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Transition Period of the Dairy Cow Revisited: I. Homeorhesis and Its Changes by Selection and Management

Holger Martens[1]

[1] Institute of Veterinary Physiology, Freie Universität Berlin, Berlin, Germany

Correspondence: Holger Martens, Institute of Veterinary Physiology, Freie Universität Berlin, Oertzenweg 19b, 14163 Berlin, Germany. Tel: 049-30-831-1805. E-mail: holger.martens@fu-berlin.de

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Abstract

The transition period of the dairy cow involves the end of pregnancy, parturition, and the onset of lactation. Multifaceted and rapid changes occur during this time, and in particular, the increase of milk secretion requires the large-scale reorientation of metabolism. The underlying mechanisms of this metabolic regulation are collectively named homeorhesis, a process that governs milk production during this phase and that exhibits (A) a chronic nature, (B) the simultaneous inclusion of multiple tissues, and (C) altered responses to homeostatic signals, but (D) no direct feedback mechanisms for possible control or limitation. Priority of milk production is one important consequence of this homeorhetic regulation with possible constraints on other physiological functions. These general properties of the homeorhetic regulation of milk secretion are specifically characterized by a) milk production according milking (suckling) frequency, b) a natural but inadequate dry matter intake, c) the mobilization of fat acids + glycerol from adipose tissue and of amino acids from protein, d) the partitioning of metabolites, IgG, and dietary nutrients to the mammary gland, e) the stimulation of milk production by high protein intake, and f) a negligible negative energy balance (NEB) at low milk production. Such a combination assures the optimal milk yield for the nutrition of the calf and for its successful survival but without a metabolic challenge or health risk for the cow. However, selection for higher milk production (uncoupled from calf nutrition) and management have changed the above-listed properties, and the regulation of homeorhetic milk production of the modern high-producing dairy cow is nowadays mostly characterized by a) increasing and maximal milk production at increased milking frequency and, under certain circumstances, the uncoupling of the GH-IGF-1 axis, b) enduring insufficient dry matter intake in relation to requirement, c) the mobilization of energy (lipolysis) and release of non-esterified fatty acids (NEFA) above the acute requirement, d) the mobilization of amino acids, e) the partitioning of metabolites, IgG, and dietary nutrient to the mammary gland, f) the potential enhanced partitioning of energy to the mammary gland at high CP intake, g) a sudden and long-lasting NEB, and h) possibly lower weight gain or even net loss of energy during the entire lactation period. These altered and often unfavorable characteristics of high milk production are, furthermore, still regulated by homeorhesis and are thus also given top priority, lack feedback control, and possibly ensue at the expense of other functions without regard for health risks. Hence, the promotion of milk yield by breeding or management might cause metabolic overload, imbalances, or even antagonisms and makes possible health hazards evident. The high incidence of various diseases, the untimely culling rates, and the increasing number of dead cows during early lactation support the assumption of general health threats at high milk production. For this reason, more attention should be paid to the physiological mechanisms of homeorhetic-regulated milk production, its indisputable alterations by breeding and management, and the resulting health risks.

Keywords: homeorhesis, nutrition, negative energy balance, production diseases

1. Introduction

"Transition period is defined as 3 weeks prepartum and 3 weeks postpartum" (Grummer, 1995). During this time, pregnancy is completed, parturition occurs, and milk production begins. "There is no other time in a cow's life that is more tumultuous" (Grummer et al., 2004). The most tumultuous part of this whole process is probably the

increase in the nutrient requirement of the cow postpartum (p.p.) as a result of rapid increasing milk production. "At parturition, demand for all nutrients doubles within a few days and within a few weeks can be 3-5 times as high as in mid gestation" (McNamara, 2004).

These rapid and multiple changes during early lactation require a complex regulation of metabolism for the successful adaptation of the cow to its new challenges and have been excellently outlined in detail by Bauman and Currie (1980), Vernon and Pond (1997), Chilliard et al. (1998), Bauman (2000), Renaville et al. (2002), Boisclair et al. (2006), Baumgard et al. (2017). Two mechanisms dominate regulation. The first, viz., homeostasis, is an acute and short minute-by-minute regulating system for keeping constant internal conditions such as body temperature, blood glucose concentration (Bauman & Currie, 1980), or pH and acid-base metabolism. The second has been coined by Bauman and Currie (1980) as "homeorhesis" and involves the "orchestrated or coordinated changes for the priorities of a physiological state, *i.e.*, coordination of metabolism in various tissues to support a physiological state"; the homeorhetic "physiological state" of the cow p.p. being milk production.

Homeorhesis is a wide-spread control system in biology and includes the regulation of growth, pregnancy, or hibernation (Bauman, 2000); it has (A) a chronic nature, influences (B) simultaneously multiple tissues, and mediates (C) altered responses to homeostatic signals (Bauman, 2000). Surprisingly, homeorhesis of milk production does not exhibit (D) a direct feedback mechanism, although this essential step of regulation and control is well known in other homeorhetic systems such as growth, pregnancy or hibernation. Instead, the appetite of the calf is proposed as a biological and indirect feedback and "control mechanism" for (limiting) milk yield. These *general* characteristics of homeorhesis (A-D) provide the framing conditions for understanding the regulation of lactation and metabolism and include *specific* mechanisms that together determine optimal milk production for the nutrition and survival of the calf: a) milk production according milking (suckling) frequency, b) inadequate dry matter intake, c) the mobilization of fat acids + glycerol from adipose tissue and of amino acids from protein, d) the partitioning of metabolites, IgG, and dietary nutrients to the mammary gland, e) the stimulation of milk production by high protein intake, and f) a negligible negative energy balance (NEB) at low milk production. However, the *general* and *specific* mechanisms of lactational homeorhesis have been substantially changed by selection for higher milk yield (MY) and by management.

The intention of these reviews (part 1 and 2) is to summarize the physiological background of the homeorhetic regulation of milk production and metabolism, to indicate the changes to this background by genetic and by management, and to point out some of the challenges of these changes as possible health risks. This approach will show possible interactions and connections between the issues of homeorhesis, negative energy balance, metabolic challenge, and health risks. These reviews are not systematic (O'Connor & Sargeant, 2014) and primarily do not contain details (for these, see the links to corresponding reviews); hence, they are not presented with any claim to completeness but aim at disclosing an unbroken ("red") thread that links these topics and at encouraging a discussion of these topics as a complex rather than as a single or separate event (Sordillo & Mavangira, 2014; see part 2).

2. Homeorhetic Mechanisms and Changes

2.1 Milk yield

The "physiological (homeorhetic) state" p.p. includes milk production with a high priority and the possible drawbacks generated by neglecting other physiological functions. Originally, this process was dedicated to the nutrition of (only) one calf with some 500-1000 l per lactation. Such production can hardly be considered as a metabolic challenge because the energy requirement at the end of pregnancy accounts to 38 MJ_{NEL}/d for the maintenance of the cow and 18 MJ_{NEL}/d for the calf, uterus, and mammary gland (Gesellschaft für Ernährungsphysiologie-GfE, 2001), with 18 MJ_{NEL}/d being sufficient for some 5 l milk after parturition. Indeed, body weight increases after the parturition of Hereford cows producing milk only for their suckling calves (Hart et al., 1975).

Milk production during early lactation can be stimulated by the frequency of milking (Wall and McFadden, 2012), and naturally, the appetite and the suckling frequency of a calf represent a regular trigger for the feedforward increase of milk production. Conversely, the feeling of satiety in the calf serves as an indirect feedback mechanism for limitation. Hence, the regulation of the amount of milk is the combination of feedforward (frequency) and feedback (appetite) mechanisms and guarantees an optimal amount for the nutrition of the calf *and* the metabolic capacity of a cow with minor health risks.

2.2 Changes of Milk Yield

Milk production per lactation has increased in Germany by a factor 3 from 2600 kg in the 1950s to almost 8000 kg in 2017 (mean of all breeds) (BRS, 2018). Single cows or herds frequently produce much more milk, and MY of 11000-12000 kg or even more are often produced in countries with intensive milk production aims. The genetic trend for milk production has increased in the USA from 37 kg in the 1960s to 116 kg per lactation in the 1990s (Hansen, 2000), and production has further increased to 10328 kg in 2016 (Baumgard et al., 2017). Levels of 11970 kg have been reported in Israel (Israeli Dairy Board, 2017).

MY shows high heritability in early lactation (Hüttmann et al., 2009) and a high priority in the breeding index of Holstein Friesian (HF) cows in Germany (Deutsch Holstein, 2017). Further increases of MY will probably occur in the future.

The increase of milk production by frequent milking during early lactation has been studied by Bar-Peled et al. (1998). The authors have observed an increase of milk production from 38 kg/d to 52 kg/d at a higher frequency of milking or at milking (3 times) combined with suckling (3 times); this suggests the stimulation of the feedforward signal by frequency and the offset of biological feedback, *i.e.*, limitation by the appetite of the calf. The optimal MY for calf nutrition has been replaced by maximal MY without a corresponding dry matter intake.

2.3 Dry Matter Intake (DMI)

Feed intake declines before parturition and slowly increases afterwards compared with the rapid enhancing milk production, thereby causing a gap between output and input or a negative energy balance (NEB). Many studies of this topic have been undertaken and reported (Chilliard et al., 1998; Ingvartsen & Andersen, 2000; Grummer et al., 2004; Dann et al., 2006; Allen & Piatoni, 2013; Van Saun & Sniffen, 2014). The genetic correlation between DMI before parturition and 30 d p.p. is high (Shonka et al., 2015), but the periparturient decline remains poorly understood (Allen, 2000; Kuhla et al., 2016).

However, some biological explanations have been suggested. The decline of DMI before parturition might be explained by the growing calf in the uterus and the resulting space limitation in the abdomen. This physical restriction is eliminated at parturition and should permit the rapid increase of DMI, but this does not occur. Gravert (1985) mentions “that the curve of feed intake (p.p. the author) still corresponds to the non-domesticated cow in according with the requirement of the suckling calf while the lactation curve has been altered by artificial selection for higher milk yields”. Vernon and Pond (1997) have discussed this problem from a comparative and biological point of view (not directly for the cow) and stated: “The inappetance around parturition is probably a throwback to the wild state when mothers would need to remain at the nest for a period of time and at this time would be unable to feed”. The wild cow (*Bos taurus*) would go into the underwood during parturition with a reduced appetite and would not forage because it was primarily taking care of the new born calf. Knight (2001) has made some suggestions in the same (biological) direction and pointed to some advantages of a reduced DMI “Energetically, mobilization confers approximately 80% efficiency, whilst digestion is only 60% efficient”, and “there is less need for physical activity (foraging).” “Perhaps, the real conundrum is why so much effort is exerted by agricultural nutritionists and dairy farmers in trying to persuade the early-lactation cow to eat more. She knows better!” Indeed, this is probably the case. Milk production (homeorhesis) has priority over DMI, and milk secretion for the nutrition of the calf is (at least) partially independent of it. This could be important under conditions in the wild. A harsh climate in spring during calving and the poor nutritional circumstances consequently do not challenge milk production and the nutrition of the calf. The reduced time for seeking or eating grass increases the period for the cow to attend the calf and to improve its survival rate.

The biological suggestions of Gravert (1985), Vernon and Pond (1997), and Knight (2001) are strongly supported by genetic studies. A negative genetic correlation exists p.p. between milk yield and dry matter intake (Karacaören et al., 2006; Liinamo et al., 2012; Manzanilla-Pech et al., 2014). It confirms the early observation of Veerkamp and Thompson (1999) that feed intake is negatively correlated with milk yield and with blood oxytocin concentration (Bar-Peled et al., 1998). Recently, a positive genetic correlation between MY and DMI has been described by Krattenmacher et al. (2019). The correlation is low at the onset of lactation (= 0.09 at d 11 in milk) and consequently, a strong negative genetic correlation has been found between MY and energy balance. The insufficient DMI around parturition is primarily of biological (genetic) origin: “She (the cow, the author) knows better” (Knight, 2001). This conclusion is supported by the studies of Grummer et al. (2004). Parity, body condition score, and various diet components only explain 18% of the variation of DMI at 3 weeks before parturition (Grummer et al., 2004). This does not exclude other management factors but clearly indicates the physiological (genetic) background that primarily determines the dip in DMI around parturition. A consequence is NEB and loss of body weight. “These patterns could not be accounted for by environmental factors such as

constrained intake or condition score at calving" (Friggens et al., 2007) and strengthen the influence of the genetic and biological background.

2.4 Changes of Dry Matter Intake

Selection for high MY has not involved selection for higher DMI according to requirement p.p., because recording DMI for individual cows is impractical (Banos & Coffey, 2010) and hence, the equivalent parameters are not known. As a consequence, milk production in the USA has been increased by some 57% over 23 years (1980-2003), but DMI unproportionally only by some 20% (Eastridge, 2006) and "this results in a more or less extended negative energy balance and increased mobilization of body reserves". DMI intake during lactation was recently determined in an international co-operation between Austria, Switzerland, and Germany. The regression coefficient between MY and DMI was only by 0.1 kg DM per kg milk during early lactation (Gruber et al., 2006) and agrees with the low correlation observed by Krattenmacher et al. (2019). The low coefficient of DMI per kg milk clearly indicates that this additional DMI does not cover the MY-dependent increase in requirement and means that the NEB increases with higher milk production after parturition, despite the positive correlation with (low) DMI per kg milk. It is worth mentioning that, in a recent review about DMI, the model of Gruber et al. (2006) predicted the DMI (total mixed ration) most accurately (Jensen et al., 2015). Interestingly, the low coefficient between milk production and DMI (0.1) was noted decades ago by Bines (1976), and the same magnitude of 0.12-0.14 was observed by Veerkamp and Koenen (1999). Furthermore, these authors summarized data from three studies with high- and low-producing cows. Milk production increased in high-producing cows without an equivalent increase of DMI. Hence, during early lactation, MY is relatively uncoupled from DMI, as was mentioned as early as 1985 (Brandt et al., 1985). The current breeding index with its high priority for MY is thus outpacing adequate nutrition and exacerbating NEB. As a consequence, a negative genetic correlation is observed between MY and energy balance (Spurlock et al., 2012), particularly during early lactation (Krattenmacher et al., 2019).

In order to compensate for low DMI and to alleviate the NEB and mobilization, diet density has been increased mainly by the addition of starch, and ≈ 7.0 MJ_{NEL}/kg DM or even more is now typical for high-producing dairy cattle. However, "attempts to abolish lipid mobilization in early lactation by feeding energy rich diets are in general not successful" (Friggens & Newbold, 2007), as have been observed by Andersen et al. (2003): "Milking multiparous cows three times a day compared with two times daily for the first 8 weeks of lactation, whether fed a low or a high concentrate level diet, increased milk yield by 8% without a corresponding increase in feed intake. As expected, cows milked three times a day had the biggest weight loss, which was most pronounced in cows fed the low concentrate level diet". However, a glucogenic vs. a lipogenic diet with almost an identical MJ_{NEL}/kg DM of the diet improves the energy balance during early lactation and reduces the fat liver content (van Knegsel et al., 2007a).

Starch stimulates fermentation and the production of propionate, which unfortunately has hypophagic effects caused by the hepatic oxidative theory leading to decreased DMI (Allen & Bradford, 2012; Gualdrón-Duarte & Allen, 2017), *i.e.*, the exact opposite to the required higher DMI. A further metabolic change probably contributes to low DMI. A decrease of DMI occurs before parturition, and concentrations of NEFA increase a.p. with a peak p.p. (Sheehy et al., 2017). Flow of NEFA to the liver during high lipolysis also has hypophagic effects (Scharrer, 1999; Allen & Piantoni, 2013), as does beta-hydroxybutyrate (BHB) (Scharrer, 1999), thereby confirming the negative correlation between BHB and DMI (Lean et al., 1992). In agreement with these anorexic findings for BHB, the BHB concentration increases in the cerebrospinal fluid of cows p.p., although the underlying anorexic mechanism is not clear (Laeger et al., 2013).

Energy density has an additional side impact. Increasing concentrate competitively decreases roughage intake (Faverdin et al., 1991). The compensation of the genetically dependent dip of DMI by energy density seems to lead to hypophagic effects and to inadequate roughage intake with the risk of displaced abomasum (Cameron et al., 1998).

The insufficient DMI p.p. also includes the under-nutrition with protein (Bell et al., 2000). In practice, the protein deficit is compensated by the increased crude protein (CP) content of the diet p.p. It enhances milk production and, in some cases, DMI (Oldham, 1984) with a possible increase of NEB (Ørskov et al., 1977; Oldham, 1984; Larsen et al., 2014). Potential side effects are discussed below.

