE = \frac{1}{N}\sum_{i=1}^{N}\left(ETi_{\,calculated} - ETi_{\,predicted}\right)^2 \quad (3)$$

The second function is the Correlation Coefficient, which proportionally clarifies the statistical cost of each element. The range of Correlation Coefficient used is from -1 to 1 (Adeloye et al., 2012).

$$R = \frac{N\sum xx' - \sum x\sum x'}{\sqrt{[N\sum x^2 - (\sum x)^2][N\sum x'^2 - (\sum x')^2]}} \quad (4)$$

The mean Absolute Errors is also taken into consideration in the model performance evaluation.

The binary FFBP-GA model is capable; even better then FFBPNN, of preparing itself for prediction depending on the stored efficiencies of the training and testing periods, and making actual prediction for any location or time interval.

4. Results and Discussion

The nonlinear relationship between weather parameters and the effect of the study area location according to latitude and altitude, in addition to other environmental factors, inspires this study to produce precise and dependable ET_0 values with time, effort, and cost consuming. The average values of weather parameters for the period 1980–2005 were employed in the FAO P-M Equation for the calculation of average monthly ET_0 as shown in Figure 3. It shows the inequality of the ET rates at different periods along the year. The design of the water-used system should accommodate peak values, which are in June, July, and August, to satisfy the minimum supply of water according to its monthly consumptive use.

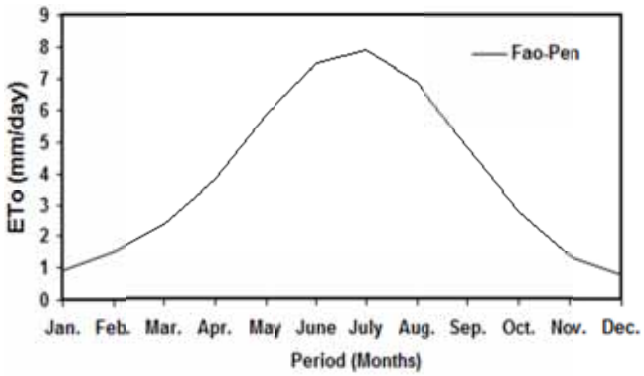

Figure 3. Average monthly P-M ET_0 at Mosul Station for the period 1980–2005

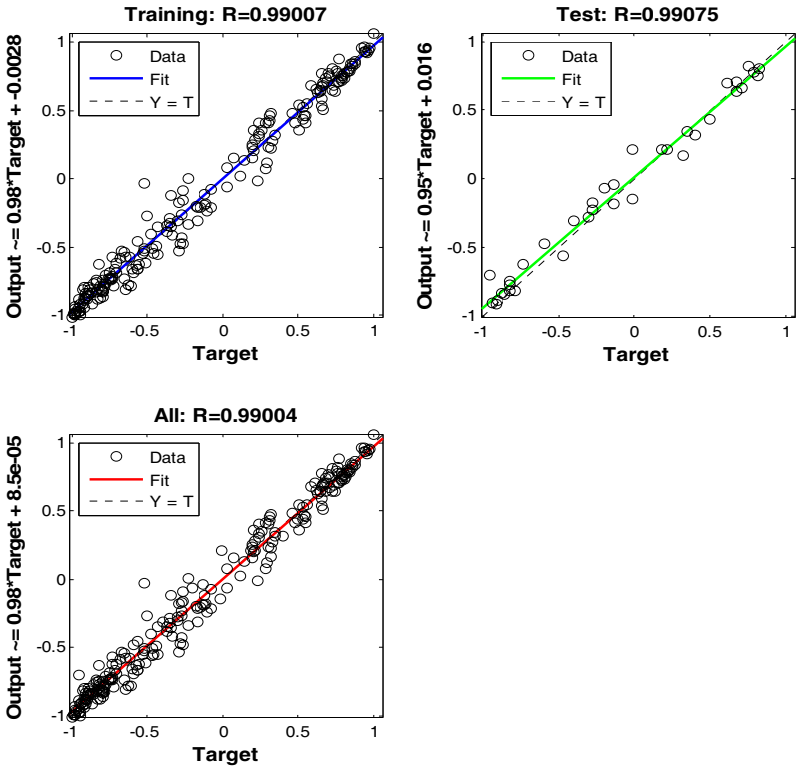

Figure 4. Relationship between targeted FAO ET_0 and ANN predicted ET_0 values for training period at Mosul station

The results of ET obtained from application of FAO P-M Equation were employed in the FFBPNN model to test the capability of the network system in predicting ET_0. The best results, obtained at 233 0f designed 1000 epochs, are shown in Figures 4 and 5 were encouraging. Eighty percent of total observed weather variables, together with their related output data, were employed in the system training, whereas the other 20% were considered in the testing period. In the training period, the data will again be classified into training, testing, and evaluation, as shown in Figure 4.

The ANN model performance at both training and testing periods was evaluated by functions of MSE, MAE and R^2 as presented in Table 1, which indicate the efficiency of the proposed ANN model in predicting ET_0.

Table 1. The performance functions for training and testing periods in ANN Model for years 1980–2005

Data Set	MSE (mm2/day^2)	MAE (mm/day)	R^2
(1) Training 80%	0.0084	0.0679	0.9900
(2) Testing 20%	0.0139	0.0971	0.9900

The correlation between calculated ET_0 using FAO P-M Equation and ANN predicted ET_0 is illustrated in Figure 5, which reveals a significant consistency between the values.

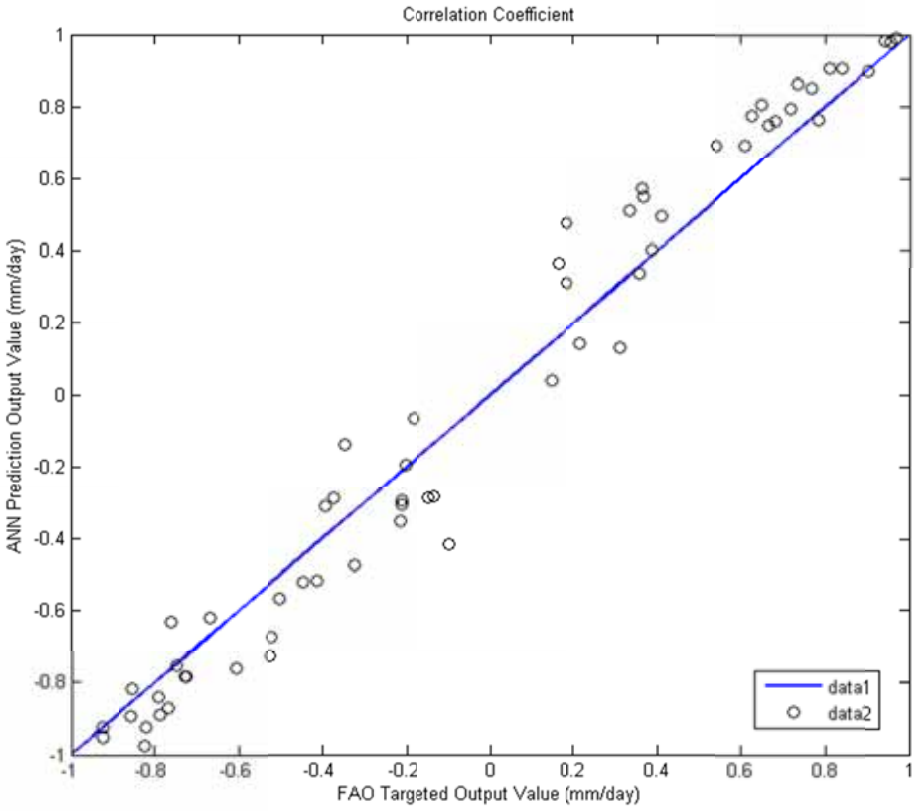

Figure 5. The correlation coefficient between calculated ET_0 using FAO P-M Equation and ANN Predicted ET_0 at Mosul Station

The ANN model was then implemented to investigate the efficiency of predicting P-M ET_0; when the input data are only the records of weather parameters employed previously, and to exclude the data of common latitude and altitude parameters.

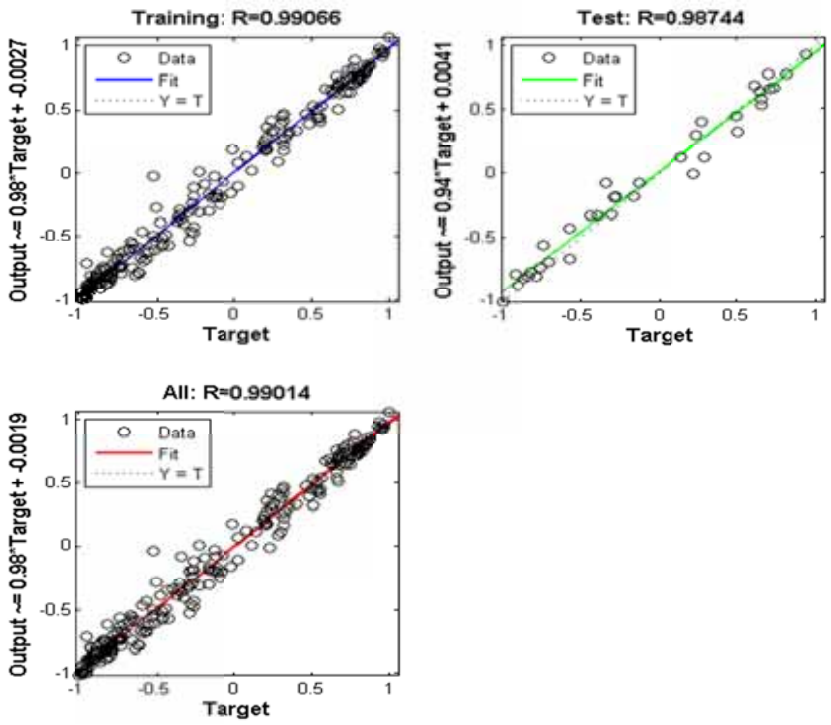

Figure 6. Relationship between targeted FAO ET_0 and ANN predicted ET_0 values without location data for training period at Mosul station

The ANN model performance at both training and testing periods was evaluated also by functions of MSE, MAE and R^2 as presented in Table 2, which indicate the efficiency of the proposed ANN model in predicting ET_0 without location data.

Table 2. The performance functions for training and testing periods in ANN Model without altitude and latitude data for years 1980–2005

Data Set	MSE ($mm2/day^2$)	MAE (mm/day)	R^2
(1) Training 80%	0.0083	0.0674	0.9900
(2) Testing 20%	0.0139	0.0971	0.9897

The correlation between calculated ET_0 using FAO P-M Equation and ANN predicted ET_0, with weather data only, is illustrated in Figure 7.

The results of implementing ANN model with only 5 inputs (weather data) and P-M ET_0 as output; show slight effect on both training and testing evaluation functions.

The same values of ET results obtained by application of FAO P-M Equation were also used against a hybrid model of FFBPNN with a Binary GA (ANN-GA). In this hybrid ANN-GA model, the maximum number of iteration, after which no significant changes were noticed, is found to be one hundred (100); population size is found to be twenty (20), and mutation rate is 0.15. The optimization function used is the cost function; by minimizing the MSE, The best cost function is 0.0067828 at generation number of 100. The hybrid of ANN-GA enhanced the model performance and minimized the fitness function represented by the Cost, which is the Mean Squared Error.

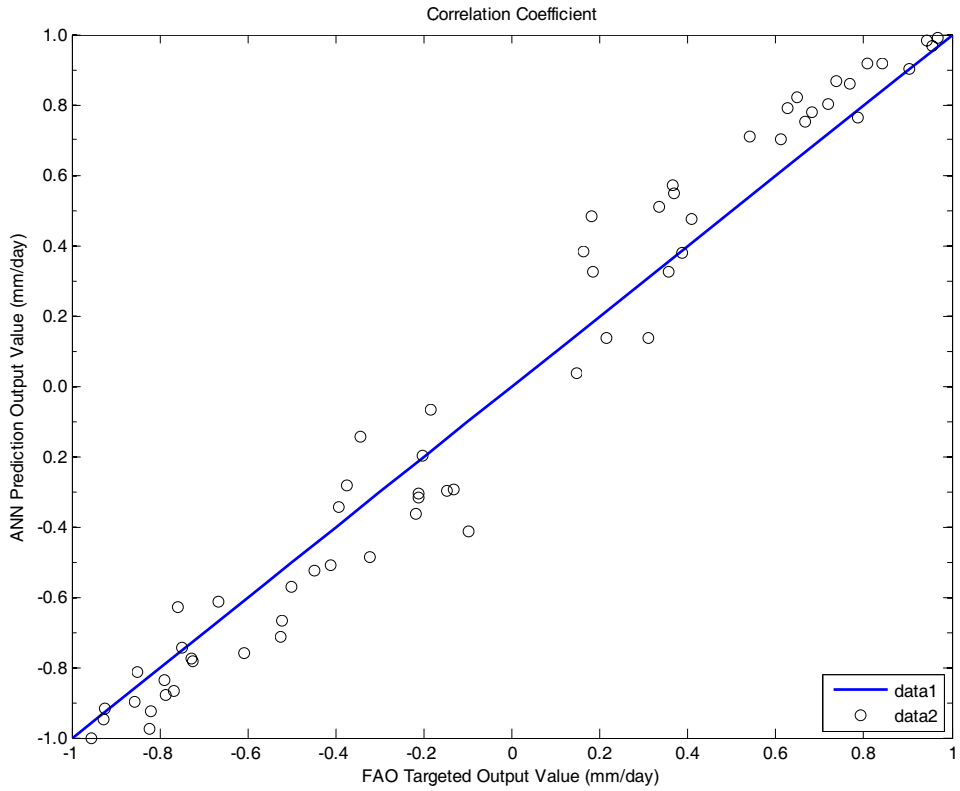

Figure 7. The correlation coefficient between calculated ET_0 using FAO P-M Equation and ANN Predicted ET_0 with weather data only at Mosul Station

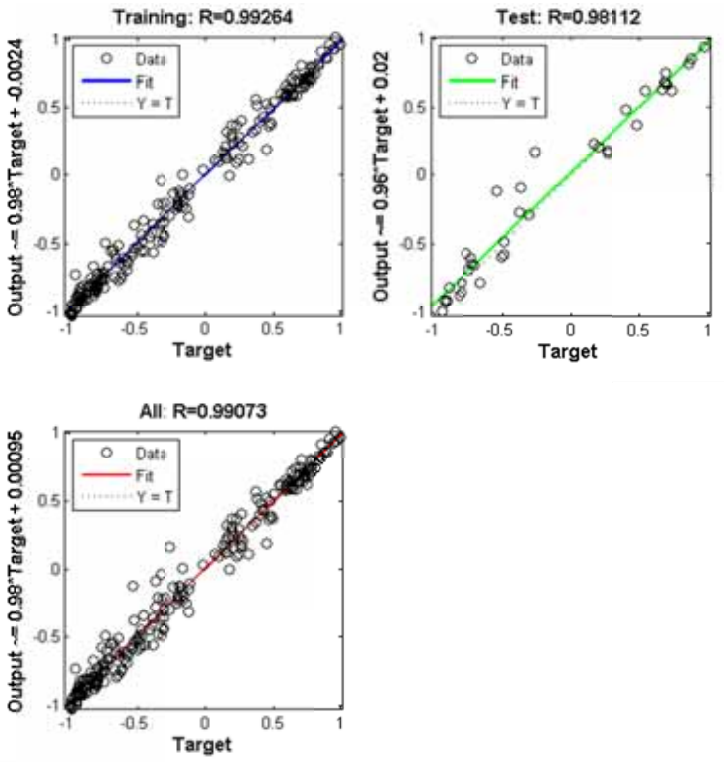

Figure 8. The relationship between targeted FAO ET_0 and Hybrid ANN-GA predicted ET_0 values for the training period at Mosul station

Figure 8 shows that the Hybrid ANN-GA simulation has modify the result of the Correlation Coefficient (R^2). It exhibited a higher consistency rather than using ANN by itself.

The main obstacle in the application of P-M Equation is the comprehensive and rich weather data needed for specific location or project in the design and operation stages. Latitude and altitude are to be specified as well; however, as the location specifications have only slight effect on implementation of intelligent models; these models would allow using historical records of weather data available in any alternative location for training the system and using the limited records available of the exact location for testing the system and even predicting actual values of ET_0 for any period.

5. Conclusion

ET_0 is the key parameter in designing any irrigation project. Building an intelligent model of ANN and Hybrid ANN-GA would provide an effective alternative to the empirical method used in the estimation of ET. The proposed Artificial Intelligence models have significant superiority as compared to the limitations embedded in the traditional empirical FAO P-M Equation method. This study shows that both ANN and Hybrid ANN-GA models can be used for prediction of ET_0 in Iraq, taking into consideration the unstable and sudden changes of weather conditions.

The normalization of input and output parameters would give the researchers a chance to overcome the limitations of application of relevant data to a specific location. In P-M Equation, both latitude and altitude values are considered properties of the study location and have great effect on ET_0 values. Normalization of input and output parameters in the FFBPNN and FFBPNN-GA models will eliminate the effect of those two parameters as they are constants; shared in all time intervals; the AI model could be recommended to be used without restrictions of location specifications.

References

Adebayo, J., Adeloye, A. J., Rustum, R., & Kariyama, I. D. (2012). Neural computing modeling of the reference crop Evapotranspiration. *Journal of Environmental Modelling & Software, 29*, 61-73. http://dx.doi.org/10.1016/j.envsoft.2011.10.012

Allen, R. G., Pereira, L., Raes, S. D., & Smith, M. (1998). *Crop Evapotranspiration, guidelines for computing crop water requirements*. FAO Irrigation and Drainage Paper No. 56, Rome, Italy.

Azzini, A. (2007). *A New Genetic Approach for Neural Network Design and Optimization*. Ph.D. thesis, Universita Degli Studi Di Milano, Italy. Retrieved from http://www.dti.unimi.it/azzini/wwwmat/TesiAzziniAntonia.pdf

Badde, D. S., Gupta, A. K., & Patki, V. K. (2013). Cascade and Feedforward Backpropagation Artificial Neural Network Models for Prediction of Compressive Strength of Ready Mix Concrete. *IOSR Journal of Mechanical and Civil Engineering (IOSR-JMCE),* 01-06.

Doorenbos, J., & Pruitt, W. O. (1977). Guidelines for predicting crop water requirements. *FAO Irrigation and Drainage Paper 24 (revised), part I, 1*. Rome, Italy.

El-Baroudy, A. Elshorbagy, Carey, S. K., Giustolisi, O., & Savic, D. (2010). Comparison of Three Data—Driven Techniques in Modelling the Evapotranspiration Process. *Journal of Hydro Informatics, 12*(4), 365-379. http://dx.doi.org/10.2166/hydro.2010.029

Kerr, R. A. (1998). Sea-Floor Dust Shows Drought Felled Akkadian Empire. *Science Magazine, 279*(5349), 325-326. Retrieved from http://www.sciencemag.org/content/279/5349/325

Khoshhal, J., & Mokarram, M. (2012). Model for Prediction of Evapotranspiration Using MLP Neural Nettwork. *Internetional Journal of Environmental Sciences, 3*(3), 1000-1009.