2.5 Partitioning and Mobilization

A key issue of the homeorhetic regulation of early lactation is the partitioning of nutrients. The high demand of nutrients for milk production at insufficient DMI requires careful allocation, which is (amongst others) predominantly regulated by changes of GH, insulin, and IGF-1 concentrations and insulin resistance (IR) in

selected tissues such as striated muscle and fat tissue (for details, see Bauman & Currie, 1980; Bell et al., 1987, Vernon, 1989; Vernon & Pond, 1997; Chilliard et al., 1998; Vernon, 1998; Bauman, 1999; Etherton & Bauman, 1998; Bauman, 2000; McNamara, 2015; Collier & Bauman, 2017; Baumgard et al., 2017). As a consequence of these hormonal adjustments, flux rates of important nutrients are changed with the priority being milk production. Briefly, the following alterations are observed.

Glucose: Gluconeogenesis is stimulated by GH (Bauman et al., 1988) and increases four-fold in superior lactating cows p.p. (Bell & Bauman, 1997). The uptake of glucose by non-mammary tissue is reduced, and the oxidation of glucose is diminished, both of which direct the spared glucose to milk production (Bauman, 2000). These modifications in glucose metabolism are caused by altered hormonal concentrations and responses to homeostatic signals. Growth hormone (GH) is increased p.p. causing insulin resistance in muscle and fat tissue (Vernon, 1989; Bell et al., 2000). IR is pronounced in adipose tissue in cows with high weight loss, but IR does not occur in the liver (Zachut et al., 2013). A growing body of evidence suggests that inflammation is involved in p.p. IR, and inflammation-dependent IR is thought to be an adaptive response of homeorhetic adaptation in early lactation (Farney et al., 2013; Montgomery et al., 2019).

IR is accompanied by low insulin concentrations (Vernon, 1989; Bell et al., 2000). Hart et al. (1978) observed an inverse relationship between insulin and MY; this has been confirmed by a strongly negative correlation between insulin and MY (Wathes et al., 2007). Recently, Zinicola and Bicalho (2019) have found that, in dairy cows during early p.p., low insulin concentrations are related to higher milk production. Furthermore, low insulin is associated with a high loss of BCS and higher NEFA concentrations.

NEFA: A further step in partitioning is the allocation of mobilized fat. The energetic deficit is compensated by the mobilization of NEFA (+ glycerol) from adipose tissue. Plasma NEFA concentrations are used for oxidation and particularly for milk synthesis and are highly correlated with their irreversible loss rate (Bauman et al., 1988).

Lipolysis is stimulated by GH and by an increased response and sensitivity to catecholamines via the increased number of β-receptors (Vernon & Pond, 1997; McNamara, 2015; Contreras et al., 2017). An aspect that is very often overlooked is that insulin is integrated into the liporegulatory feedback mechanisms by NEFA and ketone bodies (Herdt, 2000). Fatty acids containing 3-8 carbon molecules, namely propionate or butyrate, stimulated insulin secretion in ruminants (Horino et al., 1968), and lipolysis with an increase of NEFA in mice triggers the release of insulin (Heine et al., 2018). Insulin stimulates lipid synthesis and inhibits lipolysis (Herdt, 2000), thereby representing a negative feedback on lipolysis. However, this possible effect is reduced because the high demand of the mammary gland for glucose at reduced glucose concentrations decreases the insulin concentration, which is associated with IR p.p. (Vernon & Pond, 1997) (for details of IR: see De Koster & Opsomer, 2013). Further, the effect of insulin on lipid synthesis is inhibited in the presence of GH (Vernon, 1998). Hence, the mobilization of NEFA is a combined effect of the stimulation of lipolysis by GH and the inhibition of lipogenesis by low insulin concentration and IR and represents a transformation from an anabolic status a.p. to a catabolic one p.p. Mobilization occurs above the acute requirement with an increase of NEFA concentrations in the blood and ectopic fat deposition in the liver, muscle, and other organs (Reid & Roberts, 1982).

"The observed mobilization in early lactation is largely genetically driven" (Friggens & Newbold, 2007), and high genetic merit dairy cows exhibit an increased sensitivity of adipose tissue for lipolysis (McNamara & Hillers, 1986). A genetic background for mobilization is suggested by the data of Bertics et al. (1992). An increase of NEFA a.p. and +1 d relative to calving has also been observed to the same extent in both control and experimental cows, although the experimental cows were maintained a.p. at the same level at DMI by force-feeding the refusals via the rumen fistula or without the dip of DMI before parturition (Bertics et al., 1992).

Amino acids: Proteolysis occurs in cows p.p. for the limited time of 23 days (Bell et al., 2000) or sometimes 5 weeks and at an amount of 4.6 and 21 kg p.p. (Tamminga et al., 1997; Komaragiri & Erdman, 1997). The amino acids are used for gluconeogenesis and milk protein synthesis (Bell et al., 2000). Some evidence suggests that the period of negative protein balance is extended. Metabolizable protein balance is still negative 6 weeks p.p. (Mann et al., 2016). Proteolysis via proteasome activity occurs before calving and is upregulated p.p. (Mann et al., 2016). Furthermore, an increase of macro-autophagy as a further mechanism of proteolysis has been observed p.p. The authors integrate these proteolytic pathways into the homeorhetic adaptation of NEB (Mann et al., 2016) and suggest that this explains the longer proteolytic period p.p. NEB and proteolysis might be connected. NEFA at parturition is positively correlated with the concentration of 3-methylhistidine, an amino acid produced by protein degradation (Akamatsu et al., 2007). Furthermore, BHB stimulates chaperone-mediated

autophagy (proteolysis) in a cell culture system (Finn & Dice, 2005). The increase of BHB is not restricted to the early phase p.p. Mahrt et al. (2015) have concluded that the risk of hyperketonemia last at least until week 6 p.p.

Perhaps low IGF-1 p.p. indirectly aids proteolysis because the stimulating effect of IGF-1 on protein synthesis and the inhibition of degradation are reduced (Etherton, 1982). In fact, IGF-1 is a potent supporter of muscle growth (Schiaffino et al., 2013) and correlates with striated muscle growth (Velloso, 2008).

Immunoglobulin IgG: An important but very often overlooked aspect of partitioning is the transfer of immunoglobulins to colostrum. Van Knegsel et al. (2007b) determined natural antibodies in blood and colostrum and hypothesized "that the partitioning of natural antibodies between plasma and milk parallels the partitioning of energy". Herr et al. (2011) have corroborated this transfer. The IgG decline in blood a.p. is positively correlated with the IgG concentration in colostrum and supports the tendency for an inverse relationship between natural antibodies in plasma and milk (van Knegsel et al., 2007b). The transfer of IgG is independent of high or low concentrate supplementation before parturition (Eger et al., 2017) and confirms the priority of homeorhetic milk production with an IgG transfer uncoupled from energy intake. The decrease of IgG before parturition and the slow recovery p.p. (Herr et al., 2011) might significantly contribute to the immunosuppression of dairy cows during transition (Aleri et al., 2016). A decrease of IgG around parturition has not been observed in horses (Warko & Bostedt, 1993).

Infection and partitioning: Evidence indicates that "immunostimulation" (infection, the author) homeorhetically alters systemic metabolism in a coordinated effort to meet the energetic demands of leukocytes (Horst et al., 2018). Glucose is obligatory for the energy metabolism of immune cells, and a shift to its metabolism occurs with a requirement of approximately 1 g glucose/kg $BW^{0.75}$ per hour after an LPS challenge (Horst et al., 2018) or of > 1 kg glucose within 720 min (Kvidera et al., 2017), an effect that will impair other functions.

2.6 Changes of Partitioning and Mobilization

An increase of GH and a decrease of insulin and IGF-1 concentrations are the predominant hormonal alterations occurring at this phase, and insulin resistance (IR) represents a change of a set point p.p. as a general characteristic of homeorhesis (Vernon, 1998; Etherton & Bauman, 1998; Bauman, 2000; Bell et al., 2000; Baumgard et al., 2017). Partitioning and mobilization are directly or indirectly influenced by this adjustment. GH, insulin, IGF-1, and IR display a complex network of interactions and antagonisms, all of which are obviously related to milk production.

GH-IGF-1 axis: GH is released from the pituitary and has various effects in multiple tissues (Bartke et al., 2013). The actions of GH are mediated via growth hormone receptors (GHR), which are found in many tissues and have the highest abundance in the liver and adipose tissue (see references in Lucy et al., 2001). Transcription is mediated by three promoters in cattle resulting in GHR 1A, GHR 1B, and GHR 1C (Jiang et al., 1999). GHR 1A expression is specific for the liver and is generally regulated by development and nutrition (Lucy, 2004).

The binding of GH to GHR activates a complex signal cascade (Bartke et al., 2013), which leads, in the liver, via GHR 1A to the stimulation of IGF-1 synthesis and its release into the blood (Kobayashi et al., 1999). IGF-1 inhibits, as a feedback mechanism, the release of GH from the pituitary (Bartke et al., 2013). This GH-IGF-1 axis via GHR is inhibited by suppressors of cytokine signaling (SOCS) (Bartke et al., 2013). Furthermore, the expression of GHR 1A in the liver is increased by insulin (Butler et al., 2003; Lucy, 2004; Rhoads et al., 2004), possibly explaining the highly positive correlation between insulin and IGF-1 (Wathes et al., 2007; Fenwick et al., 2008; Cheng et al., 2015). Indeed, hyperinsulinemic-euglycemic clamp in dairy cows increases IGF-1 and reduces GH by a re-established feedback (IGF-1) and NEFA and BHB concentrations (Mashek et al., 2001).

This fine-tuning system of regulation obviously changes around parturition. During early lactation, GH in blood is augmented (Gross & Bruckmaier, 2015), the insulin concentration is decreased (Vernon, 1989), glucose-dependent insulin release from the pancreatic islands is blunted (= IR) (Rhoads et al., 2004), and mRNA of SOCS is increased (Winkelman et al., 2008). SOCS are negative regulators of growth factor signaling (Linossi & Nicholson, 2015), belong to insulin receptor inhibitory proteins (Haeusler et al., 2018), and might exacerbate the effects of a low insulin concentration or IR on the expression of GHR 1A. These alterations are related to lower GHR 1A expression, absent effects of GH on the liver with reduced IGF-1 release, and a diminished feedback of IGF-1 on GH discharge from the pituitary and hence to the uncoupling of the GH-IGF-1 axis with increased GH and low IGF-1 concentrations (Lucy et al., 2001; Winkelman et al., 2008). Consistent with this conclusion is the reciprocally increased mRNA expression of SOC2 and decreased mRNA expression of IGF-1 in dairy cows p.p. (Osorio et al., 2014).

Uncoupling of the GH-IGF-1 axis: An uncoupling of the GH-IGF-1 axis in dairy cows was first suggested by Ronge and Blum (1989), because GH injection did not increase IGF-1 during early lactation. Uncoupling occurs before calving (Lucy, 2004), and some correlations have been described. The decrease in GH binding and GHR 1A mRNA coincides with a decrease of IGF-1 mRNA (Radcliff et al., 2003). Furthermore, a lower IGF-1 and higher GH in blood have been observed (Radcliff et al., 2003) suggesting a reduced feedback on GH release. The control of GHR 1A expression might be the principal mechanism of uncoupling (Radcliff et al., 2003) and partially depends on DMI (Radcliff et al., 2006), NEB (Fenwick et al., 2008), and probably on the NEFA and BHB concentration (Du et al., 2018). The authors have demonstrated *in vitro* that the addition of NEFA or BHB inhibits GHR 1A mRNA and markedly reduces IGF-1 mRNA expression in calf hepatocytes. This confirms the earlier observation of the strong negative correlation between mRNA GHR 1A expression and blood NEFA and BHB concentrations (Fenwick et al., 2008).

The degree of the uncoupling of the GH-IGF-1 axis seems to vary with selection for MY, because it has been observed to the same extent in two dairy breeds (Holstein and Guernsey) (Okamura et al., 2009), but not in Angus beef cattle (Jiang et al., 2005). Differences have been confirmed in a study with diverse genetic strains (HF USA versus HF NZ) and indicate a genetic effect of the degree of uncoupling of the GH-IGF-1 axis with MY (Lucy et al., 2009). Hence, "uncoupled" and high-producing cows are characterized by high GH and IR and by low insulin and IGF-1 concentrations p.p. Furthermore, low concentrations of IGF-1 in "uncoupled" Holstein cows appear to be associated with less sensitivity to IGF-1 (Mendonca et al., 2013).

The metabolic consequences of this hormonal framework have been corroborated by the studies of Gross and Bruckmaier (2015). Holstein cows were retrospectively ranked according to their highest NEFA concentrations. These cows exhibited during early lactation, in addition to high NEFA, higher BHB, lower glucose, higher GH, lower (numerical) insulin, lower IGF-1 concentrations, higher milk yield at less DMI, and consequently lower NEB. The authors relate these changes to the uncoupled GH-IGF-1 axis with the obvious consequences for mobilization and partitioning for higher MY. The changes of metabolites p.p. (NEFA > 1.2 $mmol \cdot l^{-1}$ and BHB > 1.5 $mmol \cdot l^{-1}$) predispose animals to ketosis, and an uncoupled GH-IGF-1 axishas been observed in ketotic cows (Du et al., 2018) (see part II). Furthermore, the low IGF-1 concentration might promote and extend proteolysis (see above). Vice versa, the removal of metabolic load by once-a-day milking p.p. leads to higher glucose, insulin, and IGF-1 and lower NEFA and BHB concentrations in plasma and abolishes BCS loss (Kay et al., 2013).

Fibroblast growth factor 21 (FGF21) and GH-IGF-1 axis: FGF21 is a peptide hormone with systemic, paracrine, and autocrine effects and is induced in the liver of mice by starvation, a ketogenic diet, and protein deprivation (Fisher & Maratos-Flier, 2016; Laeger et al., 2014). FGF21 is involved in the regulation of fatty acid oxidation (Fisher & Maratos-Flier, 2016), ketogenesis, and gluconeogenesis for adaptation during starvation (De Sousa-Coelho et al., 2012), although the effect of FG21 on glucose handling is contradictory (man versus mice) (Staiger et al., 2017). Remarkably, FGF21 reduces glucose and insulin and IR in obese mice (Xu et al., 2009) and diabetic monkeys (Kharitonenkov et al., 2007), but in dairy cows, the application of FGF21 does not change blood glucose or insulin and does not act as an insulin sensitizer (Krumm et al., 2019).

However, interactions between lipid metabolism and FGF21 are also known in cattle. In the liver of cows, mRNA for FGF21 is negatively correlated with energy balance and blood glucose concentrations and is positively correlated with NEFA concentrations (Carriquiry et al., 2009). This correlation has been confirmed by Schoenberg et al. (2011). Plasma FGF21 concentration has been correlated with energy deficit, hepatic triglyceride, and NEFA. Furthermore, intralipid infusion in non-lactating non-pregnant cows increases NEFA, thereby causing a rapid increase of mRNA for FGF21 in the liver and of FGF21 concentration in blood (Caixeta et al., 2017). NEFA is obviously a potent secretagogue of FGF21 in the cow (Caixeta et al., 2017). In agreement with these data is the raised mRNA for FGF21 in dairy cows p.p. (Schlegel et al., 2013; Ha et al., 2017) and of FGF21 concentrations in plasma p.p. and during feed restriction in late-lactating cows (Schoenberg et al., 2011). Vice versa, FGF21 bolus application lowered NEFA, but this effect vanished during FGF21 infusion over 9 d (Caixeta et al., 2019). Despite this transient effect, triglyceride content in the liver was reduced by 50% (Caixeta et al., 2019).

During starvation, FGF21 has, in non-ruminants, inhibitory effects on GH in the liver and reduces the IGF-1 concentration (Inagaki et al., 2008) hinting that it has a role in GH-IGF-1 uncoupling. Dairy cows fed a.p. a high-energy diet exhibit p.p. an increased expression of FGF21 mRNA and a decrease of IGF-1 and GHR mRNA in the liver (Khan et al., 2014). "More severe NEB and NEFA results in greater hepatic FGF21", and the increase of FGF21 has been discussed as a possible reason of GH-IGF-1 uncoupling by Khan et al. (2014) and previously by Carriquiry et al. (2009). This conclusion has been confirmed by Caixeta et al. (in press). Infusion of FGF21 in

early-lactating dairy cows reveals an antagonism of GH action. “FGF21 treated cows had lower IGF-1 despite a tendency of increased plasma GH” (Caixeta et al., 2019).

Obviously, NEFA have several effects in cows. NEFA stimulates the release of FGF21 from the liver (Caixeta et al., 2017), inhibits the expression of GHR-1A in liver cells, and mediates the uncoupling of the GH-IGF-1 axis (Du et al., 2017) (see part 2). The negative association between FGF21 and IGF-1 in a cross-sectional study of man supports this conclusion (Kralisch et al., 2013) and has been confirmed *in vitro*. FGF21 significantly inhibits the release of IGF-1 in a cell culture system with HepG2 cells (human liver cancer cell line) (Kralisch et al., 2013). The possible involvement of FGF21 in the regulation of lipid metabolism such as the lowering of NEFA and triglyceride in the liver of dairy cows (Caixeta et al., 2019) or the inhibition of lipolysis in rodents (Hotta et al., 2009) and the possible effect on the uncoupling of the signal cascade of the GH-GHR-IGF-1 axis warrants further research. Does FGF21 serves as a negative feedback on lipolysis?