Kisi, Ö. (2006). Generalized regression neural networks for Evapotranspiration modeling. *Journal of Hydrological Sciences, 51*(6), 1092-1105. http://dx.doi.org/10.1623/hysj.51.6.1092

Kisi, Ö. (2008). The Potential of Different ANN Techniques in Evapotranspiration Modelling. *Hydrology Process, 22*, 2449-2460. http://dx.doi.org/10.1002/hyp.6837

Kisi, Ö., & Öztürk, Ö. (2007). Adaptive Neurofuzzy Computing Technique for Evapotranspiration Estimation. *Journal of Irrigation and Drainage Engineering,* 368-379. http://dx.doi.org/10.1061/(ASCE)0733-9437(2007)133:4(368)

Kumar, M., Raghuwanshi, N. S., Singh, R., Wallender, W. W., & Pruitt, W. O. (2002). Estimating Evapotranspiration Using Artificial Neural Network. *Journal of Irrigation and Drainage Engineering, ASCE, 128*(4), 224-233. http://dx.doi.org/10.1061/(ASCE)0733-9437(2002)128:4(224)

Landeras, G., Ortiz-Barredo, A., & Lo´pez, J. J. (2008). Comparison of artificial neural network models and empirical and semi-empirical equations for daily reference evapotranspiration estimation in the Basque Country (Northern Spain). *Agricultural water management, 95*, 553-565. http://dx.doi.org/10.1016/j.agwat.2007.12.011

Malek, M. A. (2008). *DeveloP-Ment of Rainfall Data in-Filling Model with Expectation Maximization and Artificial Neural Network*. Ph.D. thesis, Universiti Teknologi Malaysia, Malaysia.

The Ministry of Planning Central Bureau of Statistics Directorate of Environment Statistics. (2009). Environmental statistics report for Iraq for the year 2009. Retrieved from http://www.cosit.gov.iq/en/climate

Tabari, H., & Talaee, P. H. (2012). *Multilayer Perceptron for Reference Evapotranspiration Estimation in Semiarid Region*. Neural Compute & Appic. Springer-Verlag London Limited.

Trajkovic, S. (2005). Temperature-Based Approaches for Estimating Reference Evapotranspiration. *Journal of Irrigation and Drainage Engineering, ASCE, 131*(4), 316-323. http://dx.doi.org/10.1061/(ASCE)0733-9437(2005)131:4(316)

Wright, J. L. (1988). Daily and Seasonal Evapotranspiration and yield of irrigated alfalfa in southern Idaho. *Agronomy Journal, 80*, 662-669. http://dx.doi.org/10.2134/agronj1988.00021962008000040022x

Yoder, R. E., Odhiambo, L. O., & Wright, W. C. (2005). Evaluation of methods for estimating daily reference crop Evapotranspiration at a site in the humid southeast United States. *American Society of Agricultural Engineering, Soil and Water Division, 21*(2), 197-220.

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Phytochemistry, GC-MS Analysis, Antioxidant and Antimicrobial Potential of Essential Oil From Five *Citrus* Species

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Abstract

Citrus essential oils were extraction from hydro distillation technique yielding *Citrus* oil with reasonable yield. Phytochemical screening of all five *Citrus* oils showed that alkaloids, tannins, sterols, terpenoids, saponoins, flavonoids were present (50-80%). GC/MS analysis showed highest percentage of limonene (58-89%) in Citrus oils. Antioxidant study revealed that *Citrus* peel oils have strong scavenging activity (83%-91%). Antimicrobial activity was evaluated by agar well method against eight common pathogens depicted marked antimicrobial potential especially tangerine (4.9-1.9 cm inhibition zones) and grapefruit oil (4.5-1.2 cm) inhibition zones. The studies emphasized the therapeutic and commercial utilization of *Citrus* peel essential oils as food preservatives, phytomedicine and antioxidant agent.

Keywords: Citrus essential oils (EO), GC-MS analysis, phytochemicals, DPPH assay, antimicrobial activity

1. Introduction

All over the world *Citrus* is one of the widespread genus due to its prominent production. *Citrus* essential oils are naturally occurring, volatile and odoriferous oils synthesized by non woody parts of aromatic plants such as seeds, buds, leaves, flowers, stems, fruits, twigs and roots etc. and accumulated in secretory or epidermis cells and also sometimes in cavities (Ahmad, 2006). Essential oil from *Citrus* fruit peel is the fundamental product of genus *Citrus* and typically isolated by distillation or solvent extraction (Mondello et al., 2005). These are the complex mixtures of about 400 compounds of which 1-15% are non-volatile whereas 85-99% is the volatile constituents (Nannapaneni et al., 2009). Other organic compounds present in *Citrus* essential oils are aliphatic hydrocarbons, alcohols (linalool), aldehydes (citral), acids, esters and some aromatic compounds (Sharma & Tripathi, 2006). Svoboda & Greenaway (2003) reported the chief chemical constituent of *Citrus* essential oils is limonene and have a range of 32 to 98%. *Citrus* essential oils act as natural antioxidants because flavanone glycosides namely naringin, narirutin, hesperidin and neohesperidin are valuable phenolic compounds found in *Citrus* peel oil which make them liable to avert rancidity of food (Anagnostopoulou et al., 2006).

Essential oils of *Citrus* peels are medicinally very important and show variety of biological effects because they are rich in flavonoids (flavone, flavonol and flavanone), terpenes, carotenes and coumarines which are responsible for antimicrobial activity (Tepe et al., 2005). Consequently *Citrus* essential oils are extensively used in pharmaceutics as an antimicrobial, anti-diabetic, antioxidant, insect repellent, carminative, larvicidal, antiviral, antihepatotoxic and antimutagenic agent (Kanaze et al., 2008).

The rapidly growing importance of *Citrus* based essential oils in food, pharmaceuticals, perfumes, flavor and fragrance has forced Pakistan to import increasing amounts of *Citrus* oils despite its rich variegated acreage of *Citrus* fruits and one of the largest *Citrus* fruits producing country of the world. These factors provide the

opportunity for the production of highest grade essential oils from *Citrus* fruit peel. So there was an urgent need to focus on the extraction of essential oils as solid waste management and to improve our economy. This study was aimed for the assessment of phytochemical constituents, antioxidant as well as antimicrobial activities of five *Citrus* species, *Citrus* sinensis (L.) var. Malta, *C*. sinensis (L.) var. Mousami, *C*. reticulate (L.) var. Tangerine, *C*. reticulate (L.) var. Mandarin and *C*. paradisi (L.) Grapefruit.

2. Materials and Methods

2.1 Collection and Identification of Citrus Fruit Peels

Peels of five varieties of *Citrus* fruits were collected from local *Citrus* juice shop near University of the Punjab, Quaid-e-Azam campus, Lahore, Pakistan during the month of January & February 2012. Voucher specimen number PU. HHC.901, PU.HHC.902, PU.HHC.903, PU.HHC.904 and PU.HHC. 905 were assigned to Malta, Mousami, Tangerine, Mandarin and Grapefruit respectively.

2.2 Extraction of Citrus Essential Oil

Essential oils of selected *Citrus* species was extracted by hydro distillation unit for 3-4 hours extraction. Mixture of *Citrus* oils and water was incorporated which was separated, in two liquid layers which was isolated. . Hydro-distilled pure oil obtained was stored in dark brown sealed vials at 4°C until analysis.

2.3 Physicochemical and Phytochemical Investigation of Citrus EO

Physicochemical characteristics of *Citrus* essential oils including refractive index, optical rotation, specific gravity, color, odor and solubility were analyzed by the method of AOAC (2005). The chemical tests were carried out for screening of bioactive compounds present in *Citrus* essential oils using standard methods (Sofowora, 1993; Trease & Evans, 1989).

2.4 GC-MS Analysis of Citrus EO

Citrus essential oils were analyzed for their chemical composition by GC-MS analysis. GC/MS JOEL model JMS-A × 5050 H mass spectrometer (JOEL, Japan) Hewlett Packard 5890 Gas Chromatograph (JOEL, Japan). Helium as carrier gas, split ratio 1:100, electrical energy 70 eV, ionization current 200 μA, ionization temperature 250°C, column temperature with 6°C/min rise to 230°C. The chemical constituents were identified by their retention time and compared with known spectrum deposited in the National Institute Standard and Technology (NIST) library (NIST147.LIB).

2.5 DPPH Assay

Antioxidant potential was assessed by evaluating scavenging effect of each of five varieties of *Citrus* peel oils on DPPH. 500 μL of each essential oil was added in 3 ml of 0.002% methanolic solution of DPPH and shaken well. Absorbance was noted at 517 nm for all sample solutions and blank (contain only DPPH) after a stay time of 30 min in dark (Amin et al., 2006). All measurements were performed in triplicates. Scavenging potential of *Citrus* peel oils was determined in terms of percentage inhibition (I %) of DPPH by given formula:

$$\text{Percent inhibition} = (A_{blank} - A_{sample}/ A_{blank}) \times 100$$

Where A_{blank} represents absorbance of DPPH only at 517 nm and A_{sample} represents absorbance of sample under investigation at 517 nm.

2.6 Antimicrobial Activity of Citrus Oils

2.6.1 Test Organisms

Antimicrobial activity of *Citrus* peel essential oils were studied against the two Gram positive bacteria *Listeria monocytogenesn* and *Corynebacterium minutissimum* and three Gram negative bacteria *Escherichia coli, Yersinia* sp. and *Klebsiella planticola* whereas three fungal strains named as *Aspergillus flavus, A*. *fumigates* and *A*. *niger* were used. All microorganisms were obtained from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences, University of the Punjab, Lahore.

2.6.2 Agar Well Diffusion Method

Antimicrobial potential of *Citrus* peel essential oils was assessed using agar well method (Kim et al., 1995). Each microbial concentration was made 10^6 CFU/ml. Wells (8 mm) were prepared in plates (single well in case of fungi whereas four wells in case of bacteria). About 60 μL of the essential oil was dripped into the wells. Water was used as control. The inoculated plates were incubated for 24 hours at 37°C for bacterial isolates and for 72 h at 27°C for fungi isolates. Study was conducted in triplicates. Biostatic efficacy against test organisms was investigated by measuring the inhibition zones in comparison to a control.

3. Results and Discussion

3.1. Yield of Citrus EO by Hydro Distillation

For *Citrus* oil extraction by the hydro distillation, five batches of *Citrus* species were carried out to determine average productivity of *Citrus* oils (Table 1). *Citrus* oils yield were in the range of (0.28-0.45%) for 3-4 hours extraction, comparable and better in some varieties as compared to other extraction techniques reported in literature (Minh Tu et al., 2002; Lota et al., 2000). The highest yield among all *Citrus* essential oil was calculated for *C. paradise* Grapefruit 0.45% followed by *C. sinensis* var. Malta 0.37%, *C. reticulate* var. Mandarin 0.33%, *C. sinensis* var. Mousami 0.30%, *C. reticulate* var. Tangerine 0.28% (Table 1). According to the previous work of Minh Tu et al. (2002) the yield of orange essential oil was 0.13% and tangerine essential oil was 0.25%. The essential oil yielded from various cultivars of mandarin was reported, 0.1% to 0.45% (Lota et al., 2000). Varying yields of essential oil are due to different extraction methods, units, soil and climatic conditions (Huet, 1991).

Table 1. Percentage yield of *Citrus* essential oils by fabricated unit

Citrus species Essential oils (EO)	Raw Material Input (g)	Time extract (minutes)	Oil volume (ml)	Productivity (ml/2000g) %
C. paradisi	2000	215	9.0	0.45
C. sinensis var. Malta	2000	230	7.5	0.37
C. reticulate var. Mandarin	2000	210	6.6	0.33
C. sinensis var. Mousami	2000	235	6.0	0.30
C. reticulate var. Tangerine	2000	220	5.6	0.28

3.2 Physicochemical Characterization

Specific gravity of *Citrus* essential oils in present work was ranged from 0.842-0.858, refractive indices between 1.465-1.476 and all essential oils were found optically active (Table 2). These results were in line with preceding work on essential oils of *Citrus* species (Guenther, 1948).

Table 2. Physicochemical properties of *C. reticulate* var. Mandarin, *C. sinensis* var. Mousami, *C. paradisi, C. sinensis* var. Malta, *C. reticulate* var. Tangerine

Physical Parameters	*Citrus* oils				
	Mandarin	Mousami	Grapefruit	Malta	Tangerine
Color	Yellow	Light yellow	Light yellow	Pale yellow	Light yellow
Odour	Pleasant, Intense	Pleasant, less Intense	Pleasant, less Intense	Pleasant, Intense	Pleasant, less Intense
Refractive index (25^oC)	1.465	1.471	1.476	1.471	1.468
Optical rotation (25^oC)	+86	+91	+93	+89	+88
Specific gravity (25^oC)	0.844	0.849	0.858	0.847	0.842
Solubility					
Water	Insoluble	Insoluble	Insoluble	Insoluble	Insoluble
Ether	Soluble	Soluble	Soluble	Soluble	Soluble
Acetone	Fairly Soluble	Fairly Soluble	Fairly Soluble	Fairly Soluble	Fairly Soluble

3.3 Phytochemical Investigation

Phytochemical screening of all tested *Citrus* peel oils gave presence of alkaloids, tannins, terpenoids, saponins and combined anthraquinones in significant amounts (25-80%). Flavonoids and sterols were highly present in five *Citrus* essential oils (>80%). Anthraquinones were moderately present (50-80%) in essential oils of *C. sinensis* var. Mousami and Malta whereas highly depicted in *C. paradisi, C. reticulate* var. Tangerine and var. Mandarin (>80%). In all *Citrus* essential oils, alkaloids were in the range of 50-80%. Only *C. paradisi* peel oil depicted the presence of coumarin whereas all *Citrus* peel oils gave negative test for phlobatanins (Table 3). Mondello et al. (2005) explored flavonoids, terpenes, coumarins and carotenes as the major phytochemicals of *Citrus* essential oils. Likewise, Okwu et al. (2007) screened phytochemicals of five *Citrus* species and revealed the presence of saponins, tannins, flavonoids, alkaloids and phenols.

Table 3. Phytochemical Constituents of *C. reticulate* var. Mandarin, *C. sinensis* var. Mousami, *C. paradisi, C. sinensis* var. Malta, *C. reticulate* var. Tangerine

Phytochemicals	Citrus oils				
	Mandarin	Mosammi	Grapefruit	Malta	Tangerine
Alkaloids	++	++	++	++	++
Tannins	+	+	++	+	+++
Sterols	+++	+++	+++	+++	+++
Terpenoids	++	+	++	++	+++
Saponins	++	+	+	++	++
Flavonoids	+++	++	+++	+++	+++
Coumarins	-	-	+	-	-
Anthraquinones	+++	++	+++	++	+++
Combined anthraquinones	++	+	++	+	++
Phlobatanins	-	-	++	-	-

(-)=absent, (+) = present, > 50 %, compared with control, (++) = 50% < 80%, (+++) =>80%.

3.4 Chemical Composition by GC-MS Analysis

Limonene was identified as the key element of *Citrus* peel oils. GC-MS analysis of *Citrus* peel oils revealed that among five *Citrus* essential oils, Grapefruit essential oil displayed highest concentration of limonene (89.84%) followed by essential oils of Malta (88.57%), Mousami (87.84%), Mandarin (87.45%) and Tangerine (58.50%). Other chemical constituents identified were limonene oxide, α-terpineol, carvone, carveol, eugenol, spathulenol and caryophyllene oxide. α-Terpineol (12.55%) was the second major component of all five *Citrus* essential oils with Mandarin peel oil containing highest of it (Table 4). These results were in line with the former work on Brazillian tangerinesin which limonene was the major component (Feger et al., 2003). Similarly, Espina et al., (2011) reported limonene (85.50%) and α-terpineol (0.36%) as chief component of *Citrus* essential oils. In present study, apart from limonene (88.57%) and α-terpineol (8.45%), Malta peel oil also contained eugenol (0.58%), spathulenol (0.55%), caryophyllene oxide (0.99%), n-hexadecanoic acid (0.86%). Tangerine peel oil showed presence of carvone (16.97%), carveol (8.77%), 3-cyclohexene-1-methanol (8.91%) and limonene oxide (6.85%) in addition to limonene (58.50%). In Mousami peel oil, α-terpineol (12.16%) was major component after limonene (Table 4). Most of the compounds identified in *Citrus* peel oils were found to be hydrocarbons in nature (Ayoola et al., 2008). The major constituents of *Citrus* peel oils investigated by Vekiari et al., (2002) were limonene, neral, geranial, β-pinene, β-caryophyllene and neryl acetate. Preceding work stated limonene (86.27%), γ-terpinene (2.11%) and α-pinene (1.26%) in Grapefuit peel essential oil and limonene (76.28%), β-pinene (5.45%), linalool (2.32%), citral (1.74%) and α-pinene (1.26%) in Mousami peel oil (Ahmad et al., 2006). The different chemical constituents of different *Citrus* species are assumed may be due to different genetic characteristics.

Table 4. Chemical constituents of *Citrus* oils (GC-MS Analysis)

Citrus Essential oils (EO)	Retention time	Compounds	% Area
Mousami EO	7.215	Limonene	87.84
	8.867	α-terpineol	12.16
Malta EO	7.210	Limonene	88.57
	8.895	α-terpineol	8.45
	10.693	Eugenol	0.58
	11.417	Spathulenol	0.55
	11.486	Caryophyllene oxide	0.99
	13.935	n-Hexadecanoic acid	0.86
Grapefruit EO	7.215	Limonene	89.84
	8.871	α-terpineol	10.16
Mandarin EO	7.216	Limonene	87.45
	8.893	α-terpineol	12.55
Tangerine EO	7.218	Limonene	58.50
	8.345	Limonene oxide	6.85
	9.096	Carveol	8.77
	9.332	Carvone	16.97
	10.152	3-Cyclohexene-1-methanol	8.91

3.5 Antioxidant Activity

Antioxidant study revealed that all the *Citrus* peel oils have strong potential to reduce DPPH radical to DPPH-H (83%-91%). Highest antioxidant activity was shown by *C. raticulata* var. Mandarin (91.1%) followed by *C. reticulate* var. Tangerine (88.0%), *C. paradisi* (87.2%), *C. sinensis* var. Malta (86.0%) and *C. sinensis* var. Mousami 83.2% for 500 µl/ml oil (Figure 1). Antioxidant efficacy of *Citrus* peel oils in decreasing order was as follows: Mandarin >Grapefruit> Tangerine > Malta >Mousami. These results were in accordance with work of Kamal et al. (2013) that *C. reticulate* var. Mandarin showed maximum antioxidant potential whereas *C. sinensis* var. Mousami showed minimum antioxidant potential. Correspondingly, Yang et al. (2010) reported limonene is a major constituent of *Citrus* peel oils having antioxidant potential equivalent to that of strong antioxidants.

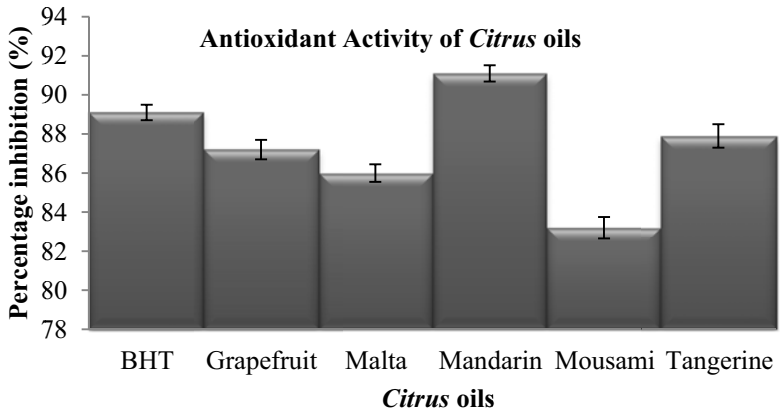
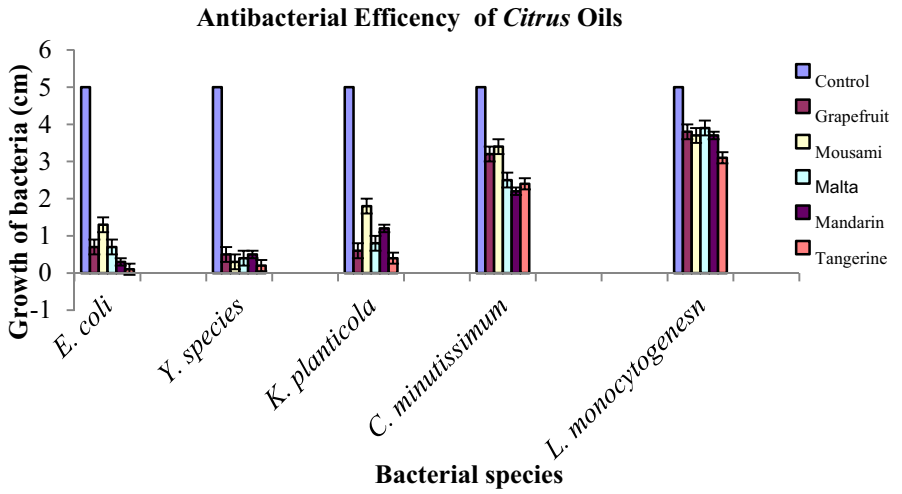