2.7 Protein Intake and Partitioning

Some evidence suggests that protein influences partitioning. A protein intake increase p.p. to cover the protein requirement favors the partitioning of available nutrients toward mammary secretion during early lactation (Oldham, 1984). The partitioning effects of CP have been studied in detail by the group of Ørskov. The infusion of casein early p.p. into the abomasum increases milk production (Ørskov et al., 1977; Larsen et al., 2014) and causes, at high casein infusion, pronounced NEB and ketosis (Ørskov et al., 1987).

The response of energy balance depends on CP intake and is not observed at high metabolizable energy (ME) intake (Ørskov et al., 1981). Extending these findings, Whitelaw et al. (1986) state that “increases in MY and milk protein in response to casein will be seen only when sufficient energy is also available either from labile body stores or from dietary sources”. The shift of energy from body stores into milk is obviously high in fat cows fed with high proportions of protein and a low dietary energy concentration (Jones & Garnsworthy, 1988). However, no side effects of high CP on energy metabolism in dairy cows have been observed by Komaragiri and Erdman (1997) and Amanlou et al. (2017). Komaragiri and Erdman (1997) discuss various explanations such as the presence of enough amino acids for lipid utilization at the low crude protein intake (16%) or the physiological limits of mobilization in high-producing cows. Obviously, the regulation of the interaction between CP intake and energy metabolism is not clear. In the short-term experiment of Oldham et al. (1978), abomasal casein infusion into Saanen goats raised the GH concentration with no significant effect on insulin or prolactin. By contrast, high casein infusion in cows leads to an increase of insulin and decrease of GH (Whitelaw et al., 1986).

3. Synopsis of Homeorhesis: Physiological Background and Changes

The publication of Bauman and Currie (1980) was the breakthrough for understanding the regulation of milk production and metabolism in the dairy cow p.p. The general characteristics of homeorhesis result in advantages for both the calf and its mother. The complex genetic interactions between the priority of MY, low DMI, mobilization, and partitioning secure the nutrition and protection of the calf with a marginal challenge to the metabolism or the health of the cow: An optimal biological network at low MY is shown in Figure 1.

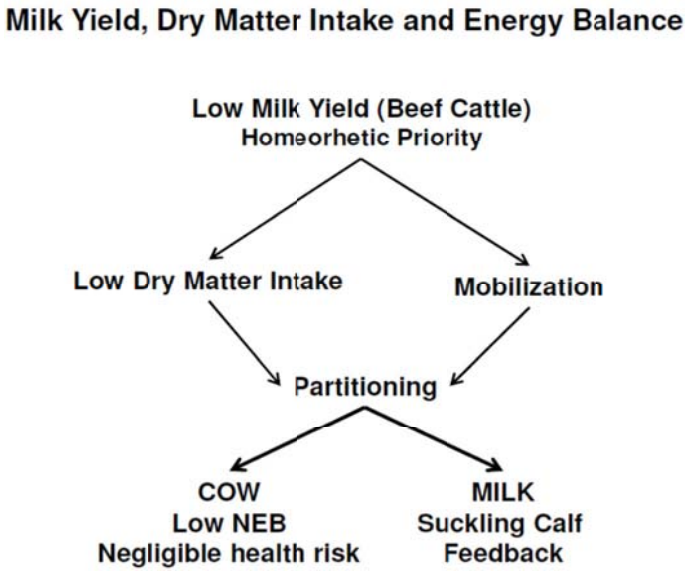

Figure 1. Scheme of network for homeorhetic regulation of milk secretion, which is genetically correlated with low DMI and with the capability of mobilization for compensating any possible gap of nutrients. At low MY (beef cattle), the interdependency between MY, DMI, mobilization, and partitioning to both milk and cow guarantees nutrition and assistance of the calf and improves its survival without a health risk for the cow. The possible NEB is of no importance

However, selection for higher MY, frequent milking, diets with high energy density, and high CP intake have increased milk production, and some of the initial optimal homeorhetic characteristics have been turned into unfavorable attributes. A major problem during early lactation is the discrepancy between the rapid increase of MY and the delay of the increase in DMI.

The insufficient DMI during early lactation (Karacaören et al., 2006; Liinamo et al., 2012; Manzilla-Pech et al., 2014; Krattenmacher et al., 2019) causes a negative genetic correlation between MY and energy balance (Spurlock et al., 2012; Brade, 2013; Krattenmacher et al., 2019) and results in the mobilization of reserves. Unsurprisingly, "it can be concluded that continued selection for high milk production will lead to a further increase in the p.p. energy deficit" (Buttchereit et al., 2011). The discrepancy between input and output was realized decades ago by Arendonk et al. (1991), and the authors proposed "an additional trait in the selection goal to avoid an increase in negative energy balance during early lactation". This confirmed the earlier observation of McNamara and Hillers (1986) that lipolysis is more pronounced in cows of high genetic merit and is independent of energy restriction p.p.; this clearly underlines the homeorhetic regulation of energy metabolism p.p. Milk production has priority over DMI with genetically driven mobilization (Friggens & Newbold, 2007). Consequently, a "consistent correlation between (milk) yield and live-weight changes" is found (Veerkamp & Koenen, 1999) and MY in Holstein cows "relies to a greater extent on mobilization of body reserves" (Gruber et al., 2014).

Furthermore, the partitioning of dietary energy contributes to under-nutrition. "High genetic merit animals put the extra energy from the concentrate-based diets into milk, rather than reducing the energy gap" (Agnew & Yan, 2000; Veerkamp et al., 2003; Patton et al., 2006), an observation that agrees with that of Friggens and Newbold (2007) in Holstein Friesian cows, namely that "it is difficult to argue, given that these cows were on the same feed throughout their productive life, that intake (14 d p.p., the author) was constrained, when there was substantial energy mobilization".

Hence, under these conditions, milk production can be regarded as "a metabolic burden imposed by the synthesis and secretion of milk" (Knight et al., 1999). Taken together, the modern high-producing dairy cow p.p. is mostly characterized by,

- increasing milk production with partial or total uncoupling of the GH-IGF-1 axis and less sensitivity to IGF-1,
- inadequate and enduring low DMI in relation to an increased requirement for milk production and maintenance,

- mobilization of NEFA above requirement with ectopic deposition of fat,
- mobilization of amino acids from protein,
- preferential partitioning of metabolites, IgG, and dietary nutrient to the mammary gland,
- a sudden and long-lasting NEB,
- at high CP intake, partitioning of dietary or mobilized energy to the mammary gland with potential increase of NEB,
- less weight gain or possibly net loss of energy during the entire lactation period.

These unfavorable (to a certain extent) characteristics of high milk production during early lactation are furthermore regulated by homeorhesis with its high priority and lack of feedback control, irrespective of the impairment of other functions. A potential constraint of health by homeorhetic priority of milk production can easily be learnt from the pathogenesis of milk fever. Ca is secreted into milk even with a risk of periparturient paresis and further constraints of health. Higher milk production is associated with reduced blood Ca concentration at d 1 in milk (Neves et al., 2018). Hence, the promotion of MY by breeding and management has revealed possible restrictions of health (Figure 2).

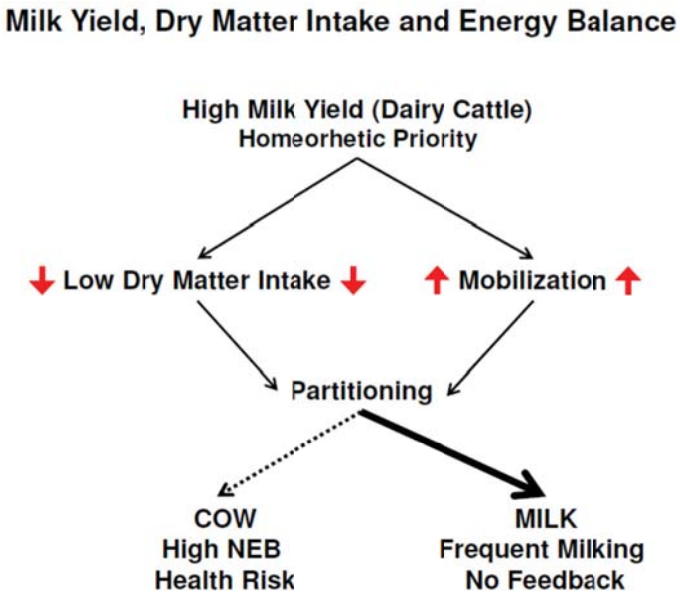

Figure 2. The well-balanced network of milk yield, DMI, mobilization, and partitioning of nutrients at low MY (Figure 1) has been disturbed by selection and management for high milk production. Milk yield is negatively genetically correlated with DMI and positively correlated with high mobilization and with the partitioning of metabolites and nutrients primarily to the mammary gland. The major consequence is an exacerbating NEB, which is often observed before parturition. The rapid and long-lasting NEB in combination with the high metabolic rates and changes of hormones and metabolites can cause maladaptation and is a major health risk. The arrows indicate amplification (up) or mitigation (relative decrease) (down)

One major and general shortcoming for health is the discrepancy between input and output and the resulting NEB, which simply represents under-nutrition, but concomitantly includes alterations of hormones and metabolites and a sustained increase of metabolic rate.

4. The Negative Energy Balance: NEB

4.1 Past and Present

The difference between the requirement for MY and DMI in dairy cows p.p. was mentioned many decades ago (Broster, 1972), with Bines (1976) stating: “Food intake, especially in early lactation, is a major factor limiting production from dairy cattle.” Increase of MY at insufficient DMI is the major reason for NEB, although a growing body of evidence suggests that the energy requirement for maintenance has been increased in

high-producing cows (Moares et al., 2015; Erdmann et al., 2019) and is related to the change of body composition with reduced fat and the enlarged size of metabolically active organs (Agnew & Yan, 2000). Because Hart et al. (1975) have observed no loss of body weight (BW) p.p. in beef cattle with low MY (suckling calves), the NEB is related to MY and the higher maintenance requirement of high-producing cows.

In the 1980s, the total NEB accounted for some hundreds of MJ_{NEL} and was apparent over a few weeks (Gravert et al., 1986; Berglund & Danell, 1987). Since that time, the extent and duration of NEB has been increased. Tamminga et al. (1997) observed a NEB of 1284 MJ_{NEL} for 8 weeks p.p., and Sutter and Beever (2000) determined a deficit of 1786 MJ for 8 weeks. This increase of NEB has continued: 2676 MJ (mean) for 83 days with high variations and a much higher (maximal) NEB (Banos & Coffey, 2009). The degree of NEB obviously and almost linearly depends on MY: 580 MJ_{NEL} at MY < 25 kg/d, 1323 MJ_{NEL} at MY 25-30 kg/d, and 1956 MJ_{NEL} at MY > 30 kg/d within 11 weeks p.p. (Brandt et al., 1985). The duration of NEB linearly increases with milk production (Steinwidder & Gruber, 2002) and can last for > 100 days (Bulang et al., 2006).

The NEB causes mobilization, which amounts to 41.6 kg empty body weight (BW) (Tamminga et al., 1997) or some 41 kg BW (Sutter & Beever, 2000). However, higher rates of loss of BW (114 kg in 4 weeks) have been determined by Van den Top et al. (2005) in over-conditioned cows. Most of the BW loss occurs during the first few weeks p.p. and exhibit a wide variation from calving to the nadir of BW: 0-185.1 kg (parity 1), 0-198.0 kg (parity 2), and 0-269.2 kg (parity ≥ 3) (van Straten et al., 2008).

Live weight changes increase with MY with rising lactations, and the NEB is more pronounced during the 2nd and 3rd lactation in high genetic merit cows (Coffey et al., 2004). Further, the milk production peak is reached earlier in the 3rd lactation without equivalent DMI (Coffey et al., 2004), and for this reason, the decline of BCS p.p. increases with increasing parity from 0.3 (1st lactation) to 0.9 (≥ 4 lactation; 5-point scale) (Waltner et al., 1993).

The change of BW is related to corresponding changes of body condition score (BCS). A ratio of 80-84.6 kg BW/unit BCS (5-point scale) has been discussed in the recommendations for nutrient requirements (National Research Council [NRC], 2001); a decrease of 1-unit in BCS for a cow with a BW of 650 kg and BCS of 4 (5 point scale) would provide 1743 MJ_{NEL} or 2126 MJ tissue energy (0.82 conversion from tissue energy to MJ NEL) (NRC, 2001). This magnitude of MJ of 1 ΔBCS is within the range of NEB in the study of Sutter and Beever (2000) but below 2676 MJ (Banos and Coffey, 2009). At parturition, a BCS of 3.0 (5-point scale) is recommended, and cows should not lose more than 0.5 (Roche, 2006) or 0.5 to 1.0 units p.p. (Roche et al., 2009). A decline of 0.5-1.0 BCS means a loss of body weight of some 40-80 kg (NRC, 2001). Hence, the current NEB and loss of BCS probably exceeds the recommended magnitude of 0.5-1.0 BCS.

A further aspect of NEB should be discussed. It is an underlying supposition that the NEB in early lactation is compensated by body weight gain during the later lactation and dry period. “In a gross sense, intake across the whole of a lactation correlates reasonably well with output. It would be a surprise if this situation was not so” (Knight, 2001). However, Veerkamp and Koenen (1999) have stated that “selection for high milk yield results in less live-weight gain”, and Coffey et al. (2004) have observed, in high genetic merit cows during 3 lactations on a low concentrate diet, a decrease of BCS of 0.54 or a loss of 1148 MJ (calculated according NRC, 2001). The calculations of Schröder and Staufenbiel (2006) indicate a loss of 1075 MJ (ΔBCS 0.54 = 5.4 mm back fat thickness or 27 kg body fat [1 kg body fat = 39.8 MJ]).

A net loss of energy is not restricted to high-producing cows. Hurley et al. (2018) have observed, in cows in an extensive production system in Ireland (mainly grass), a strong phenotypic correlation between residual energy intake (REI) and energy balance. Hence, “animals genetically selected to have a lower REI (*i.e.*, more efficient; the author) resulted in cows who consumed less net energy intake but were also in negative energy balance throughout the entire lactation”. A continuous loss of energy and long-lasting NEB without compensation appears to be an additional health risk.

4.2 NEB in Biology and Dairy Cows

Periods of under-nutrition and even severe NEB occur in mammals and short or longer phases of food deprivation (*adaptive fasting)* are known and are related to hibernation, mating, molting, migration, or care of young (Secor & Carey, 2016; Martinez & Ortiz, 2017). *Adaptive fasting* is “an inherent survival tactic and adaptation and is distinct from starvation” (Martinez & Ortiz, 2017) and “an innate component of many organisms” (Secor & Carey, 2016). An example is the elephant seal, which loses some 42% of body weight after parturition during 4 weeks without feed intake (Costa et al., 1986).

This loss is much more than that seen in dairy cows during the same time period (Tamminga et al., 1997; Sutter & Beever, 2000), suggests a possible adaptation in dairy cows too and, at first glance, the NEB in dairy cows resembles a "physiological (biological) reaction or adaptive fasting". However, a comparison of the current NEB of the dairy cow with the biological NEB of other species appears to be questionable. First, adaptive fasting is the result of long adaptation over thousands of years of evolution. This was obviously the best strategy of survival for the relevant species. Such an adaptation appears to be unlikely in dairy cows. The current duration and extent of NEB is a result of selection for high milk production during the last 60-80 years. Secondly, the elephant seal produces milk for only one pup. The amount of milk for one calf would hardly cause a significant NEB in cows (Hart et al., 1975). Finally, and very importantly, the milk of the elephant seal does not contain sugars (Oftedal, 1993), which are mainly produced by gluconeogenesis in the cow and are the bottle neck of metabolism for milk production and for health during early lactation (Bell, 1995). Therefore, although an allusion of NEB in biology (seal, whale) appears to be attractive (Bauman, 2000), it is probably not very helpful. Dairy cows probably do not exhibit biologically adaptive fasting, but rather the current extent and duration of NEB is an involuntary shortage, "exhibits characteristics of chronic under-nutrition" (Vernon, 1998), and cannot be classified as "natural". The present prolonged and very deep magnitude of NEB with its consequences for hormonal and metabolic changes and high metabolic rates displays symptoms of pathophysiology or insufficient adaptation.

4.3 NEB and Adaptation of Metabolism

The rapid increase of MY and of metabolism p.p. requires a comprehensive homeorhetic adaptation of various organ systems such as those for digestion and liver metabolism or in the mammary gland (Bauman, 2000). Adaptation of a cell, organ, or whole organism is the reaction to an internal or external stimulus (Broom, 2006). For instance, the immediate multiplication of metabolism in the dairy cow p.p. is accompanied by heat production and the possible increase of body temperature. Mechanisms are activated to dilate subcutaneous blood vessels for dissipating this heat. Body temperature is normalized, and feedback mechanisms are triggered to complete the previously activated mechanisms of heat dissipation. The organism succeeds in coping with the stimulus, and ideally, other functions are not impaired: "Coping means having control of mental and bodily stability" (Fraser & Broom, 1990) and involves "the physiological capability to respond properly and thus maintain homeostasis" (Siegel, 1995).