Figure 1. Antioxidant activity of *Citrus* oils by DPPH assay using BHT as standard. Data expressed as ±standard error bars

(A)



(B)

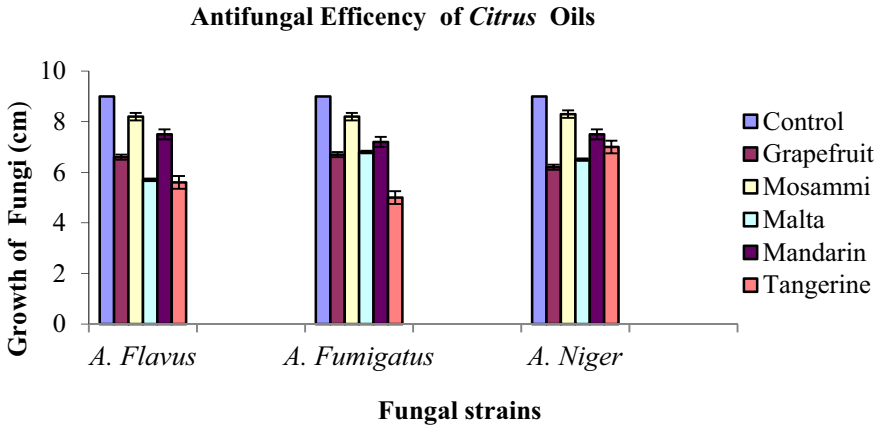


Figure 2. Antimicrobial activity of *Citrus* oils (A) Antibacterial activity of *Citrus* oils against five bacterial species (B) Antifungal activity of *Citrus* oils against three fungal pathogens. Error bars specify the mean ±standard error

3.6 Antimicrobial Activity

The antimicrobial activity of *Citrus sinensis*(L.) var. Malta and Mousami, *Citrus reticulate* (L.) var. Tangerine and Mandarin and *Citrus paradise* (L.) grapefruit was examined on five bacterial strains *L. monocytogenesn, C. minutissimum*, *E. coli,Y.* sp. and *K. planticola* (Figure 2A). The essential oils had various degrees of inhibition potential against the five bacterial strains. Tangerine oil depicted maximum bacteriostatic activity against all the test bacteria i.e., *E. coli, Y.* sp. *K. planticola*, *L. monocytogenesn* with inhibition zones (4.9 cm, 4.8 cm and 4.6 cm and 1.9 cm, respectively) except *C. minutissimum* var. Mandarin essential oil revealed second highest inhibitory potential against *E. coli* and *C. minutissimum* (4.7 cm and 2.8 cm) while Mousami oil against both *L. monocytogenesn* (1.3 cm) and *Y.* sp. (4.7 cm). Grapefruit essential oil displayed marked bactericidal activity against *K. planticola* depicting the inhibition zone of 4.4cm. Least antibacterial effect was shown by Mousami in case of *C. minutissimum*, *E. coli* and *K. planticola* (1.6 cm, 3.7 cm and 3.2 cm, respectively), whereas Grapefruit and Mandarin against *Y.* sp. (4.5 cm each). The growth of *L. monocytogenesn* was least affected by Malta oil (1.1 cm). *C. minutissimum* and *L. monocytogenesn* were least susceptible to growth inhibition by *Citrus* essential oils. The antimicrobial activities of *Citrus* species are strongly related with chemical constituents like flavonoids and phenols (Viuda-Martos et al., 2008). The active ingredients responsible for antimicrobial potential of *Citrus* peel oils are monoterpene components (Pavithra et al., 2009). D-limonene, linalool or citral are major attributors for the antimicrobial capacity of *Citrus* peel oils. Previous work revealed that the inhibitory influence of *Citrus* peel essential oils is owed to the presence of linalool rather than limonene (Fisher & Phillips, 2006). However it has

been found that antimicrobial activity is not only produced by one particular major component but also due to the antagonistic and synergistic effects of variety of compounds (Deba et al., 2008).

The essential oils of Grapefruit, Mousami, Malta, Mandarin and Tangerine showed the tendency to impede the growth of molds *A. flavus, A. fumigates* and *A. niger* (Figure 2B). In all molds, Tangerine and Grapefruit oils revealed great antifungal potentials. In case of *A. flavus* and *A. fumigatus*, Tangerine peel oil was the best growth inhibitor (3.4 cm-4.0 cm). Followed by Grapefruit and Malta peel oils, which showed almost equal reduction in growths for these two molds. Mandarin and Mousami showed the lowest growth reductions of *A. flavus* and *A. fumigatus*. The mycelium growth of *A. niger* was most susceptible to grapefruit essential oil with inhibition zone (2.8 cm) while least affected by mousami peel oil with inhibition zone (0.7 cm). Viuda-Martos et al. (2008) studied inhibitory influence of mandarin, orange and grapefruit peel essential oils on four fungal pathogens. *A. flavus* growth was best prevented by Mandarin essential oil whereas *A. niger* growth was most susceptible to Orange peel oil. Grapefruit essential oil was the most efficient against *P. verrucosum* and *P. chrysogenum.*

4. Conclusion

The results of present investigation are the basis for extraction of essential oil of *Citrus* by cheaper methods to design pilot plant to extract EO for industrial production. Some major restraints in viable industrial exploitation of medicinal plants are due to the poor agricultural practices, quality control trials, strain in marketing and dearth of research on process and product development. Coordination among various institutes and organizations of the country can lead for sustainable commercial utilization.

References

A. O. A. C. (2005). *Official methods of Analysis* (15th ed.). Association of Official Analytical Chemists, Washington DC.

Ahmad, M. M., Rehman, S., Iqbal, Z., Anjum, F. M., & Sultan, J. I. (2006). Genetic variability to essential oil composition in four *Citrus* fruit species. *Pakistan Journal of Botany, 38*, 319-324.

Amin, I., Norazaidah, Y., & Emmy Hainida, K. I. (2006). Antioxidant activity and phenolic content of raw and blanched *Amaranthus*species. *Food Chemistry, 94*, 47-52. http://dx.doi.org/10.1016/j.foodchem.2004.10.048

Anagnostopoulou, M. A., Kefalas, P., Papageorgiou, V. P., Assimopoulou, A. N., & Boskou, D. (2006). Radical scavenging activity of various extracts and fractions of sweet orange peel (*Citrus sinensis*). *Food Chemistry, 94*, 19-25. http://dx.doi.org/10.1016/j.foodchem.2004.09.047

Ayoola, G. A., Johnson, O. O., Adelowotan, T., Aibinu, I. E., Adenipekun, E., Adepoju-Bello, A. A., … Odugbemi, T. O. (2008). Evaluation of the chemical constituents and the antimicrobial activity of the volatile oil of *Citrusreticulata*fruit (Tangerine fruit peel) from South West Nigeria. *African Journal of Biotechnology, 7*(13), 2227-2231.

Deba, F., Xuan, T. D., Yasuda, M., & Tawata, S. (2008). Chemical composition and antioxidant, antibacterial and antifungal activities of the essential oils from *Bidenspilosa* Linn. Var. Radiata. *Food Control, 19*, 346-352. http://dx.doi.org/10.1016/j.lwt.2007.04.011

Espina, L., Somolinos, M., Lorán, S., Conchello, P., García, D., & Pagán, R. (2011). Chemical composition of commercial *Citrus* fruit essential oils and evaluation of their antimicrobial activity acting alone or in combined processes. *Food Control, 22*, 896-902. http://dx.doi.org/10.1016/j.foodcont.2010.11.021

Feger, W., Brandauer, H., & Ziegler, H. (2003). Analytical investigation of Murcott (Honey) tangerine peels oil. *Journal of Essential Oil Research, 15*(3), 143-147. http:// dx.doi.org/10.1080/10412905.2003.9712097

Fisher, K., & Phillips, C. A. (2006). The effect of lemon, orange and bergamot essential oils and their components on the survival of *Campylobacter jejuni, Escherichia coli* O157, *Listeria monocytogenes, Bacillus cereus* and *Staphylococcus aureus in vitro* and in food systems. *Journal of Applied Microbiology, 101*, 1232-1240. http://dx.doi.org/10.1111/j.1365-2672.2006.03035.x

Guenther, E. (1948). The Essential Oils. *The examination and analysis of essential oils, synthetics, and isolates* (pp. 229-367). New York: Van D. Nostrand Company Inc.

Huet, R. (1991). Les huillesessentielles d' agrumes. *Fruits, 46*(5), 551-576.