The current exposure of metabolism during early lactation is a challenge for the coping and physiological capabilities and adaptation of the dairy cow. In effect, adaptation is overloaded causing metabolic disorders (see comprehensive review by Sundrum, 2015) and is a "hampered process" with impaired reproductive performance (Jorritsma et al., 2003). Insufficient adaptation coincides with the priorities of homeorhetic "milk secretion allowing them to proceed at the expense of other metabolic processes even to a point that a disease is created" (Bauman & Currie, 1980) or "even a pathological state" (Vernon, 1998). The metabolic load during this period is a further challenge and could finally cause "metabolic stress" (Knight et al., 1999), as "that amount of metabolic load (or metabolic burden) which cannot be sustained (or tolerated), such that some energetic processes (which could include those maintaining general health) must be down regulated". A metabolic stress can easily be imagined: "For a typical dairy cow producing 40 L of milk/d, the metabolic energy requirements for milk production are about 200 MJ/d, whereas only about 65 MJ/d is needed for maintenance. An equivalent metabolic demand for humans is running 3 marathons per day" (Sheldon et al., 2018). 40 L of milk/d is not the upper limit: "An early lactation cow will produce 50 to 100 kg of milk per day" (Lucy, 2016).

The homeorhetic priority of milk production, impaired adaptation, and metabolic stress promote restrictions of other functions, although the sequence and cause and effect are not always clear. However, the abnormal incidence of metabolic disorders during early lactation (Drackley, 1999; Ingvartsen et al., 2003; Moyes et al., 2013; Carvalho et al., 2019) and the high culling rate and number of dead cows during the first weeks p.p. (Dechow & Goodling, 2008) before or almost parallel to the peak of lactation support the conclusion of a "pathological state": The "down regulation" of the processes "maintaining general health" causes and exacerbates health risks with fatal consequences, as shown by the increasing death rates with increasing MY (Miller et al., 2008).

5. Conclusion

"Milk secretion is a characteristic feature of all mammalian species" (Oftedal, 2012) and is regulated by a complex of general and specific mechanisms termed homeorhesis. This regulation includes milk secretion uncoupled from DMI, the mobilization of reserves, and the partitioning of the nutrients to the mammary gland. The appetite of the calf represents a unique combination of a feedforward stimulation of milk production by

suckling frequency and of a feedback mechanism for limiting milk production by satiety. As a result, the demand of the growing calf is covered, and the mammary gland and metabolism of the mother are preserved, despite a small NEB. Indeed, "the success of these regulatory processes is essential to ensure the well-being of the lactating mother and survival of the nursing young" (Bauman, 2000).

Unfortunately, this balanced system has been involuntarily overlooked and unknowingly abrogated. First, milk MY has been increased over decades by intense selection for this trait and is further enhanced by frequent milking. The cutting off of the limiting feedback for MY by the uncoupling of milking from calf nutrition has exacerbated output >> input. The lack of consideration of the antagonism between MY and DMI have resulted in severe and long-lasting NEB in high-producing cows with distinct alterations in their hormones and metabolites. Secondly, the gap of nutrients has been closed by the mobilization of reserves and is accompanied by an increase of NEFA concentrations in blood above the actual requirement and capacity of metabolism. NEFA causes, among other effects, IR and ectopic fat deposition, particularly in the liver with the incidence of ketosis. Thirdly, milk production with an increasing imbalance between input and output continues to have homeorhetic priority and possibly proceeds at the expensive of other functions with a subsequent impairment of health. Hence, *optimal* MY for calf nutrition has been replaced by *maximal* milk production for economic reasons with evidence of critical off-target effects and with the down regulation of the processes maintaining general health. The unintended consequences of high MY (≈ NEB) are obvious maladaptation, metabolic stress, and indeed health risks. These health risks have clearly been expressed by Lucy (2016): "Unfortunately, years of genetic selection for milk production without consideration of other traits lead to problems in health, reproduction and longevity in modern dairy cows." (see part 2).

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Transition Period of the Dairy Cow Revisited: II. Homeorhetic Stimulus and Ketosis With Implication for Fertility

Holger Martens[1]

[1] Institute of Veterinary Physiology, Freie Universität Berlin, Berlin, Germany

Correspondence: Holger Martens, Institute of Veterinary Physiology, Freie Universität Berlin, Oertzenweg 19b, 14163 Berlin, Germany. Tel: 049-30-831-1805. E-mail: holger.martens@fu-berlin.de

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Abstract

Dairy cows have been selected during the last century primarily for milk production, which has been increased by a factor 3-5 per lactation during this period without a concomitantly adequate increase of dry matter intake (DMI). This discrepancy between input and output is caused by a negative or minutely positive genetic correlation between milk yield and DMI and leads, in high-producing dairy cows in early lactation, to a severe and long-lasting negative energy balance (NEB) with distinct hormonal and metabolic alterations. Milk production during this period is regulated by homeorhesis with high priority for this trait, which is relatively uncoupled from DMI, and hence with possible restrictions of other functions. The extent and duration of the current NEB is a health risk and is probably one of the reasons for genetic correlations between milk yield and disease. The gap between input and output is closed by the mobilization of reserves characterized by a rapid increase of non-esterified fatty acids (NEFA) above the acute requirement, in turn leading to ectopic fat disposition in the liver and other organs. Therefore, fat liver and ketosis occur during early lactation within a phase of the priority of the homeorhetic (genetic) regulation of milk production at insufficient DMI. Ketosis is correlated with an impairment of fertility. The correlation between an early cause (ketosis) and a later effect (impaired fertility) cannot be explained satisfactorily, but possible epigenetic alterations look promising for future research. The revealed connection between homeorhesis, fat liver and ketosis, and the impairment of fertility provides an approach for discussions of these topics as a complex. The convergence between these issues should furthermore be extended to other production diseases. Since the genetic background of this interaction must not be neglected, the current breeding system should include further health traits with a predominant emphasis on parameters of metabolism and energy balance.

Keywords: homeorhesis, ketosis, fertility

1. Introduction

Milk production of dairy cows has been increased by a factor 3-5 per lactation during the last century. In Germany, production per lactation has been improved from 2600 kg in the 1950s to almost 8000 kg in 2018 (mean of all breeds) (BRS, 2019). Single cows or herds produce much more milk, and milk yields (MY) of 11000-12000 kg (or even beyond that amount) are very often attained in countries with intensive milk production. Similarly, the trend for milk production has been genetically increased in the USA, and production from some 2000 kg in the 1920s has risen to 10328 kg in 2016 (Baumgard et al., 2017) and to 11970 kg in Israel (Israeli Dairy Board, 2017). This increase in production is the result of intense genetic selection primarily for milk yield, improved nutrition, proper management, and the careful prevention and treatment of diseases by veterinarians.

The postpartum delay between the rapid increase of MY and maximal dry matter intake (DMI) causes a period of negative energy balance (NEB), high metabolic rates, and changes of hormones and metabolites, which is exacerbated with increasing MY (see part 1) and the prevailing negative genetic correlation between MY and DMI (Karacaören et al., 2006; Liinamo et al., 2012; Manzanilla-Pech et al., 2014), although a positive energy balance has recently been described (Krattenmacher et al., 2019). The deficit of nutrients occurs during a time of

homeorhetic (genetic) regulation of MY with high priority, with the rapid mobilization of reserves, possible metabolic stress at current performance, and hence, potential constraints and the impairment of health. Probable consequences were discussed as early as 1988 by Emanuelson who concluded: "The situation is further complicated by results showing that a genetic antagonism probably exists between production traits and disease resistance".

The possible genetic interaction between production and health has been analyzed by Berry et al. (2011) and Price et al. (2016) in comprehensive reviews. Heritability estimates of disease are low (< 5%) (Pryce et al., 2016), but genetic correlations between MY and diseases are evident (Berry et al., 2011). For instance, genetic correlations have been demonstrated between MY and ketosis (Simianer et al., 1991; Uribe et al., 1995), MY and mastitis (Koeck et al., 2014b; see review Martin et al., 2018), MY and lameness (Gernand et al., 2012; Koeck et al., 2014b), MY and retained fetal membranes (Heringstad et al., 2007), and MY and impairment of fertility (Pryce et al., 1997; Royal et al., 2002), together with an antagonistic (genetic) relationship between MY and days of productive life (Pritchard et al., 2013). Body weight changes during early lactation (≈ NEB) exhibit genetic correlations with ketosis, metabolic and infectious diseases (Frigo et al., 2010), and fertility (Dechow et al., 2002). Furthermore, in a recent study concerning the genetic analysis of cow mortality and milk production, Tsuruta et al. (2017) concluded that "The increase in reliability with genomic information is particular high for cow mortality" and "the existence of a common region on *Bos taurus* autosome 14 affecting both traits (MY and mortality, the author) may indicate a major gene with a pleiotropic effect on milk and mortality".

Beilharz et al. (1993) approached possible genetic health risks at high MY from a theoretical point of view: resource allocation. The authors discussed some potential restrictions of fitness at continued selection for one trait at limited resources and concluded that "unless the environment (resources, the author) is being improved, antagonisms between traits will start to develop as soon as production traits are selected" (Beilharz & Nitter, 1998). An analogous conclusion has been made by Berry et al. (2011): "Although there were a few exceptions, selection for increased milk production alone without cognizance of other traits is expected to increase the incidence of mastitis, lameness, cystic ovaries, ketosis and metritis".

The performance of farm animals requires the allocation of resources for maintenance, growth, production, and reproduction (Huber, 2017; see also resource allocation of Beilharz et al., 1993) and "genetic changes in nutrient partitioning towards production and away from other life functions are expected (in cows, the author)" (Friggens & Newbold, 2007). Hence, possible restrictions of fitness in dairy cows are facilitated by this major shift and the partitioning of nutrients for milk production at limited resources (NEB).

The genetic correlations between MY and diseases (see above) and the objections of Emanuelson (1988), Beilharz et al. (1993), Beilharz and Nitter (1998), Friggens and Newbold (2007), and Berry et al. (2011) do not offer a causal explanation, although the study of Tsuruta et al. (2017) indicates that closer genetic and possible underlying information will become available in future. MY during early lactation is correlating with NEB, and NEB clearly means limited resources and a *statistical* risk for health during a period of homeorhetic priority of milk secretion. However, NEB with its changes of hormones and metabolites offers some *causal* explanations of fresh cow or productions diseases.

The intention of this review is to promote the discussion of two types of production diseases in more detail: fat liver and ketosis as a consequence of the genetic correlation between increasing milk production and insufficient DMI at homeorhetic priority of milk production, and the impairment of fertility as a result of an explicit "primarily physiological antagonism" between production and reproduction at limited resources.

2. Physiological and Genetic Background of Fat Liver and Ketosis as Well as Fertility

2.1 Fat Liver

Pathogenesis of fat liver: Metabolic conditions of the pathogenesis of fat liver (and ketosis) have been outlined in detail by Danfær (1994), Herdt (2000), Vernon (2005), Ametaj (2005), Geelen and Wensing (2006), with fine biochemical points being made by White (2015). NEFA are released from the adipose tissue, increase in the blood, are taken up by the liver proportional to the concentration (Emery et al., 1992; Reynolds et al., 2003), and are partly metabolized including the release of β-hydroxybutyrate (BHB). The remaining NEFA are converted to TAG (triacylglycerol) and, if the re-synthesis to TAG exceeds the export as VLDL (very low density lipoprotein), accumulation occurs in liver cells and finally causes fat liver (Emery et al., 1992; Drackley et al., 2001; Katoh, 2002; Bobe et al., 2004; Ingvartsen, 2006) and further ectopic fat deposition (EFD) in non-adipose tissue (Roberts et al., 1981). The NEFA concentrations are probably associated with the expression of the mRNA of genes that are involved in the development of periparturient fat liver (Loor et al., 2005). Moreover, EFD causes

cellular dysfunction (lipotoxicity) via potential oxidative stress and proinflammatory pathways (Montgomery et al., 2018), although the knowledge of this system in ruminants is limited (McFadden & Rico, 2019).

DMI is also related to fat liver. Dairy cows with low DMI intake before (Bertics et al., 1992) and after (Hammon et al., 2009) parturition have a higher liver fat content. The prepartum diminished DMI can be used as "an early indicator of subclinical ketosis" (Goldhawk et al., 2009).

In addition to the classic scheme of the pathogenesis of fat liver, inflammation might contribute or accompany fat liver. Ohtsuka et al. (2001) have reported higher concentrations of the pro-inflammatory cytokine TNF-α in cows with moderate and high fat liver content. Ametaj (2005) has discussed the potential role of endotoxins and observed increased TNF-α, serum amyloid A, and haptoglobin in cows with fat liver. Infusion of TNF-α into lactating dairy cows causes, within the liver, a significant increase of TAG and a decrease of transcript abundance of enzymes involved in gluconeogenesis (Bradford et al., 2009). The induction of ketosis by feed restriction causes a several fold increase of pro-inflammatory IL-6 expression in the liver, which "might contribute to development of liver lipidosis, ketosis, and insulin resistance" (Loor et al., 2007).

Human fat liver: Non-alcoholic fatty liver disease (NAFLD) in mankind is an extremely common disease in western societies, with NAFLD being considered as the primary event followed by oxidative stress, production of pro-inflammatory cytokines, and lipotoxicity (Gong et al., 2017). Studies of the pathophysiology of NAFLD have revealed remarkable parallels with the fat liver of cows. A growing body of evidence suggests that low growth hormone (GH) is involved in the initiation and progression of NAFLD (Gong et al., 2017). This correlation has led to experiments with GH receptor (GHR) knockout mice (= uncoupling GH-IGF-1 axis), which exhibit notable changes from the wild-type: fat liver, a 4-fold increase of GH and very low IGF-1 concentrations, increased free fatty acids concentrations, and insulin resistance (IR) (Fan et al., 2009). These results have been confirmed by Liu et al. (2016). Furthermore, the restoration of liver IGF-1 expression in mice via hepatic IGF-1 transgene normalizes some of the changes mentioned above but does not significantly reduce liver fat content and is insufficient to resolve fat-induced oxidative stress and inflammation in the liver (Liu et al., 2016). Additionally, the uptake of free fatty acids into the liver cells via fatty acid translocase (also known as CD36) is upregulated in mice with impaired GH action in the liver (Barclay et al., 2011). The mRNA of CD36 is increased in high-yielding cows in early lactation (McCarthy et al., 2010) during a period of NEB and an uncoupled GH-IGF-1 axis (Lucy, 2004). This suggests that an increase of CD36 and NEFA uptake into the liver cells occurs, if the liver is refractory to GH. These data remain puzzling but should be integrated and, more importantly, experimentally established in ruminants: are CD36 expression and NEFA uptake (down)regulated by GH and (up)regulated by the uncoupling of the GH-IGF-1 axis?

TAG and impairment of liver function: TAG accumulation impairs liver ureagenesis (Strang et al. 1998), the conversion of ammonia to urea (Zhu et al., 2000), and glucose production (Murondoti et al., 2004), which could explain the significant correlation of fat liver with lower blood glucose concentration (Gröhn et al., 1983). This fits together with the effects of TNF-α infusion, namely TAG accumulation and a decrease of transcription of enzymes involved in gluconeogenesis (Bradford et al., 2009). Furthermore, fat liver is associated with a decrease of blood albumin concentration (Reid, 1982) and is correlated with multiple diseases such as the displacement of the abomasum, mastitis, metritis, and impaired immunoreactivity. According to the compilation of data by Bobe et al. (2004), the incidence of moderate fat liver (5-10% TAG of wet weight) varies from 20-65% and of severe fat liver (> 10% TAG) from 5-24%.

2.2 Ketosis

Homeorhesis and ketosis: The possible risk of ketosis at low DMI during early lactation has been characterized by Baird (1977): "The cow will attempt to maintain milk production despite food deprivation and as a result will become ketotic" and "cows are only susceptible to the disorder (primary ketosis) during early lactation, when the homeorhetic stimulus to lactate is at a maximum" (Baird, 1981). These statements of Baird clearly underline the priority of milk production above possible health risks and suggest ketosis as a homeorhetic and genetic disease.

Fat liver and ketosis: Fat liver is believed to precede the increase of ketones in the blood (Grummer, 1993; Katoh, 2002), and hence, fat liver is accompanied p.p. by *ketosis prone metabolic circumstances*: high NEFA, increased production and concentrations of BHB, low glucose concentration at high demand, and probably inflammation. The proinflammatory cytokine IL-6, which regulates the hepatic synthesis of acute phase proteins, is positively related with NEFA and BHB at d 8 p.p. (Mansouryar et al., 2018) and is increased in ketotic cows (Loor et al., 2007; Rodriguez-Jimenez et al., 2018). Methylglyoxal (MGO), which is commonly used as a diagnostic parameter in diabetes type 2 in man, is significant higher in subclinical ketotic cows ($1.2\ mmol \cdot l^{-1} \geq BHB \leq 3.0\ mmol \cdot l^{-1}$) and is positively correlated with acute phase protein haptoglobin in lactating cows (Li et al., 2018a).

The possible role of proinflammatory cytokines correlates with the association of inflammatory biomarkers (serum amyloid A, haptoglobin, lipopolysaccharide binding protein) in ketotic cows (Abuajamieh et al., 2016).

The usually tested blood parameters can be extended by analogous data in milk. BHB and the fat-to-protein ratio in milk are genetically correlated with clinical ketosis (Koeck et al., 2014a), and the data of Bach et al. (2019) suggest milk BHB and NEFA as promising indicators of diseases.

The metabolic alterations that occur during inflammation are accompanied by hormonal changes. Ketotic cows exhibit a significantly decreased insulin concentration (Hove, 1974; Sakai et al., 1993) and a more pronounced decrease of insulin early p.p. (Kerestes et al., 2009). The insulin response to glucose is significantly lower in cows with ketosis (Sakai et al., 1993; Kerestes et al., 2009) suggesting IR. Insulin stimulates the peripheral utilization of ketones (see the literature, especially Brockman, 1979; Hayirli, 2006) and decreases hepatic ketone production (Hayirli, 2006), which may be impaired at low insulin concentration. The treatment of ketotic cows with glucose (day 1-5) or glucose + insulin (day 2-4) supports this outcome. NEFA and ketone bodies were lower and glucose higher at d 6 in a group of cows receiving glucose + insulin compared cows treated with glucose alone (Sakai et al., 1993). The low insulin concentration as a health risk in ketotic cows has been emphasized as early as 1976 by Brockman and 1986 by Giesecke.

Lower IGF-1 and higher GH concentrations have been measured in cows with clinical ketosis (Du et al., 2018), and the application of GH causes high NEFA and ketone concentrations (Kronfeld, 1965). The latter author suggests "excessive secretion of endogenous GH could conceivably play a role in the pathogenesis of naturally occurring bovine ketosis". This suggestion agrees with a recent observation. Holstein and Jersey cows were treated a.p and p.p with recombinant bovine somatotropin (rbST). This treatment did not influence blood BHB concentration but numerically increased ketosis (Silva et al., 2017a). However, a further study of periparturient treatment with rbST resulted in a decreased p.p. BHB concentration (Silva et al., 2017b).

Uncoupling of the GH-IGF-1 axis: Ketotic cows exhibit, in addition to the discussed metabolic and hormonal changes, an uncoupling of the GH-IGF-1 axis (Du et al., 2018): an increase of GH and a decrease of IGF-1. Furthermore, in calf liver cells *in vitro*, NEFA or BHB causes a reduction of GHR 1A and IGF-1 mRNA expression (uncoupling of GH-IGF-1 axis) (Du et al., 2018) and explains the earlier observation of a negative correlation between NEFA as well as BHB and IGF-1 concentrations (Wathes et al., 2007; Cheng et al., 2015).

The uncoupled GH-IGF-1 axis raises a problem involving the control of gluconeogenesis by GH when "the liver is refractory to GH" (Lucy, 2008), which may cause some health risks: "The consequences of inadequate GHR 1A expression are serious. The liver remains unresponsive to GH and various GH-dependent processes (including gluconeogenic mechanism) are not initiated. This may predispose the cow to fat liver and ketosis and preclude the normal hepatic mechanism for nutrient partitioning during increased lactation" (Lucy et al., 2001). This concern regarding the uncoupled GH-IGF-1 axis and ketosis has indirectly been confirmed. The infusion of insulin + glucose recouples the GH-IGF-1 axis (Butler et al., 2003; Rhoads et al., 2004), and the treatment of ketotic cows with glucose + insulin reduces NEFA and ketone bodies and raises glucose compared with the treatment of ketotic cows with glucose alone (Sakai et al., 1993). This suggests the stimulation of gluconeogenesis by GH after the recoupling of the GH-IGF-1 axis by glucose + insulin, as has been shown by Butler et al. (2003).

The likely pivotal role of insulin within this complex of uncoupled GH-IGF-1 axis, fat liver, and ketosis can be further deduced from the treatment of cows with a slow-release insulin (SRI). An increasing single dose of SRI at d 3 p.p. decreases the percentage increase in liver triglyceride (TG), and on d 5, the average liver TG tends to be lower than that in the control (Hayirly et al., 2002). In agreement with these data are the observations of Smith et al. (2009). Prepartum administration of 2,4-thiazolidinedione, a ligand of the peroxisome proliferator-activated receptor-ɣ (PPAR-ɣ), which potentiates the action of peripheral insulin in several species (see reference by Smith et al., 2009), reduces p.p the concentration of NEFA and liver fat content in dairy cows (Smith et al., 2009). Prevention of fatty liver by glucagon is probably mediated by an increase of glucose and insulin and the resulting decrease of NEFA (Nafikov et al., 2006).

The refractory behavior of the liver to GH with consequences for gluconeogenesis is probably worsened by BHB. Ketogenesis depresses glucose synthesis (see Danfær, 1994), hyperketonemia in ewes reduces endogenous glucose production (Schlumbohm and Harmeyer, 2004), and intravenous BHB infusion for 48 h in cows reduces the glucose concentration in the blood (Zarrin et al., 2013). This antagonism explains the significant negative correlation between glucose and BHB in dairy cows (Harrison et al., 1990) and the reciprocal concentration of high BHB and low glucose in ketotic cows (Li et al., 2012; Du et al., 2018) or of glucose and acetoacetate (Hove,

1974). This antagonism agrees with the inhibition of glucose synthesis from propionate *in vitro* by BHB in sheep hepatocytes (Demigne et al., 1986).

The reasons for the BHB effect on glucose synthesis are not clear, but histone lysine can be post-translationally modified by butyrylation or β-hydroxybutyrylation, and hence, gene expression can be modulated by metabolites such as ketone bodies, as shown in experimental animals (Li et al., 2018b). A growing body of evidence shows that BHB is a signaling metabolite in monogastric animals with effects on energy expenditure, IR, and feeding behavior (Rojas-Morales et al., 2016).

The health risks of NEFA and BHB are not restricted to the uncoupling of the GH-IGF-1 axis and the inhibition of gluconeogenesis because both metabolites have hypophagic effects and decrease DMI (Scharrer, 1999), thereby confirming the negative correlation between BHB and DMI (Lean et al., 1992) and explaining the decline of DMI in cows with clinical ketosis (Baird, 1977, 1982). In addition, increasing evidence suggests that BHB induces hepatocyte adoptosis in dairy cows with fat liver (Tharwat et al., 2012) and add to immunosuppression. BHB reduces bovine neutrophil and bactericide activity against mammary pathogenic *E. coli* (Grinberg et al., 2008).

Genetic background and ketosis: The heritability of ketosis was determined very early by Dohoo et al. (1984) at 0.31 and by van Dorp et al. (1998) at 0.39. A recent compilation of heritability by Pryce et al. (2016) summarized much lower values. The significant genetic correlations between MY and ketosis support a genetic background for this disease. In heifers of Norwegian cattle, genetic correlations of 0.65 (Simianer et al., 1991) and, in Canadian Holstein cows, of 0.77 (Uribe et al., 1995) have been determined, although lower correlations have been published (Lyons et al., 1991). The genetic background of ketosis is mirrored by data concerning the high heritability of the BHB and NEFA concentrations (0.3-0.4) (Oikonomou et al. (2008b) and of the fat-to-protein ratio (0.3) in milk during early lactation (Buttchereit et al., 2012). Consistent with the correlation between MY and ketosis is the observation regarding a genetic correlation (0.49) between milk BHB ($\geq$ 0.2 mmol·l^{-1}) and the fat-to-protein ratio of milk in Canadian Holsteins (Koeck et al., 2014a).

The genetic correlations can be deduced from the (genetic) homeorhetic priority of milk production, which is relatively uncoupled from DMI (= NEB), and the resulting uncoupling of GH-IGF-axis: high NEFA concentrations, impaired gluconeogenesis in the fat liver, and the production of ketone bodies. Adaptation is overloaded (= ketosis), and milk production proceeds at the expensive of other physiological functions with an accompanying impairment of health.

Ketosis and secondary diseases: The complex interactions and side effects of BHB might explain the greater chances of subclinical ketosis with metritis ($\geq$ 1.4 mmol·l^{-1} BHB), displacement of the abomasum ($\geq$ 1.2 mmol·l^{-1} BHB), lameness ($\geq$ 1.5 mmol·l^{-1} BHB) (Suthar et al., 2013), association with impaired fertility (Baird, 1982), early culling (Seifi et al., 2011), and reduced milk production (Duffield et al., 2009). This general interaction between ketosis and diseases has been confirmed by Berge and Vertenten (2014): "Ketosis was associated with significant higher odds of all common fresh cow diseases: metritis, mastitis, displaced abomasum, lameness and gastrointestinal disorders".

A comprehensive meta-analysis by Raboisson et al. (2014) has confirmed the general health risk of subclinical ketosis with reduced milk production and longer calving-to-first-service and calving-to-conception interval. The predisposing effects of BHB concentrations as a health risk have been cautiously analyzed by McArt et al. (2013) with a cut-point of BHB with 0.8 mmol·l^{-1} before and 1.2-1.4 mmol·l^{-1} after parturition and have, among others, been carefully summarized in a wide-ranging review by Overton et al. (2017).

Incidence of ketosis: The high demand of energy with hormonal and metabolic alterations promotes metabolic stress, which is obviously wide spread in high-producing cows throughout the world. The incidence of subclinical ketosis is calculated to be up to 40% (see review by McArt et al., 2013) and, in European dairy farms, at 39% based on a milk test for ketones $\geq$ 100 µmol/l (Berge & Vertenten, 2014) or 21.8% with a BHB threshold in blood of $\geq$ 1.2 mmol/l (Suthar et al., 2013). The incidence of subclinical ketosis at 72% is much higher in cows with more than three lactations (threshold $\geq$ 1.2 mmol·l^{-1} BHB in blood) (Kaufman et al., 2018) and agrees with the findings of McArt et al. (2013) that advanced parity is a major predictor of hyperketonemia during early lactation. This high incidence in older cows coincidences with the possible net loss of energy with increasing numbers of lactations, MY, and deeper NEB (Coffey et al., 2004) and suggests an exacerbation of ketosis during long-lasting energy shortage and the lack of replenishment within the current lactation. This is in agreement with the increasing decline of BCS p.p. with rising parity (Waltner et al., 1993) and consequently with higher chances of diseases in ketotic cows of parity 2 or more (Berge & Vertenten, 2014).

Pathogenesis of ketosis: Taken together, the current data allow an extended explanation of the pathogenesis of ketosis. Selection for higher milk production at insufficient DMI leads to the stepwise uncoupling of the GH-IGF-1 axis (high GH and low insulin, IR, and IGF-1 concentrations, see above), the rapid mobilization of NEFA, and increase of NEFA concentration, fat liver, the augmented hepatic production of BHB, the probable inhibition of gluconeogenesis at increased demand, and low glucose concentrations. The uncoupling of the GH-IGF-1 axis by high NEFA and BHB obviously appears to introduce a "vicious circle" (Du et al., 2018) with further risks of increase of fat liver, ketosis, and secondary diseases (Figure 1).

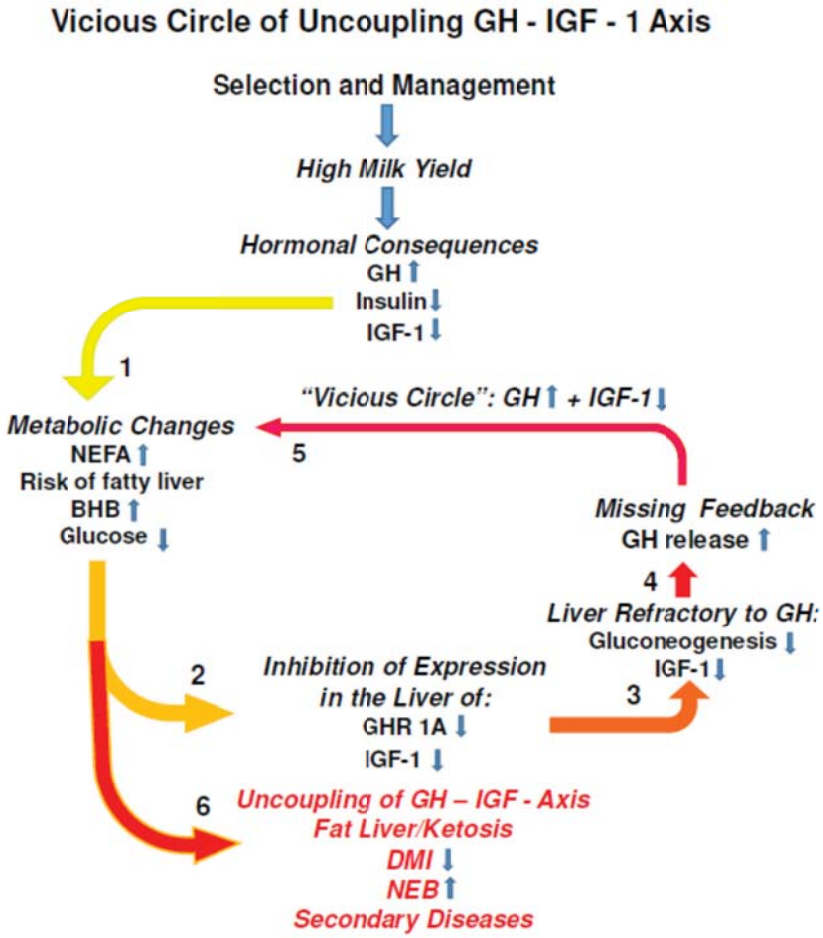

Figure 1. Schematic flow diagram of the pathogenesis of ketosis. The discrepancy between input and output with hormonal alterations such as high GH, low insulin, insulin resistance, and low IGF-1 causes (1) mobilization of NEFA, risk of fat liver with increase of BHB production, impaired gluconeogenesis, and decrease of glucose concentration. The metabolites NEFA and BHB (2) decrease the expression of IGF-1 and GHR 1A in the liver, leading to the liver becoming refratory to GH (3). Gluconeogenesis and IGF-1 synthesis in the liver are further decreased, as is the feedback of IGF-1 on the inhibition of GH release from the pituitary. GH is further increased. These hormonal alterations (5) with metabolic consequences of high NEFA and BHB exacerbate the uncoupling of the GH-IGF-1 axis resulting in the "vicious circle" of ketosis (Du et al., 2018) with reduced DMI and increased NEB and risk of secondary diseases (6)

The relationship between the uncoupling of the GH-IGF-1 axis and the pathogenesis of ketosis in dairy cows is intriguing because this uncoupling is not observed in sows p.p. (Lucy, 2008), and ketosis is not known in pigs.

The homeorhetic priority of milk production together with the genetic disposition of high-producing cows is a challenging adaptation and triggers metabolic disorders and impairs fertility. Ketosis (Baird, 1982) and subclinical BHB early p.p. (Walsh et al., 2007) are correlated with impaired fertility. This negative interaction is corroborated by the genetic correlation between ketosis and cystic ovaries (see review Pryce et al., 2016).

2.3 Fertility

Decline of fertility and reproduction: Impairment of fertility has been the major cause for disease-dependent culling of dairy cows in Germany for many years (BRS, 2019). Butler (2003) and Dobson et al. (2008) have summarized the reciprocal correlation between an increase of MY and the decrease in conception rate during the last few decades. "Management, nutrition, production, and genetics are the main reasons for the decline in fertility in modern dairy cows" (Chagas et al., 2007); this is related to the delayed resumption of ovarian cyclicity p.p., disarranged ovarian activity as a persistent corpus luteum and prolonged luteal phase, low progesterone and estradiol concentrations, reduced estrus behavior, metritis, low oocyte quality, and early embryonic mortality. Possible reasons for these aberrations have been discussed in several reviews and original publications: genetics (Berry et al., 2003; Berry et al., 2016; Hazel et al., 2017; Cai et al., 2019), management (Beever, 2006; Roche, 2006; van Saun & Sniffen, 2014), nutrition in general (Diskin et al., 2003; Lucy, 2003; Beever, 2006; Roche, 2006; Dann et al., 2006; Chagas et al., 2007; Friggens et al., 2010; van Saun & Sniffen, 2014; Drackley & Cardoso, 2014; Rodney et al., 2018), infection and fertility (Sheldon et al., 2009), NEB, health risks, perturbed immune function, and inflammation (Esposito et al., 2014), lipid reserves (Friggens et al., 2003), protein intake and fertility (Butler, 2000; Tamminga, 2006; Lean et al., 2012), protein and urea (Cheng et al., 2015), metabolic status and fertility (Pushpakumara et al., 2003; Wathes, 2012), "mismatch between metabolism and fertility" (Leroy et al., 2008), hormonal changes and fertility (Lucy, 2003; Santos et al., 2016), enhanced clearance of steroids in high-producing cows (Sangsritavong et al., 2002; Wiltbank et al., 2006), decreased progesterone synthetic capacity of lower corpus luteum volume (Moore et al., 2014a), and from a veterinarian point of view, "causes of poor fertility" (Walsh et al., 2011).