Kamal, G. M., Ashraf, M. Y., Hussain, A. I., Shahzadi, A., & Chughtai, M. I. (2013). Antioxidant potential of peel essential oils of three Pakistani *Citrus* species: *Citrus reticulata*, *Citrus sinensis*and *Citrus paradisii. Pakistan Journal of Botany, 45*(4), 1449-1454

Kanaze, F. I., Termentzi, A., Gabrieli, C., Niopas, I., Georgarakis, M., & Kokkalou, E. (2008). The phytochemical analysis and antioxidant activity assessment of orange peel (*Citrus sinensis*) cultivated in Greece-Crete indicates a new commercial source of hesperidin. *Biomedical Chromatography, 23*, 239-249. http://dx.doi.org/10.1002/bmc.1090

Kim, J., Marshall, M. R., & Wei, C. I. (1995). Antibacterial activity of some essential oil components against five food borne pathogens. *Journal of Agriculture and Food Chemistry, 43*, 2839-2845. http://dx.doi.org/10.1021/jf00059a013

Lota, M. L., De Rocca Serra, D., Tomi, F., & Casanova, J. (2000). Chemical variability of peel and leaf essential oils of mandarins from *Citrus reticulata* Blanco. *Biochemical Systematics and Ecology, 28*, 61-78. http://dx.doi.org/10.1016/S0305-1978(99)00036-8

Minh Tu, N. T., Thanh, L. X., Une, A., Ukeda, H., & Sawamura, M. (2002).Volatile constituents of Vietnamese pummelo, orange, tangerine and lime peel oils. *Flavour and Fragrance Journal, 17*, 169-174. http://dx.doi.org/10.1002/ffj.1076

Mondello, L., Casilli, A., Tranchida, P. Q., & Dugo, P. (2005). Comprehensive *two*-dimensional GC for the analysis of *Citrus* essential oils. *Flavour and Fragrance Journal, 20*, 136-140. http://dx.doi.org/10.1002/ffj.1506

Nannapaneni, R., Chalova, V. I., Crandall, P. G., Ricke, S. C., Johnson, M. G., & O'Bryan, C. A. (2009). *Campylobacter* and *Arcobacter* species sensitivity to commercial orange oil fractions. *International Journal of Food Microbiology, 129*(1), 43-49. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.11.008

Okwu, D. E., Awurum, A. N., & Okoronkwo, J. I. (2007). Phytochemical composition and *in vitro* antifungal activity screening of extracts from *Citrus* plants against *Fusarium Oxysporum* of Okra plant (*Hibiscus esculentus*). *African Crop Science Conference Proceedings, 8*, 1755-1758.

Pavithra, P. S., Sreevidya, N., & Verma, R. S. (2009). Antibacterial activity and chemical composition of essential oil of *Pamburusmissionis. Journal of Ethnopharmacology, 124*, 151-153. http://dx.doi.org/10.1016/j.jep.2009.04.016.

Sharma, N., & Tripathi, A. (2006). Effects of *Citrus sinensis*epicarp essential oil on growth and morphogenesis of *Aspergillusniger* Van Tieghem. *Microbiological Research, 10*, 1016-1020.

Sofowora, A. E. (1993). *Medicinal Plants and Traditional Medicine in Africa* (p. 288). Ibadan, Nigeria: Spectrum Books Ltd.

Svoboda, K. P., & Greenaway, R. I. (2003). Lemon scented plants. *International Journal of Aromatherapy, 13*(1), 23-32. http://dx.doi.org/10.1016/S0962-4562(03)00048-1

Tepe, B., Daferera, D., Sokmen, A., Sokmen, M., & Polissiou, M. (2005).Antimicrobial and antioxidant activities of the essential oils and various extracts of *Salvia tomentosa*Miller (Lamiaceae). *Food Chemistry, 90*, 333-340. http://dx.doi.org/10.1016/j.foodchem.2003.09.013

Trease, G. E., & Evans, W. C. (1989). *Pharmacognosy* (2nd ed.). Braille Tiridel and Macmillan Publishers.

Vekiari, S. A., Protopapadakis, E. E., Parthena, P., Dimitrios, P., Panou, C., & Vamvakias, M. (2002). Composition and seasonal variation of the essential oil from leaves and peel of a Cretan lemon variety. *Journal of Agricultural Food Chemistry, 50*(1), 147-153. http://dx.doi.org/10.1021/jf001369a

Viuda-Martos, M., Ruiz-Navajas, Y., Fernandez-Lo´pez, J., & Perez-Alvarez, J. (2008). Antifungal activity of lemon (*Citrus* lemon L.), mandarin (*Citrus* reticulataL.), grapefruit (*Citrus*paradisiL.) and orange (*Citrus*sinensisL.) essential oils. *Food Control, 19*, 1130-1138. http://dx.doi.org/10.1016/j.foodcont.2007.12.003

Yang, S. A., Jeon, S. K., Lee, E. J, Shim, C. H., & Lee, I. S. (2010). Comparative study of the chemical composition and antioxidant activity of six essential oils and their components. *Natural Product Research, 24*, 140-151. http://dx.doi.org/10.1080/14786410802496598

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Essential Oil Variation and Trace Metals Content in Garden Sage (*Salvia officinalis* L.) Grown at Different Environmental Conditions

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Abstract

This study was conducted to determine the essential oil variation and concentrations of certain trace metals (Co, Cu, Fe, Mn and Zn, Pb, Cd) in the sage plant (*Salvia officinalis* L.) grown in their different geographic natural environment. The samples of *Salvia officinalis* were collected from Jeresh and Ajloune, Ma'an and Tafilah, and Amman located in their natural original regions in the north, south, and middle locations of Jordan, respectively. The results showed a wide variation of essential oil contents among *S. officinalis*grown in different variable natural environment. The range varied from 0.87% in Amman to 2.8% in Jarash. In general, the oil content in *S. officinalis* grown in the north regions was higher than recorded in the middle and south regions.The essential oil content in *S. officinalis* grown in Jarash and Ma'an were higher than recoded in other investigated groth regions. Trace metal concentrations in all investigated samples were varied.The most toxic trace metals Co, Pb, and Cd were not detectable in all studied samples. Fe metal recorded the highest concentration which varied from 834.5 mg/kg in Ajloun to 1743 mg/kg in Ma'an. Cu recoded the lowest mean levels among all detected metals and varied from 6.60 mg/kg in Amman to 9.25 mg/kg in Ajloune .The highest mean levels of Mn were recorded in the southern regions in Tafilah and Ma'an (53.7 and 50.4 mg/kg, respectively), while the lowest was recorded in the middle region in Amman (26.10 mg/kg). Zn concentration varied from 27.80 mg/kg in Ma'an to 42.72 mg/kg in Tafillah. All of detected metals were within the range of permissible limit for medicinal plants and lower than that detected in *S. officinalis* originated from other local and global habitats. The essential oil and trace metals contents in *S. officinalis* were mainly affected by variable natural climatic conditions. Moreover, the current study showed that *S. officinalis* grown in some locations of Jordan are characterized by low trace metals contents and can safely be used for pharmaceutical and edible purposes without any hazardous effect on human health.

Keywords: essential oil, jordan, sage, trace metals

1. Introduction

Garden sage (*Salvia officinalis* L.) is a perennial medicinal plant with woody stems, and grayish leaves belonging to the family Lamiaceae that comprises over 900 species all over the world. It's native to the Mediterranean region in which twenty species of Salvia were found grow wildly in Jordan (Al-Eisawi, 1996). *S. officinalis* is a popular plant in Jordanian folk medicine and used for edible consumption and herbal remedy as a source of therapeutically active essential oil (Amr & Đorđević, 2000). Sage extracts, mostly essential oil, are also widely used in the drug, beverage, cosmetic and fragrance industries. Various studies have proved that the essential oil extracts of garden sage exhibited antimicrobial activities against some pathogenic microorganisms and food borne bacteria (Hayouni et al., 2008; Klaus et al., 2008). Moreover, the individual secondary metabolites of sage could exhibit various pharmacological activities such as anesthetic, antihistaminic, antirheumatic, diuretic, expectorant, insecticidal, purgative (monoterpenes); analgesic, antiarhythmic, antiepileptic, spasmolitic, anthelmintic, antiinflammatory, antitumorous, hypotensive, and sedative (Velickovic et al., 2003).

Trace metals of *S. officinalis* have an important biological role in human, animal and plant health. Trace metals content in medicinal plant research conducting is of great interest because of their effect on the biological active compounds in medicinal plants and may cause serious effects on human health. Thus, the maximum permissible limits of toxic metals in medicinal plants such as cadmium and lead were reported by World Health Organization,

(2007). In Jordan, a recent study was conducted to determine Pb concentration in seventy nine popular samples of medicinal plants (Alomary et al., 2013), they found that Pb concentration in the most samples was higher than recommended values by World Health Organization. The mean Pb levels were ranged between 13.1 and 16.9 μg/g in the most and less commonly used herbs (Alomary et al., 2013). In addition, many worldwide research studies have been conducted to evaluate the content of heavy metals in certain medicinal plants consumed by popular or used in pharmaceutical industries (Amr & Đorđević, 2000; Malencic et al., 2003; Chan, 2003; Angelova et al., 2005; Szentmihályi & Then, 2007; Blagojević et al., 2009; Abu-Darwish et al., 2010).

The content of essential oil and heavy metals in medicinal plants can be affected by environmental conditions (Maksimovic et al., 2007) and geochemical characteristics of soil or location in which the plant is grow (Chan, 2003; Blagojević et al., 2009; Abu-Darwish et al., 2010). Therefore, the present study was conducted to find out the effect of environmental conditions on the yield of essential oil and certain trace metal contents in *S. officinalis* grown naturally in different geographical locations of Jordan.

2. Materials and Methods

2.1 Plant Material

Aerial parts of *S. officinalis* were collectedin vegetative period during April, in five localities of Jordan: Ma'an (South Jordan); Tafillah (South Jordan); Jerash (North Jordan); S5- Ajloun (North Jordan); and Amman (Middle Jordan). Voucher specimens were deposited at the Herbarium of Shoubak University College- Al-Balqa Applied University. The plant material was dried in draughty place at about 20°C, all specimens were identified on the basis of macroscopic characteristics by comparison with authentic sample and a voucher specimen was deposited at the Herbarium of Al-Shoubak University College. The dried samples of *S. officinalis* were separately crushed and mild into small pieces and sieved through (0.5 mm) mesh sieve.

2.2 Determination of Essential Oil

Essential oil contents were extracted from dried aerial parts of the all collected samples of Sage plant by the hydro-distillation method using a Clevenger-type apparatus (British Pharmacopeia, 1998) similar to (European Pharmacopoeia, 2007), using 50 g of the dried mild and sieved plant and 500 mL of water in 1000 mL round bottomed flask. Distillation time was 2 h at a rate of 2-3 min^{-1} the values reported are the mean of at least three distillations and three replications for each specimen.