Signal cascade of fertility and reproduction: The detrimental effects on fertility occur at each step of the signal cascade of regulation of fertility and pregnancy and include a) the hypothalamus/pituitary and release of gonadotropins, b) blood metabolites and steroid concentrations, c) the ovary with follicular growth and corpus luteum, d) the quality of the oocyte, or e) embryo development, as has been analyzed (a, c, d, e) in a previous review by Webb et al. (1999).

Consequently, various aspects of the signal cascade from hypothalamus/pituitary, ovary, and uterus and from fetal development and (under)nutrition or metabolism have been studied and described: the dominant role of GnRH in the hypothalamus/pituitary and the release of gonadotropins (HPG axis) (Wade & Jones, 2004; Schneider, 2004; Clarke, 2014; Hill & Elias, 2018), the effects of nutrition and metabolic status on circulating hormones such as LH, FSH, estradiol, progesterone, insulin, and IGF-1 and possible effects on pre-ovulatory follicle growth and ovulation (Diskin et al., 2003, Wathes, 2012), the somatotropic axis and follicular growth (Silva et al., 2009; Lucy, 2012), the regulation of the corpus luteum (Wiltbank et al., 2012), the role of glucose for embryonic and fetal development (Lucy et al., 2014), progesterone and early pregnancy (Spencer et al., 2015), prostaglandins and maternal recognition (Arosh et al., 2016), oocyte development and stress (Roth, 2018), embryonic and early fetal loss (Diskin & Morris, 2008), the role of lipids as regulators of conceptus development (Ribeiro, 2018), and the interaction between metabolic stress and innate immunity and inflammation of the endometrium (Sheldon et al., 2017).

It is not the intention of this overview to summarize, repeat, or emphasize the possible main topics of impaired fertility. The available data will be used for the discussion of the following hypotheses. Does a new pregnancy make sense during a phase of under-nutrition? Is the cow, from a biological point of view, interested in becoming pregnant again during the period of the homeorhetic priority for milk production at a high metabolic load and at insufficient nutrition? If so, how is the interaction between under-nutrition and fertility mediated?

Nutrition and fertility: Wade and Jones (2004) have analyzed the biological relationships between (under)nutrition and fertility (mainly in experimental animals and not in cattle) and concluded that a hierarchy of nutrient allocation occurs at insufficient nutrition:

a) with priority for *essential* processes such as cell maintenance, circulation, and neural activity,

b) with restriction of *reducible* processes such as thermoregulation, locomotion, or growth or,

c) with cessation of *expendable* processes that are not essential for survival such as fat storage or *reproduction*.

Hence, fertility is directly influenced by under-nutrition (Wade & Jones, 2004; Schneider, 2004; Clarke, 2014; Hill & Elias, 2018) and indirectly by the preference for one trait at limited resources (Beilharz et al., 1993), by metabolic stress and downregulation of other traits (Knight et al., 1999), or by the priority of homeorhetic milk production (Bauman & Currie, 1980). MY should be allocated as an "essential process" according Wade and

Jones (2004) and has priority despite the prevailing NEB (under-nutrition) and the antagonism between MY and DMI (see above).

These theoretical considerations are confirmed by a growing and overwhelming set of experimental data about interactions between the shortage of nutrients and the impairment of the regulation of fertility and pregnancy. Day et al. (1986) have observed low LH concentrations and pulse frequency in heifers with restricted feed intake. Further, the LH response to GnrH is lower in animals having a restricted diet. In agreement with these results are the findings of Rutter and Manns (1987) in beef cows p.p.: the reduced availability of glucose by phlorizin treatment causes smaller and fewer large LH pulses in p.p. beef cows. Schillo (1992) has summarized, in a comprehensive review, the interaction between feed restriction and cyclicity in ruminants. Under-nutrition inhibits pulsatile LH secretion by reduced GnrH secretion in the hypothalamus. Metabolites and hormones or oxidizable fuels are suggested as possible mediators between hormones and fertility traits. In accordance with these conclusions are the observations of Canfield and Butler (1990) that "the pulsatile LH secretion is suppressed until the nadir of NEB is reached", and Canfield and Butler (1991) suggest further that the ovaries of cows under NEB are less sensitive to LH. Decreased LH secretion and reduced sensitivity is probably the cause for the positive and linear relationship between the days p.p. until the nadir of NEB and number of days to first ovulation (Beam & Butler, 1999). The increasing loss of BCS (= deeper nadir of NEB) delays ovulation (Beam & Butler, 1999) leading to the conclusion "that negative energy balance is the major nutritional link to low fertility in lactating cows" (Butler, 2005). Banos and Coffey (2010) have confirmed these observations and conclusions. The duration and sum of NEB show high genetic correlations between the numbers of days after calving and the first observed estrus.

The correlations mentioned above indicate an interaction between insufficient nutrition, metabolism, and the signal cascade of cyclicity, estrus, and finally pregnancy. The missing interface between these parameters was discussed by Canfield and Butler as early as 1990; they suggested the involvement of the hypothalamus or higher neuroendocrine control centers and proposes that the energy status is conveyed to the brain by NEFA. Indeed, the availability of oxidizable fuels, glucose, and NEFA is the "transmitter" that is detected by peripheral and central metabolic fuel sensors (Wade & Jones, 2004; Schneider, 2004).

Metabolic sensor and oxidizable fuels: The central sensor is located in the area postrema (AP) in the floor of the fourth ventricle in the hindbrain (Wade & Jones, 2004). Corresponding glucose-sensing neurons are arrayed in the brainstem and the hypothalamus (Levin, 2006). The information regarding oxidizable fuels at the fuel detector in the hindbrain is relayed to the GnRH neurons in the forebrain (details of transmitter and neurons see Wade & Jones, 2004; Schneider, 2004; Clarke, 2014; Hill & Elias, 2018). GnRH is the driver of reproduction, is secreted in a pulsatile manner from the preoptic area of the hypothalamus into the hypophysial portal system, and regulates the synthesis and pulsatile release of LH from the anterior pituitary (Clarke, 2014).

A key role as oxidizable fuel is obviously exerted by glucose at the central sensor. Glucoprivation of this fuel sensor suppresses LH pulses in rat (Nagatani et al., 1996; Murahashi et al., 1996) and in sheep occurs peripheral to the GnRH neuron (Bucholtz et al., 1996; Ohkura et al., 2000). Blood glucose concentration is reduced in cross-bred heifers at restricted feed intake with corresponding effects on LH release (Yelich et al., 1996). The LH response is obviously related to GnRH release. Growth restriction in young sheep reduces the frequency of GnRH release, and not all GnRH pulses stimulate the pulsatile secretion of LH (l'Anson et al., 2000). This confirms the data of Day et al. (1986) showing a decreased LH response to GnrH infusion in feed-restricted heifers (see above). The possible downstream effects are predictable and agree with the conclusion of Diskin et al. (2003) that NEB "adversely affects the size and the ovulatory fate of the dominant follicle". Concomitantly, the duration of estrus decreases with increasing MY (Wiltbank et al., 2006).

The decisive role of glucose on fertility was shown decades ago in cows by McClure (1968). The conception rate of cows curvilinearly increases with increasing glucose concentration in the blood and agrees with observations of Harrison et al. (1990) who found that, in cows, a low blood glucose concentration during week 1 p.p. is negatively correlated with the number of days to conception. Adverse effects of a low glucose concentration early p.p. on conception have been confirmed since then (Reist et al., 2003; Oikonomou et al., 2008a; Garverick et al., 2013; Cardoso et al., 2013), and vice versa, an increased ratio between glucose and BHB has been correlated with the higher probability of estrous expression at first ovulation (Westwood et al., 2002), and a higher blood glucose concentration is positively associated with pregnancy after first insemination, including the production of heavier fetuses (Green et al., 2012). Lucy et al. (2014) and Dupont et al. (2014) have summarized the available data on glucose and fertility in comprehensive reviews and have emphasized the outstanding peripheral role of glucose for ovarian cyclicity and luteal function, immune function and uterus health, pregnancy, and fetal development.

NEFA are also oxidizable fuels, and evidence exists with regard to the effect of lipoprivation on LH pulses in hamsters, but is uncertain in other species (Wade & Jones, 2004), and corresponding data with respect to NEFA are missing in cattle. In sheep, intravenous NEFA infusion does not change luteinizing hormone secretion (Estienne et al., 1989). By contrast, "increased serum NEFA and BHB concentrations had a detrimental effect on reproductive performance" (Opsina et al., 2010) with a NEFA threshold of ≥ 0.27 mmol·l^{-1} prepartum and ≥ 0.72 mmol·l^{-1} p.p. and a p.p. BHB value of ≥ 1.4 mmol·l^{-1}. This corroborates the data of Walsh et al. (2007): the predicted probability of pregnancy linearly decreases with increasing BHB concentrations in week 2 p.p. Similarly, Garverick et al. (2013) conclude that "postpartum monitoring NEFA and glucose could theoretically be used to identify cows at risk for infertility". The correlation with NEFA has been validated by Ribeiro et al. (2013). An elevated NEFA concentration reduces P/AI on d 65 after AI. The p.p. threshold of ≥ 0.72 mmol·l^{-1} (Opsina et al., 2010) has been confirmed by Ribeiro (2018). Cows with NEFA concentrations ≥ 0.7 mmol·l^{-1} during the first 2 weeks p.p. have reduced pregnancy after AI.

Previous *in vitro* studies (Jorritsma et al., 2004; Leroy et al., 2005, 2006) suggest a possible explanation. Jorritsma et al. (2004) have observed a negative effect of NEFA on the proliferation of granulosa cells, and fertilization and embryonic development of cumulus-oocyte-complexes are reduced by NEFA. Leroy et al. have studied the effect of NEFA (2005) and BHB and glucose (2006) on the development of bovine oocytes. NEFA and BHB during oocyte incubation have negative effects on oocyte maturation. The influence of BHB is more pronounced at a low glucose concentration. NEFA concentrations reflect NEB (Herdt, 2000; Urdl et al., 2015), and based on current knowledge, NEFA and BHB have primarily peripheral effects during the development of oocytes. Furthermore, an "impaired uterine environment" (during NEB and high NEFA, the author) also contribute to subfertility" (Ribeiro, 2018).

Metabolic hormones and fertility: The interaction between nutrition and fertility is not restricted to oxidizable metabolites and their possible effects on central or peripheral sensors. The concentrations of NEFA, BHB, and glucose reflect the changes of GH, insulin, IR, and IGF-1, and the effects of the hormones GH, insulin, and IGF-1 on the signal cascade of fertility regulation have previously been described (Beam & Butler, 1999; Roche et al., 2000; Adam et al., 2000; Lucy, 2012; Wathes, 2012; Kawashima et al., 2012, Bollwein et al., 2014). The hormonal changes of increased GH, low insulin, IR, and low IGF-1 are the framework of regulation of metabolism, mobilization, and partitioning and determine the homeorhetic priority of high milk production. Undoubtedly, these hormones and the usual hormonal combinations (and the resulting concentrations of NEFA, BHB, and glucose, see above) have effects on reproduction.

The somatotropic axis includes GH and IGF-1 (see part 1). The possible role of GH and IGF-1 on follicular growth has been reviewed by Silva et al. (2009) and by Lucy (2012) who concludes that the effect of GH is mainly indirect via metabolism and the control of IGF-1 by GHR 1A in the liver. The role of IGF-1 has been outlined in detail by Velasquez et al. (2008), Wathes (2012), and Kawashima et al. (2012). mRNAs for IGF-1 have been detected in bovine follicles and luteal cells, and IGF-1 stimulates the proliferation and steroid-genesis in granulosa cells (for details and references, see Velasquez et al., 2008; Wathes, 2012; Kawashima et al., 2012). Consequently, cows with lower IGF-1 concentrations have significantly lower progesterone concentrations (Pushpakumara et al., 2002), and Beam and Butler (1999) have demonstrated a significant linear correlation between IGF-1 and estradiol concentrations. Further, in *in vitro* studies, the early development of the bovine blastocyst is influenced by IGF-1 (Byrne et al., 2002), which possibly acts in the oviduct (Pushpakumara et al., 2002) and in the uterus by influencing secretion and embryo growth (Velasques et al., 2008). The influence of IGF-1 is not limited to the periphery. "An intrapituitary IGF system exists in sheep and the present results are consistent with an endocrine role of the IGF-1 in nutritional modulation of LH secretion" (Adam et al., 2000). Zulu et al. (2002) have analyzed the role of IGF-1 in bovine reproduction and conclude that "IGF-1 is therefore one of the long sought factors that signal nutritional status to the reproductive axis", and that "IGF-1 plays a pivotal role in cattle fertility, acting as a monitoring signal … when nutritional conditions for successful reproduction are reached" (Velasquez et al., 2008).

The multiple modulation of the signal cascade by IGF-1 from the pituitary to ovary and to uterus might explain the relationship between IGF-1 and fertility in cows (Taylor et al., 2004). The latter authors observed that the lowest IGF-1 concentration p.p. in multiparous cows was correlated with the highest milk production, and that cows with the lowest IGF-1 concentration p.p. failed to become pregnant despite services. Gobikrushanth et al. (2018) studied in more detail the association between serum IGF-1 and fertility. Cyclic multiparous cows had a greater serum IGF-1 concentration during the first week p.p. compared with acyclic cows, whereas primiparous and multiparous cows with a high serum IGF-1 concentration had a greater P/AI than those with low IGF-1. The optimum serum IGF-1 thresholds predictive for P/AI were 85.0 ng/mL and 31.0 ng/mL for primiparous and

multiparous cows, respectively. Correspondingly, cows with a high genetic merit for fertility had greater IGF-1concentrations in their blood (Moore et al., 2014b).

Insulin is indirectly involved in the somatotropic axis, because it mediates the expression of GHR 1 A in the liver (Butler et al., 2003), and hence, insulin and IGF-1 are positively correlated (Wathes et al., 2007). Furthermore, insulin has effects on the HPG axis including the ovary with a potential influence on fertility (Beam and Butler, 1999). Despite these versatile impacts, insulin measurements do not improve predictions of fertility (Wathes et al., 2007). Wathes (2012) has analyzed this topic and suggests the diet-dependent variation of insulin concentration and, in particular, the regulation of insulin receptors as possible reasons for the missing correlation. Despite this restriction, insulin is well known to regulate ovarian activity in woman and experimental animals (Porestky et al., 1999), and cows with a good genetic merit for fertility tend to have a greater insulin concentration (Moore et al., 2014b).

The numerous direct or indirect relationships between nutrient-dependent hormones or metabolites and fertility reveal the outstanding importance of the sufficient availability of nutrients for fertility and are in agreement with the data of Oikonomou et al. (2008a). BCS, BHB, NEFA, and glucose during early lactation as parameters of energy metabolism have the highest genetic correlation with reproductive performance. Malnourishment at high demand for MY and reduced intake predispose to reduced fertility, and this negative modulation can be observed at the fuel sensor in the hindbrain, hypothalamus, pituitary, ovary, oviduct, and uterus and during early pregnancy.

The extensive set of data compiled above appears to offers a causal explanation of the impairment of fertility within the signal cascade of cyclicity regulation including pregnancy. Unfortunately, the list of hormones and metabolites sometime change before parturition, regularly immediately after parturition, and hence far before the cows fail to conceive between 60 and 100 d pp.p. This gap prompted Britt in 1992 to propose a hypothesis about possible effects on the development of a resting primordial follicle until ovulation, which requires approximately 100 days. If the growing follicle is exposed to adverse conditions during this period, gene expression might be affected (Britt, 1992) with delayed consequences for ovulation, oocyte quality, or pregnancy. Interestingly, Britt (1992) considered NEB as an adverse condition that somehow “imprinted” upon follicle development. This hypothesis was used to explain retrospectively the difference in fertility between dairy cows with a high and low change of BCS early p.p. (Britt, 1992). It confirmed the hypothesis that “events that occur early postpartum influence fertility much later” (Britt, 1992). The modulation of “gene expression” of the follicle resulting in impaired or altered development was, at that time, a hypothesis to explain early cause and later effect (Britt, 1992). The delay remains, to the author’s knowledge, miraculous, but some suggestions can be made.

Gene expression can be influenced by epigenetic gene regulation, which includes a) covalent DNA modification, b) covalent posttranslational modifications made to the tails of histone proteins, and c) noncoding RNA (Ideraabdullah & Zeisel, 2018). One of the most intriguing aspects is the acetylation of the histone proteins by the metabolites butyrate (butyrylation) or BHB (β-hydroxbutyrylation) as driven by ketogenesis under low nutrient conditions (Xie et al., 2016). The binding of metabolites to the tail of histones facilitates the accessibility of DNA, which is now more permissive for transcription, and contributes to the regulation of DNA-based processes (“gene expression”). The reaction is catalyzed by the histone acetyltransferase (HAT) and the release by histone deacetylase (HADAC) (Narita et al., 2019), which can be inhibited by butyrate and BHB (Shimanzu et al., 2013), thereby supporting the evidence that BHB is a signaling metabolite (up to now in monogastric animals, the author) with effects on energy expenditure, IR, and feeding behavior (Rojas-Morales et al., 2016). Hence, undoubtedly, metabolites have wide-spread epigenetic activity and consequences for metabolism (Li et al., 2018b; Xie et al., 2016), metabolic syndrome, and NAFLD (Ideraabdullah & Zeisel, 2018). Unfortunately, to the knowledge of the above authors, corresponding results concerning fertility are still missing, but the interaction between metabolites and the epigenetic modulation of gene expression within the signal cascade of cyclicity and pregnancy offers experimental support.

Despite the uncertainty between early causes and later effects, each level of signal cascade can be modulated (see above), and for future discussion of this topic, a hierarchy of impact is proposed: The higher possible influences in the signal cascade denote the consequences. Glucose and IGF-1 appear to be predominant factors within this arbitrary classification. Glucoprivation at the central fuel sensor in the hindbrain inhibits GnrH release and consequently LH pulses (Wade & Jones, 2004; Schneider, 2004; Clark, 2014), and IGF-1 modulates LH release in the pituitary (Adam et al., 2000) with the known peripheral effects (see above). Glucose and IGF-1 are decreased p.p., cooperate in the central nervous system, and intensify the inhibition of pulsatile LH release.

In addition to the effects at the top of the regulation cascade, the possible impact of the myriad effects downstream of GnrH and LH release appear to the author as possible "intervention sites". If the local hormonal and metabolic circumstances are not optimal, possible steps for follicle growth, steroid synthesis, estrous behavior, ovulation, or embryo development are downregulated. Hence, the whole signal cascade resembles the combination of a central control hierarchy with peripheral subsidiarity, and suggest the compound embedment of the regulation of reproduction with the goal of the optimal regulation of cyclicity and successful pregnancy or simply of avoiding malfunction.

This system allows flexible adaptation to metabolic and hormonal changes. These biological responses should above all be regarded as positive reactions, because pregnancy during under-nutrition possibly challenges the health of the cows and the development of the embryo. Hence, the possible modulation of reproduction by nutrition should be considered as a biological capability to adjust to various inputs during an optimal window for the next pregnancy. The impairment or constraint of fertility is, in this sense, not a disease but a normal transient period: "The cow knows better". Leroy et al. (2008) have discussed this problem in detail as a "mismatch between metabolism and fertility". The nutrient prioritization of the cows favors milk production over fertility (Leroy et al., 2008) or, again, endorses homeorhesis. However, this general biological background can be disrupted, go out of control, and promote, for instance, the pathogenesis of cystic ovaries with an altered HPG axis and intraovarian components (Ortega et al., 2015).

3. Interdependencies Between NEB, Ketosis, and Fertiliy

The genetic antagonism between increasing MY and DMI p.p. is a challenge for a high-producing cow with multiplied demands and should be critically contemplated because the deep and long-lasting NEB over two months, with a deficit of 1500 MJ_{NEL} or more, represents chronic under-nutrition (Vernon, 1998) and a distinct health risk, despite the original biological background. Presumably for this reason, Roche et al. (2009) have stated "It is perfectly natural for cows to lose BCS (= NEB, the author) in early lactation (homeorhesis), and this loss cannot be eliminated by improved feeding". Of course, the capability for compensating an energy deficit by mobilization and BCS loss is natural with an important biological intention (see part 1), and the possible deficit is of minor impact at MY for the nutrition of one calf (Hart et al., 1975). The congenital biological predisposition for a slight NEB and a moderate rate of mobilization has been involuntary used to fill the increasing deficit with increasing MY. Hence, the current extent and duration of NEB is, from the author's point of view, not "natural" and leads to pathophysiological consequences. The hormonal background includes high GH, low insulin, IR, and low IGF-1, finally resulting in the uncoupling of the GH-IGF-1 axis and metabolic changes such as increased NEFA, BHB, and low glucose (Table 1). These alterations challenge health and predispose to fat liver and ketosis (Table 1).

A second predisposition with health risk is the correlation between MY (≈ NEB) and impairment of fertility (Table 1). The manifold downregulation at the various levels of the signal cascade of regulation of fertility and pregnancy at impaired nutrition primarily involves a biological response.

Unfortunately, the hormonal and metabolic conditions surrounding high MY (≈ NEB) are the major reasons of fat liver, ketosis, and disturbed fertility, and this common etiology explains the correlation between these two diseases (Baird, 1982; Walsh et al., 2007).

Table 1. Compilation of hormonal and metabolic changes that enable and accompany high MY (≈ NEB) and their direct or indirect influences on the risk of fat liver and ketosis or of impairment of fertility in high-producing dairy cows

Hormonal and metabolic changes at high MY	Risk of fat liver and ketosis	Impairment of fertility
GH ↑	+	(+)
Insulin ↓	+	(+)
Insulin Resistance (IR)	+	(+)
IGF-1 ↓	+	+
Uncoupled GH-IGF-1 axis	+	(+)
Glucose ↓	+	+
NEFA ↑	+	+
BHB ↑	+	+
NEB ↑	+	+
Risk of Inflammation	+	+
Immunosuppression	-	+

Note. ↑: increase; ↓: decrease; +: increasing risk of ketosis or impairment of fertility; (+): uncertain; -: not known.

4. Suggestions and Conclusions

Homeorhesis: Homeorhesis is a term for the regulation of milk production during early lactation, although the major focus of regulation changes during the lactation period. After parturition, milk production has the absolute homeorhetic priority. With decreasing milk production after peak MY and increasing DMI, a zero energy balance is achieved. At this point, lost body weight is compensated for, and during the rest of lactation and the dry-off period, nutrients are used for the mother and the growing calf (Oldham, 1984). Below, this term is divided into phase 1 of homeorhesis from parturition until the end of NEB followed by phase 2 until the end of pregnancy.

An important conversion of the priority of nutrient partitioning occurs during these two phases. During phase 1, the homeorhetic priority of milk production occurs at insufficient DMI, and feed restriction does not influence MY (McNamara & Hillers, 1986). However, restricted feed intake during phase 2 (after the period of NEB) causes a reduction of MY (Gross & Bruckmaier, 2015), which indicates that the new priority is now for the mother and pregnancy. Remarkably, the nutrition of the calves is ensured in both phases: homeorhesis directly ensures the nutrition of calf 1 in phase 1 and of calf 2 indirectly in phase 2.

Metabolic stress: Metabolic stress is an often and broadly used term to describe the metabolic changes and challenges during early lactation. This term should thus only be used in the sense of Knight et al. (1999) with the meaning "that amount of metabolic load (or metabolic burden) which cannot be sustained (or tolerated), such that some energetic processes (which could include those maintaining general health) *must be down regulated*". A corresponding conclusion has been made by Sordillo and Mavangira (2014): "A major underlying factor responsible for the development of transition cow disorders is metabolic stress, which occurs when cows *fail to adapt* physiologically to an increase of nutrient requirement needed for parturition and the onset of copious milk synthesis and secretion".

An indication of failing adaptation and metabolic stress is the rate of mobilization above the actual requirement (Sordillo & Raphael, 2013); the increase of NEFA causes IR, fat liver, BHB production, ketosis, and secondary diseases. Two imbalances occur at the same time. DMI is lower than requirement, whereas the mobilization of NEFA is above requirement. Growing evidence indicates that the imbalances increase with MY and are probably the major health risk, not MY per se. Mobilization according the requirement and steady state at low NEFA would be a desired ambition as a future goal of selection and management.

Evidence is accumulating that metabolic stress includes inflammation (Trevisi et al., 2012; Sordillo & Raphael, 2013) and oxidative stress (Sordillo & Aitken, 2009). Vice versa, inflammation has an impact on metabolism. Infusion of the proinflammatory cytokine TNF-α in lactating dairy cows causes an increase of NEFA (Kushibiki et al., 2000), a significant increase of TAG in the liver (Bradford et al., 2009), a decrease of transcription of enzymes involved in gluconeogenesis (Bradford et al., 2009), and a decrease of insulin sensitivity (Kushibiki et al., 2000). Hence, metabolic stress involves the risk of subclinical or clinical production diseases including inflammation, and these possible hazards should be mentioned when the term "metabolic stress" is applied

NEB and replenishment: The loss of body reserves during early lactation is generally assumed to be replenished before the next parturition. Increasing evidence suggests that this compensation does not occur in all cases (Waltner et al., 1993; Coffey et al., 2004). A NEB throughout an entire lactation has been monitored by Hurley et al. (2018) in cows reared in a non-intense production system and selected for lower residual energy intake (REI). In agreement, Miglior et al. (2017) have found a negative genetic correlation "between gross feed efficiency and energy balance (from-0.73 to -0.99) indicating that selection for more efficient cows would favour a lower energy status". These findings shed further light on efforts to select cows with higher feed efficiency in order to reduce feed costs, which are the highest cost factor of milk production. If high feed efficiency means that NEB occurs without compensation by replenishment, milk production is maintained at the expense of body reserves. A lack of replenishment should be judged as milk production with abrasion, and "this gradual erosion of body energy stores may be of concern of health, welfare, and profitability viewpoints" (Coffey et al., 2004).

Relief of NEB: The NEB of early lactation is a serious health risk. The insufficient DMI at the onset of lactation (Karacaören et al., 2006; Liinamo et al., 2012; Manzanilla-Pech et al., 2014; Krattenmacher et al., 2019) and the primarily partitioning of nutrients to the mammary gland in high-producing cows (Agnes & Yan, 2000) make the limitation of the NEB with a further increase of MY unlikely, despite manifold efforts of the periparturient nutritional management (see above). Hence, Lacasse et al. (2018) have discussed the possibility of reducing the gap by a *decrease of output*: a) inhibition of milk fat synthesis, b) prepartum milking, c) once-a-day milking, d) incomplete milking, or e) inhibition of lactating signal. They propose, based on current knowledge, incomplete milking for 5 or 6 days p.p. as a tool to reduce the metabolic load or even stress. This would provide load removal at the present but is not a solution for the future if the selection for higher MY continues.

Production and reproduction: The reciprocal relationship between MY (≈ NEB) and fertility (Butler, 2003; Dobson et al., 2008) is usually considered as "antagonism". However, the downregulation of the whole signal cascade of regulation of fertility and pregnancy should be assessed as a biological reaction to insufficient nutrition: "Biologically, it is a predictable response, part of a normal function" (Knight et al., 1999). Any intervention for possible improvement has limitations under these circumstances. Non-observance of these interactions might explain the low conception rate and early embryo death in dairy cows, particularly if hormones (GnrH, prostaglandin) and consecutive AI are applied according to a fixed time scale regardless of energy status or BCS.

Lucy (2001) has published an excellent review with the title "Reproductive loss in high-producing cows: Where will it end?" This urgent question is still valid, because the improved understanding of cyclicity and pregnancy, the more accurate diagnosis by ultrasound, and the application of synchronization programs does not and can hardly lead to any ameliorating improvements without the integration of biological mechanisms.

Separate disease or syndrome? The relationships between production and health risks have been debated for decades (Ott, 1996; Rauw et al., 1998; Knaus, 2009; Ingvartsen, 2006: Oltenacu & Broom, 2010). The underlying under-nutrition with maladaptation and the downregulation of traits that maintain general health (Knight et al., 1999) can be correlated with numerous diseases. Previously, the diseases have been examined and studied primarily as separate events. Studies restricted to one topic will indeed lead to a better understanding of physiological, biochemical, endocrinological, or immunological sequences but have limitations if the pathogenesis of the topic is not embedded into the complex of metabolic stress, inflammation, and immunosuppression. An approach for a better understanding of this complex has been proposed by Sordillo and Mavangira (2014), namely that "a link may exist between metabolic and immune pathways during times of altered nutrient metabolism that can increase the risk of diseases" and suggests a possible common etiology, at least for some of the production diseases. The possible interdependencies are cognizable by the positive genetic correlations of metabolic diseases with other diseases (Pryce et al., 2016; Heringstad et al., 2007). For instance, the strong genetic correlation between ketosis and displaced abomasum (Pryce et al., 2016). Heringstad et al. (2007) has shown that selection against mastitis causes correlated selection responses to other diseases, thereby suggesting a potential common etiology.

As a first step, our knowledge of physiology, biochemistry, immunology, nutrition, veterinary medicine, animal husbandry, and the possible pathophysiological changes and diseases during transition should be summarized and pooled in order to discern possible interactions. The complexity of interactions as implied by Sordillo and Mavangira (2014) should be further studied by an approach considering production diseases as a complex under a common umbrella of "hormonal and metabolic challenges, inflammation, and immunosuppression". This will probably raise the "chicken and egg" issue, but suggestions for solving one problem in isolation without consideration of the whole complex have obvious limitations.

Management: Proper management, including all environmental factors, do indeed determine the success of a dairy herd production and health. However, the genetic disposition to various health risks in high-producing cows remains, and good management can reduce or perhaps even obviate these risks. Vice versa, insufficient management will exacerbate these risks and increase the incidence of production diseases (Figure 2).

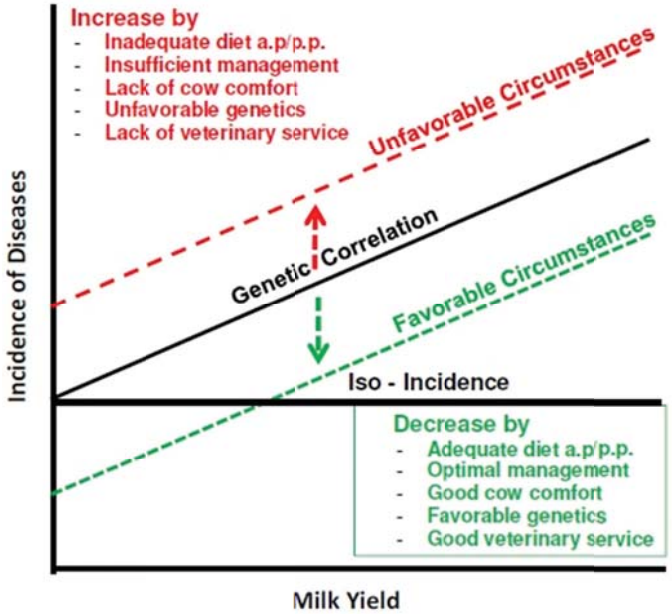

Figure 2. Scheme of correlation (possibly not linear) between milk yield (≈ NEB) and incidence of diseases. This relationship exhibits wide variation, and the incidence of diseases depends on the individual circumstances and might even be absent (iso-incidence). (Modified from Martens, 2016)

Figure 2 highlights the dominant impact of management and might explain the controversy seen in the literature. Fleischer et al. (2001) have demonstrated an exponential increase of various diseases with raising MY, but this is absent in the study of Wangler and Sanftleben (2007). The possible health risks are probably compensated by proper management and confirm the allocation theory of Beilharz et al. (1993). Selection for one trait is possible with no risk for fitness at increased resources and allocation for all traits. However, the primary obligation of optimal management or of good veterinary service does not involve compensation for the genetic disposition of health risks. Further, the major relevance of good management does not eliminate genetic disposition.

An analysis of the etiology of production diseases should therefore distinguish between the cause (genetic disposition) and effects of management. Unfortunately, the differentiation between cause and effect is difficult in practice (except for severe failures of management). However, most of the diseases in dairy cows are observed within the first month p.p. (Carvalho et al., 2019) and therefore during NEB and in association with the corresponding metabolic stress that occur during milk production. The high incidence of subclinical ketosis during early lactation, its heritability (Dohoo et al., 1984; van Dorp et al., 1998), and the genetic correlation between MY and ketosis (Simianer et al., 1991; Uribe et al., 1995) support this conclusion. Hence, genetic disposition is suggested to be the dominant player in the pathogenesis of diseases during this period.

Breeding index: During most of the last century, the primary selection traits for dairy cows were higher production and conformation (for details, see Miglior et al., 2017). This has fundamentally changed during last decades. In 1978, the total merit index (TMI) with balanced breeding goals was introduced for the selection of Norwegian Reds and led to the genetic improvement of production, fertility, and health (https://www.norwegianred.com/Start/Norwegian-Red/about-norwegian-red/Norwegian-Red-Total-Merit-Index/). The implementation of the additional traits was possible, because corresponding data had been available since 1975 from the national health recording system in Norway. This integration has resulted in a significant reduction of clinical mastitis in Norwegian Red cows (Heringstad et al., 2000; Heringstad & Østerås, 2013). Mastitis resistance in the TMI has been frequently adopted by other Scandinavian countries: Finland and Sweden in 1983 and Denmark in 1990 (Heringstad et al., 2000).