2.3 Determination of Heavy Metals Content in Sage Plant Samples

The content of Co, Cu, Fe, Mn and Zn, Pb, and Cd in particular sage samples were analyzed using Atomic AbsorptionFlame Emission Spectrophotometer Varian Spectro AA.200as described by (Al-Alawi & Mandiwana, 2007). Cathode lamps used as radiation source. Air acetylene gas was used for all the experiments. The Absorption wavelength for the determination of each metal together with its liner working range and correlation coefficient of calibration graphs are given in Table 1. The plant samples were oven dried at 70°C for 24 h until the dry weight was constant. The dried samples were then ground and passed through a 0.2 mm plastic sieve. Then, 0.5 g of plant sample was wet digested with an Ultra-pure nitric acid (HNO_3 (10-15 mL) in a polyethylene test tube using a heating block digestion unit at 120°C. The final solution was filtered into a 25 or 50 mL volumetric flask through a 45-μm filter paper and diluted to the mark with ultra-pure water. Ultra-pure water was used for all dilutions and sample preparation. Analytical results have evaluated by statistical analysis system. The standard error values of the means were calculated to compare the site categories.

Table 1. Operating parameters for working elements

Elements	Wave length(nm)	Lamp intensity (mA)	Slit width(nm)	Correlation coefficient(r)
Pd	217.0	5 mA	0.2	0.998
Cd	228.8	4 mA	0.5	0.998
Zn	213.9	5 mA	1.0	0.998
Cu	234.7	4 mA	0.5	0.998
Fe	248.3	5 mA	0.2	0.998
Mn	279.5	5 mA	0.2	0.998

3. Results and Discussion

Means of essential oil contents in *S. officinalis* L. are listed in Table 2. The results revealed a wide variation in the values of essential oil yielded from sage plant grown in different environmental locations. The variation range of oil content varied from 0.87 to 2.80%, reaching minimum and maximum values in Amman and Jeresh located in the middle and northern regions of Jordan, respectively. The results showed that the oil content in all specimens of *S. officinalis*, except that cultivated in Amman (Middle of Jordan) were found to satisfy the requirements of Pharmacopeias such as European Pharmacopoeia, which requires a yield of oil should be ≥ 1.2% v/w. calculated with reference to the anhydrous (dried) drug (European Pharmacopoeia, 2007). In south regions, Essential oil was ranged from 1.8 to 2.2% in specimens collected from Tafillah and Ma'an respectively, while it was increased from 1.20 to 2.8% in the northern growth areas of Ajloun and Jeresh, respectively. These results were in agreement with the results obtained by Amr and Đorđević (2000), who found that *S. officinalis* collected during the vegetative period from some middle locations of Jordan, yielded 1.18-1.32%.

Table 2. Averages of ambient temperatures(°C), rainfall (mm), and relative humidity (%), and means of essential oils yield (%), and moisture content (%) of garden sage cultivated in different environmental regions in Jordan

Place of Sage growth	Altitude (m)	Ambient temp. min. – max. (°C)	Seasonal means of rainfall (mm)	Relative humidity (%)	Moisture (%)	Oil (%)
Ajlune	800-1150	10.1 – 18.6	582.2	80.20	4.9%	1.20±0.17
Jarash	520-750	8.5 – 18.40	445.6	68.30	3.4%	2.80±0.06
Amman	700-1050	6.8 – 23.5	490.0	51.70	3.7%	0.87±0.12
Ma`an	1069-1100	20.0 – 32.0	44.20	57.80	6.1%	2.20±0.17
Tafelah	900-1140	11.8 – 23.4	237.6	23.30	5.2%	1.80±0.17

Table 3. Concentration of heavy metals (mg/kg) *in Sage* (*Saliva Officinalis*) in various locations in Jordan

Location	mg/kg						
	Co*	Cu*	Fe*	Mn	Zn	Pb*	Cd*
Ajlune	<IDL	9.25±0.006	834.5±0.003	39.2±0.008	41.23±0.007	<IDL	<IDL
Jarash	<IDL	8.27±0.003	899±0.007	39.1±0.006	40.54±0.0001	<IDL	<IDL
Amman	<IDL	6.60±0.001	1010.5±0.00	26.1±0.001	29.71±0.0001	<IDL	<IDL
Ma`an	<IDL	7.20±0.005	1743±0.006	53.7±0.004	27.80±0.003	<IDL	<IDL
Tafelah	<IDL	7.77±0.006	714.25±0.002	50.4±0.003	42.76±0.001	<IDL	<IDL
IDL	0.005<	0.003<	0.006<	0.002<	0.001<	0.001<	0.002<

* are included in accreditation.

Maric et al. (2006) observed that the oil content in *S. officinalis* was increased in higher altitude than lower. In contrast, the present study showed that the oil content in lower altitude regions of Jarash (520-750 m ab.s.l) and Ma'an (1100-1140 m ab.s.l) was higher than those observed in higher altitudes regions of Tafilah, Ajloun and Amman (2.2-2.8%) (0.87-1.8), respectively. The same results were reported by Haider et al. (2009) and Takaloo et al. (2012) who found that the lowest oil yields were recorded in plants collected from the relatively higher altitude. However, other factors may be induced the essential oil yields in Jarash and Ma′an such as temperature, soil fertility, locality, and the high sunlight radiation level. It was reported that, the aromatic medicinal plants submitted to high level of sunlight radiation yielded higher content of essential oil than others (Letchamo & Gosselin, 1995; Silva et al., 2006). Qasaimeh (2012) showed that the average daily solar radiation for the years (2004-2010) in regions closed toMa'an was 6215 Wh/m^2·d and higher than in Tafilah 5763 Wh/m^2·d and Amman 5711 Wh/m^2·d. This is indicated by the effect of high sunlight radiation level in Ma′an on the higher yields of oil from *S. officinalis*. Li et al. (2006) who found that the highest oil yield was in sage plants grown at 45% of full sunlight. However, this variation indicates the effect of geographical growth location on the yields of essential oils. Such variations could be explained by the effect of environmental factors related to the predominant climatic and geographical differences on various growth areas (Table 3) (Perry et al., 1999; Chan, 2003; Maksimovic et al.,

2007; Abu- Darwish et al., 2010). This is confirmed by the results of oil content recorded in the worldwide regions of Egypt (1.52-1.65%) (Khalil et al., 2008), Lithuania (0.7–1.4%) (Bernotienė et al., 2007), Bosnia and Herzegovina (0.29%-1.07%) (Maric et al., 2006), Algeria 0.9% (Dob et al., 2007) and Spain 1.4-2.0 (Arraiza et al., 2012).

The concentration of Cu metal ranged from 6.6 in Amman to 9.25 mg/kg in Ajloune. These concentrations were within the average content of Cu in dry plant material which is reported to be 2.0-20 mg/kg by Malencic et al. (2003) indicating that all the investigated growth regions are not contaminated with Cu metal. These results were in agreement with the results of our previous study conducted in the south Shouback region and showed that Cu concentrations in *S. officinalis* cultivated at 15 and 30 cm inter-raw spaces were 7.32 and 7.02 mg/kg, respectively. In contrast, Amr and Đorđević (2000) found that Cu metal concentration in *S.officinalis* grown in Al Dbajbe near Amman-Sahab highway was ranged from 61 to 70 mg/kg and higher than the permissible limits.

Fe is one of the most essential elements for living organism's cells. The results showed a wide variation of Fe concentration in *S. officinalis*. It was ranged from 834.5 to 1743 mg/kg in the northern (Ajloun) and southern (Ma'an) regions of Jordan. However, they were within the concentration limits in dry plant material (1000 mg/kg) (Malencic et al., 2003). These differences in Fe concentrations could be explained by the assumption of Shad et al. (2008) who suggested that high concentration of Fe in plants may be due to the foliar absorption from the surroundings air. This is supported by the results of Fe concentrations in S.officinalis cultivated in worldwide regions which revealed a wide variation in the values of Fe concentrations. They were 297.4, 453.77 and 450 mg/kg in Turkey (Başgel & Erdemoğlu, 2006), Serbia (Malencic et al., 2003) and Romania (Ştef et al., 2010), respectively. On the other hand, Yeritsy and Economakis (2002) reported that Fe concentration in leaves could be increased by higher concentration of Fe in the growth nutrient medium indicating that the soils of Ma'an and Amman regions are rich source content of Fe.

Mn concentration in all collected samples of *S. officinalis* from different investigated regions were ranged from 26.1 mg/kg in Amman, the middle region to 53.7 mg/kg in Ma'an and Tafilah, the southern regions of Jordan. The contents of Mn in *S. officinalis* originated in the north regions were 39.2 and 39.1 mg/kg in Ajloun and Jarash, respectively. They were higher than found in the middle and lower than recorded in the south regions of Jordan. However, the concentrations of Mn in *S. officinalis* originated in the different geographical locations of Jordan were higher than the critical threshold for Mn deficiency in plant (<10 ppm), These results were in agreement with previous results obtained from *S. officinalis*growen at 30 cm inter-raw spacing and harvested during the vegetative period in the south shouback region (Abu-darwish et al., 2010).

The results of Zn metal concentration in *S. officinalis* showed a limited variation among the different geographic origin regions. The range varied from 27.80 in Ma'an to 42.76 mg/kg in Tafilah, the southern regions of Jordan. In the north, it was 41.2 mg/kg in Jarash and 40.54 mg/kg in Ajloun while it was 29.71 mg/kg in the middle region. The same findings were observed by Amr and Đorđević (2000) who found that the concentration of Zn in *S.officinalis* originated from middle of Jordan varied from 22 to 29 mg/kg. On the other hand, Malencic et al. (2003) reported that the concentration of Zn in plant may vary between 30-150 mg/kg, but usually it is between 20-50 mg/kg. In general, the low contents of Zn in all studied samples indicated to non polluted soil environment and/or in locations faraway from industrial and heavy traffic activities which were reported the main factors of plant growth soil pollution with heavy metals (Angelova et al., 2005; Blagojević et al., 2009; Stancheva et al., 2009). In conclusion, the results of the present study revealed that *S. officinalis* growen in Jordan is a safe medicinal plant containing sufficient amount of a biological active essential oil that is clearly influenced by metrological and environmental factors. *S. officinalis* originated within investigated southern, middle and northern regions of Jordan are free from hazardous heavy metals such as Cd, Pb. Moreover, the surrounding environments of *S. officinalis* original locations are not polluted with heavy metals. Further studies on the composition of *S. officinalis* essential oil, grown in different Jordan environments are recommended.

References

Abu-Darwish, M. S., Abu-Dieyeh, Z. H., Al-Fraihat, A. H., Al-dalain, S. Y., Afifi, F. U., & Al-Tabbal, J. A. (2010). Determination of essential oils and heavy metals accumulation in *Salvia officinalis*cultivated in three intra-raw spacing in ash-shoubak, Jordan. *International Journal of Agriculture& Biology, 13*, 981-985.

Al-Alawi, M. M., & Mandiwana, K. L. (2007). The use of Aleppo pine needles as a bio-monitor of heavy metals in the atmosphere. *Journal of Hazardous Materials, 148*, 43-46. http://dx.doi.org/10.1016/j.jhazmat.2007.02.001

AL-Eisawi, D. M. (1996). *Vegetation of Jordan*. Book published by UNESCO (ROSTAS). Cairo Office: Cairo.

Al-Omary, A., El-Jamal, E., Al-momani, I., Attiyat, A., & Obeidat, S. (2013). Pb in medicinal plants from Jordan. *Enviro Chem Lett, 11*, 55-63. http://dx.doi.org/10.1007/s10311-012-0378-y

Amr, S., & Đorđević, S. (2000). The investigation of the quality of Sage (*Salvia officinalis*) originating from Jordan. FACTA Universitatis Series: *Working Living Environmental Protection, 5*, 103-108.

Angelova, V., Ivanov, K., & Ivanov, R. (2005). Heavy metal content in plants from family Lamiaceae cultivated in an industrially polluted region. *Journal of Herbs, Spices & Medicinal Plants, 11*, 37-46. http://dx.doi.org/10.1300/J044v11n04_05

Arraiza, M. P., Arrabal, C., & López, J. V. (2012). Seasonal Variation of Essential Oil Yield and Composition of Sage (*Salvia officinalis*L.) Grown in Castilla - La Mancha (Central Spain), *Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 40*(2), 106-108.

Başgel, S., & Erdemoğlu, S. B. (2005). Determination of mineral and trace elements in some medicinal herbs and their infusions consumed in Turkey. Science of the Total Envir*onment, 359*, 82-89. http://dx.doi.org/10.1016/j.scitotenv.2005.04.016

Bernotienė, G., Nivinskienė, O., Butkienė, R., & Mockutė, D. (2007). Essential oil composition variability in sage (*Salvia officinalis* L.). *CHEMIJA, 18*(4), 38-43.

Blagojević, N., Damjanović-Vratnica, B., Vukašinović-Pešić, V., & Đurović, D. (2009). Heavy Metals Content in Leaves and Extracts of Wild-Growing *Salvia Officinalis*from Montenegro. *The Polish Journal of Environmental Studies, 18*(2), 167-173.

British Pharmacopeia. (1998). *Her Majesty's Stationery office, London, 2*, 137-138.

Chan, K. (2003). Some aspects of toxic contaminants in herbal medicines. *Chemosphere, 52*, 1361-1371. http://dx.doi.org/10.1016/S0045-6535(03)00471-5.

European Pharmacopoeia. (2007). Strasbourg, France: *Council of Europe 2* (6th ed). Kelp, monograph 1426.

Haider, F., Narendra, K., Banerjee, S., Naqvi, A. A., & Bagchi, G. D. (2009). Effect of altitude on the essential oil constituents of *Artemisia roxburghiana* Besser var. *purpurascens* (Jacq.) Hook. *Journal of Essential Oil Research, 21*(4), 303-304. http://dx.doi.org/10.1080/10412905.2009.9700177

Hayouni, E. A., Chraief, I., Abedrabba, M., Bouix, M., Leveau, J. Y., Mohammed, H., & Hamdi M. (2008). Tunisian Salvia officinalis L. and Schinusmolle L. essential oils: Their chemical compositions and their preservative effects against Salmonella inoculated in minced beef meat. *International Journal of Food Microbiology, 125*(3), 242-251. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.04.005.

Khalil, M. Y., Kandil, M. A., & Swaefy, H. M. F. (2008). Effect of three Different Compost Levels on Fennel and *Salvia* Growth Character and their Essential Oils. *Res. J. Agric. Biol. Sci., 4*, 34-39.

Khan, M. A., Ahmad, I., & Ur Rahman, I. (2007). Effect of Environmental Pollution on Heavy Metals Content of *Withaniasomnifera. Journal of the Chinese Chemical Society, 54*, 339-343.

Klaus, A., Beatovia, S. D. V., Niksia, M. P., Jelaaia, S. A., Nedovia, V. K., & Petrovia, T. S. (2008). Influence of Ethereal Oils Extracted from *Lamiaceae* Family Plants on Some Pathogen Microorganisms, *Proc. Nat. Sci, Matica Srpska Novi Sad, 115*, 65-74.

Letchamo, W., & Gosselin, A. (1995). Effects of supplemental lighting and soil water levels on growth, essential oil contend and composition of two thyme (*Thymus vulgaris* L) clonal selections. *Canadian Journal of Plant Science, 75*(1), 231-238. http://dx.doi.org/10.4141/cjps95-044

Li, Y. L., Craker, L. E., & Potter, T. (1995, August). Effect of light level on essential oil production of sage (Salvia officinalis) and thyme (Thymus vulgaris). In *International Symposium on Medicinal and Aromatic Plants 426* (pp. 419-426). Retrieved from http://ww.actahort.org/books/426/426_46.htm

Maksimovic, M., Vidic, D., Milos, M., olic, M. E. S, Zic, S. A., & Siljak-Yakovlev, S. (2007). Effect of the environmental conditions on essential oil profile in two Dinaric Salvia species: S. brachyodonVandas and S. officinalis L. *Biochemical Systematic and Ecology, 35*, 473-478. http://dx.doi.org/10.1016/j.bse.2007.02.005.

Malencic, D. R., Kevesan, Z. S., & Popovic, M. T. (2003). Mineral composition of selected Salvia species growing wild in the Vojvodina province. *Proc. nat. sci. Matica Srpska Novi sad, 105*, 25-33. http://dx.doi.org/10.2298/ZMSPN0305025M

Maric, S., Maksimovic, M., & Milos, M. (2006). The impact of the locality altitudes and stages of development on the volatile constituents of Salvia officinalis L. from Bosnia and Herzegovina. *Journal of Essential Oil Research, 18*(2), 178-180. http://dx.doi.org/10.1080/10412905.2006.9699060

Misra, A., & Sharma, S. (1991). Critical Zn concentration for essential oil yield and menthol concentration of Japanese mint. *Nutrient Cycling in Agroecosystems, 29*(3), 261-265. http://dx.doi.org/10.1007/BF01052394

Perry, N. B., Anderson, R. E., Brennan, N. J., Douglas, M. H., Heaney, A. J., McGimsey, J. A., & Smallfield, B. M. (1999). Essential oils from Dalmation sage (*Salvia officinalis L.*): variations among individuals, plant parts, seasons and sites. *Journal of Agriculturaland Food Chemistry, 47*, 2048-2054. http://dx.doi.org/10.1021/jf981170m

Qasaimeh, A. (2012). Solar Energy Optimization through Seasons: Case Study in Jordan. *Smart Grid and Renewable Energy, 3*, 275-281. http://dx.doi.org/10.4236/sgre.2012.34038**.**

Shad, A. K., Lajbar, K., Iqbal, H., Khan, B. M., & Naveed A. (2008). Profile of Heavy Metals in selected Medicinal plants. *Pakistan journal of Weed Science Research, 14*, 101-110.

Silva, F., Pinto, J. E. B. P., Cardoso, M. das G., Nascimento, E. A., Nelson, D. L., Sales, J. de F., & Mol, D. J. de S. (2006). Influence of Radiation Level on Plant Growth, Yield and Quality of Essential Oil in Carqueja. *Ciênciae Agrotecnologia Lavras, 30*(1), 52-57. http://dx.doi.org/10.1590/S1413-70542006000100007.

Stancheva, I., Geneva, M., Hristozkova, M., Boychinova, M., & Markovska, Y. (2009). Essential Oil Variation of *Salvia officinalis* (L.), Grown on Heavy Metals Polluted Soil. *Biotechnology & Biotechnological Equipment, 23*, 373-376.

Ştef, D. S., Gergen, I., Traşcă, T. I., Hărmănescu, M., Ştef, L., Drugă, M., … Hegheduş-Mîndru, G. (2010). Screening of 33 Medicinal Plants for the Microelements Content.*Animal Science and Biotechnologies, 43*(1), 127-132.

Szentmihályi, K., & Then, M. (2007). Examination of microelements in medicinal plants of the Carpathian basin. *Acta Alimentaria, 36*(2), 231-236. http://dx.doi.org/10.1556/AAlim.36.2007.2.9

Takaloo, S. G., Hassani, A., Hassanpouraghdam, M. B., Meshkatalsadat, M. H., Pirzad, A., & Heidari, M. (2012). Essential Oil Content and Composition of *Thymus migricus* Klokov & Desj-Shost. Affected by Plant Growth Stage and Wild Habitat Altitude. *Romanian Biotechnological Letters, 17*(1), 6983-6988.

Velickovic, A. S, Ristic, M. S., Velickovic, D. T., Ilic, S. N., & Mitic, N. D. (2003). The Possibilities of the Application of Some Species of Sage (Salvia L.) as Auxiliaries in the Treatment of Some Diseases. *Journal of the Serbian Chemical Society, 68*(6), 435-445. http://dx.doi.org/10.2298/JSC0306435V

World Health Organization. (2007). Dept. of Technical Cooperation for Essential Drugs and Traditional Medicine.WHO guidelines for assessing quality of herbal medicines with reference to contaminants and residues. *World Health Organization.* Geneva, Switzerland.

Yeritsyan, N., & Economakis, C. (2002). Effect of Nutrient Solution's Iron Concentration on Growth and Essential Oil Content of Oregano Plants Grown in Solution Culture. *ActaHorticulturae (ISHS), 576*, 277-283. Retrieved from http://www.actahort.org/books/576/576_41.htm

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