Since that time, TMIs have been adopted by many countries (Miglior et al., 2005; Brade, 2018). Miglior et al. (2005) analyzed the selection index from 15 countries and determined, on average, values for production of 59.5%, for durability of 28.0%, and for health of 12.5%. Large variation was observed if the emphasis was on production. Brade (2018) confirmed this variation. Production accounted for 26% in the Netherlands and for 74% in Japan in 2017. Various sub-indices such as fertility, calving performance, somatic cell count, longevity (length of productive life), workability, and claw and udder health are included in TMI.

A significant shift in animal breeding occurred with the implementation of genomic selection in the United States, Great Britain, Ireland, New Zealand, Australia, the Netherlands, France, the Scandinavian countries, and Germany (Silva et al., 2014). Huge numbers of genomics markers of the livestock genome (single nucleotide polymorphisms-SNP) are available and, for instance, have been demonstrated for production traits such as lactation curve, milk, fat, and protein (Oliveira et al., 2019) and various health traits such as displaced abomasum (Zerbin et al., 2015; Lehner et al., 2018), cystic ovaries, displaced abomasum, ketosis, lameness, mastitis, metritis, retained placenta (Parker Gaddis et al., 2014), mastitis, metritis, retained placenta, displaced abomasum, ketosis, and lameness, (Vukasinovic et al., 2017), association between reproductive performance and serum IGF-1 (Gobikrushanth et al., 2018), ketosis (Parker Gaddis et al., 2018; Kroezen et al., 2018), and somatic cell score (Oliveira et al., 2019). As a consequence, Zoetis Genetic offered, for the first time, an evaluation for wellness traits of Holsteins.

Furthermore, the incorporation of future possible traits such as feed efficiency, immune response, or resistance to infections, and of environmentally friendly animals with less waste and lower greenhouse gases production are being considered (Weller et al., 2017; Miglior et al., 2017), as are the inclusion of data from sensors as a part of precise farming and from mid-infrared analyses (for references, see review Pryce et al., 2016). These new technologies and, indeed, broad extended knowledge are promising and will hopefully integrate the challenge of antagonism between MY and DMI as recently discussed by Krattenmacher et al. (2019).

Conclusions: High-producing cows pass p.p. through a period of NEB. The extent and duration of this under-nutrition has been changed dramatically during the last 3 to 4 decades and can hardly be considered as "natural". Successful adaptation in the sense of "having control of mental and bodily stability" (Fraser & Broom, 1990) and of "the physiological capability to respond properly, and thus maintain homeostasis" (Siegel, 1995) appears to be impossible in many cases. The consequences are well known: the incidences of fresh cow or production diseases and early death and the premature culling rate are high.

In the past and even today, health risks and diseases have been and are being primarily studied and treated as separate events. In spite of this better knowledge, no relief or improvements in fertility and pregnancy or in mastitis or lameness are apparent. For instance, German data show that the culling rate attributable to lameness has increased over time (Vereinigte Informationssysteme Tierhaltung-vit, 2016), and "no studies have reported a reduction in the prevalence of lameness over the last 20 years" (Heringstad et al., 2018). The incidence of clinical mastitis in the USA increased from 13% in 1996 (USDA, 1996) to 25% in 2016 (USDA, 2016). Further, the annual incidence risk of the mortality of cows has been described in a meta-analysis over 25 years: an increase from some 2% in the 1980s to 6-8% in 2008 or by 2% per decade has occurred (Compton et al., 2017). This observation hardly supports a general mitigation of health risks, although the remarkable decrease of mastitis in the Norwegian Red (Heringstad & Østerås, 2013) and the GWS (genome-wide selection) and genetic selection are promising aspects for the future.

The myriad studies of separate events have improved our knowledge, and in the current reviews (part 1 + 2), these data have been used in an attempt to create a synopsis between the physiology of homeorhesis, its changes by selection and management, high MY, broad NEB with hormonal and metabolic changes, ketosis, and fertility by means of an approach to disclose dependencies and causal sequences. The possible convergence should be extended by the addition of further topics to achieve a better understanding of the whole complexity. The integration of immunology, endocrinology (metabolism and fertility), biochemistry, physiology, nutrition, veterinary medicine, and animal husbandry needs to be deepened.

The current breeding indices should encompass further health traits having a predominant emphasis and urgency, including parameters of energy metabolism (metabolites and hormones, the balance of nutrients) for sustainable breeding goals and a long-life cycle of dairy cattle production. The negative or minutely positive genetic correlation between MY and DMI during early lactation with the resulting NEB is an underlying burden for production and health in current breeding indices. With other words: "Genetic selection for high-producing healthy cows should reverse current trends and create a future cow that can transition well and produce

successfully in high metabolic environment" (Lucy, 2016). Impaired fertility and reproduction under these conditions is primarily a physiological reaction, not a disease, and requires our special attention.

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Waste Nutrient Solution as an Alternative Fertilizer in Curled Mallow Cultivation

Chungkeun Lee[1], Dong Sub Kim[2], Yurina Kwack[3] & Changhoo Chun[1,4]

[1] Department of Plant Science, Seoul National University, Seoul, Korea

[2] Department of Plant Science, University of California Davis, Davis, California, USA

[3] Division of EcoDivision of Eco-friendly Horticulture, Yonam College, Cheonan, Korea

[4] Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul, Korea

Correspondence: Changhoo Chun, Department of Plant Science, Seoul National University, Seoul 151-921, Korea. Tel: 82-2-880-4567. E-mail: changhoo@snu.ac.kr

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Abstract

To determine the feasibility of reusing waste nutrient solution as an alternative fertilizer for vegetable production, we investigated the growth and shoot nutrient content of curled mallows (*Malva verticillata* L.) irrigated with tap water (pH 7.8, EC: 0.5 $dS \cdot m^{-1}$), nutrient solution (pH 5.7, EC: 2.7 $dS \cdot m^{-1}$) and waste nutrient solution (pH 5.0, EC: 2.2 $dS \cdot m^{-1}$ in average) collected from plant factories. Three waste nutrient solutions were applied in sequential order to a waste nutrient solution treatment and mineral compositions of irrigation solutions were analyzed. We measured the total number of leaves, fresh and dry weight, chlorophyll content (SPAD value) and total phenolic content of curled mallow shoots and analyzed shoot and soil nutrient content using inductively coupled plasma-optical emission spectrometer (ICP-OES). Curled mallows were harvested twice during the cultivation. Curled mallows irrigated with waste nutrient solutions had a similar fresh weight (yield), total phenolic content and number of leaves compared to those grown with fresh nutrient solution, and had higher fresh and dry weight, chlorophyll content (SPAD value) and percentage dry weight compared to those grown with tap water upon first and second harvest. The dry weight of curled mallows grown in waste nutrient solution was lower than that of plants grown in nutrient solution on first harvest, but there was no significant difference between the waste nutrient solution and nutrient solution groups on second harvest. Curled mallows irrigated with nutrient solution and waste nutrient solution showed similar mineral content. These results suggest that waste nutrient solution in curled mallow cultivation could be reused and provide more efficient and sustainable nutritional solutions that improve the productive yields of crops in the agriculture sector.

Keywords: fertigation, hydroponics, mineral absorption, nutrient content, greenhouse

1. Introduction

In recent years, hydroponic systems have become increasingly popular in the greenhouse industry (Savvas & Gruda, 2018). The hydroponic crop production increased up to approximately 30,000 ha in 2015 (Simpkins, Jungers, & Stimmel, 2015) and the hydroponic market is expected to have annual growth rate of 6.5% from 2018 to 2023 (Mordor Intelligence, 2018). As a consequence of the development of the hydroponics industry, the amount of waste nutrient solution discharged from hydroponic systems has also increased.

Discharged waste nutrient solution generally contains high concentrations used to increase crop yield. Nutrient solutions in closed hydroponic systems are periodically dumped during crop cultivation to prevent accumulation of salt ions, which can cause serious growth disorders (Sánchez-Guerrero, Lorenzo, Medrano, Baille, & Castilla, 2009; Savvas, Meletiou, Margariti, Tsirogiannis, & Kotsiras, 2005; Savvas et al., 2007) and plant diseases that can reduce crop yield and even eliminate all crops at once (Badgery-Parker, 2002; Grewal, Maheshwari, & Parks, 2011). Zekki, Gauthier, and Gosselin (1996) also noted that prolonged recycling of nutrient solution can cause yield reduction in closed hydroponic systems. In addition, the volume and concentration of applied nutrient solution are generally higher than those required for plant growth in order to satisfy variability in irrigation equipment and to maximize crop yield (Grasselly et al., 2005; Rouphael & Colla, 2009).

Increasing amounts of nutrient rich waste solution from hydroponic systems pose a major environmental concern. Waste nutrient solution contains high concentrations of nitrogen and phosphorus and discharge can lead to the eutrophication of lagoon water and groundwater, causing oxygen depletion and toxin release through algal blooms, severely contaminating drinking water (Kumar & Cho, 2014; Prystay & Lo, 2001). Grewal et al. (2011) reported that approximately 60% of irrigated nutrient solution could be discharged as drainage water in an open hydroponic system and that reusing waste nutrient solution could help reduce environmental pollution.

Many studies have explored crop cultivation using municipal and domestic wastewater (Cirelli et al., 2012; Urbano, Mendoca, Bastos, & Souza, 2017) and animal manure vermicompost (Gutiérrez-Miceli et al., 2007; Mowa, Akundabweni, Chimwamurombe, Oku, & Mupambwa, 2017). In these studies, reusing the nutrients from waste had no negative effects on crop growth. However, only a few studies have examined the potential use of waste nutrient solution from plant cultivation in hydroponic systems as an appropriate irrigation solution. Furthermore, most of the studies on waste nutrient solution from hydroponics focused on the different effects of irrigation solutions on plant growth, but did not analyze the mineral content of plant tissue (Choi, Lee, & Ok, 2011a; Choi et al., 2011b; Zhang, Kang, & Kim, 2006), nor did they include detailed analysis of soil and plant nutrient content (Kim et al., 2000; Park, Kim, Yoo, Ok, & Yang, 2005; Zhang, Lim, Kang, & Kim, 2010).

Curled mallow (*M. verticillata*) is a leafy vegetable popular in East Asia and known for its several pharmaceutical effects (Ko et al., 2019). The plants were harvested twice in this experiment to compare the effects of irrigation solutions over cultivation time. The objectives of this study were to investigate the growth and nutrient content of curled mallows irrigated with waste nutrient solution, nutrient solution and tap water, and to confirm the feasibility of reusing waste nutrient solution as an alternative fertilizer in curled mallow cultivation.

2. Material and Method

2.1 Plant Material and Growth Conditions

Curled mallows (*Malva verticillata* L.) were grown from May 23 to August 1, 2014 at the experimental farm of Seoul National University (Suwon, Korea, 37.3°N, 127.0°E). During the cultivation period in a greenhouse, the mean air temperature was approximately 24 °C and maximum and minimum air temperatures were 29 and 21 °C at the experimental site (Figure 1).

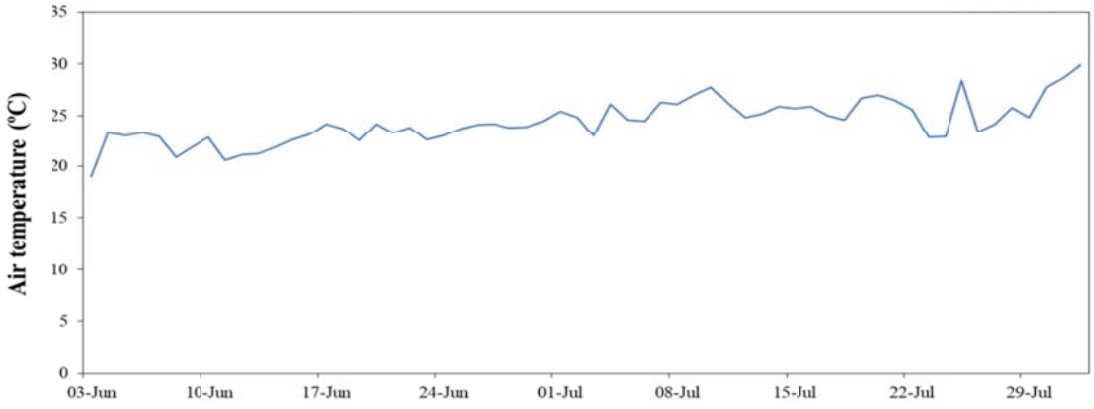

Figure 1. Air temperature during the cultivation period at the experimental site

The seedlings were grown in the plant factory using fluorescent lamps as a light source (air temperature during the photo/dark period, 25/21 °C; photo/dark period, 16/8 h; light intensity, PPF 210 $\mu mol \cdot m^{-2} \cdot s^{-1}$; CO_2 concentration, 750 $\mu mol \cdot mol^{-1}$) for 11 days in 128-cell trays. Seedlings were selected randomly and five plants were transplanted to each plastic pot (180 × 600 × 150 mm) filled with commercial soil on June 3. Pots were drip-irrigated with the same amount on each treatment when necessary considering the growth environment (Figure 2).

Figure 2. Curled mallows drip-irrigated with three irrigation solutions in a greenhouse

Nutrient solution (NS), waste nutrient solution (WNS) and tap water (TW) were used as the irrigation solution. The nutrient solution was prepared according to the Japanese standard 'Enshi' formula, which is widely used for vegetable crop cultivation (Yamasaki, Suzuki, & Shinohara, 1976). Waste nutrient solutions were collected from three different plant factories using a deep flow technique (DFT) hydroponic system cultivating spinach. Nutrient solution was applied on June 10 in NS treatment and three waste nutrient solutions were applied in sequential order from June 10 to 19 (waste nutrient solution 1), June 20 to July 20 (waste nutrient solution 2) and July 21 to August 1 (waste nutrient solution 3), respectively, to WNS treatment.

The pH and electrical conductivity (EC) levels of irrigation solution were measured with a pH and EC meter (D-54, Spectrum Technologies, Inc., Aurora, IL, USA) and the ion concentrations (NO_3, NH_4, PO_4, K, Ca, Mg, Na, Cl and SO_4) were determined via ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) equipped with a Dionex IonPac CS12A 4 mm × 250 mm column (cation) and AS20 4 mm × 250 mm column (anion). The cation and anion composition of the irrigation solution were calculated as ratios of molar concentration and positioned as a single point in trilinear coordinates using the method of De Rijck and Schrevens (1998).

2.2 Growth Analysis

Curled mallows were harvested on July 1 (28 days after transplanting, DAT, first harvest) and again on August 1 (59 DAT, second harvest) after the shoot had regenerated. Plants were harvested 20 mm from the bottom of the stem and all the remaining leaves of the curled mallows were removed at the first harvest in order to provide similar conditions for the three different treatment groups in the second cultivation. We selected nine plants randomly and measured the chlorophyll index using a chlorophyll meter (SPAD-502, Minolta Co., Ltd., Tokyo, Japan). The total number of leaves and fresh and dry weight were also recorded and the percentage dry weight was calculated as the ratio between fresh weight and dry weight.

2.3 Chemical Analysis

Soil and plant samples were oven-dried at 80 ^{o}C for at least 72 h to analyze nutrient content. Nitrogen was measured with a Kjeldahl nitrogen analyzer (Kjeltic Auto 1038 system, Tecator AB, Hoganas, Sweden) using the Kjeldahl method (Kjeldahl, 1883) and P, K, Ca and Mg were analyzed using an inductively coupled plasma-optical emission spectrometer (ICP-OES) (ICP-730ES, Varian, Mulgrave, Australia) with a modified method from Adesemoye, Torbert, and Kloepper (2008).

For soil analysis, a modified Mehlich 1 (double acid) extraction method was used (Mehlich, 1953). Each soil sample was dried and sieved using a 0.60 mm stainless steel sieve. After 1.4 g of sieved soil and 12.5 mL of Mehlich 1 extraction solution (0.05 mol·L^{-1} H_2SO_4 + 0.05 mol·L^{-1} HCl) was added to a 50 mL extraction flask, it was shaken for 5 min on a reciprocating shaker (HB-203L, Hanbaek Scientific Co., Bucheon, Republic of Korea) at 180 oscillations·min^{-1}. The solution was filtered through Whatman No. 2 filter paper and an extra 1.5 mL of Mehlich 1 extraction solution was added during the filtering process. The filtrate was sent to National Instrumentation Center for Environmental Management (NICEM, Seoul National University, Seoul, Korea) and analyzed using ICP-OES technique to determine the mineral composition of the soil sample.

For plant analysis, 0.5 g of fine dry powder of the leaf and stem tissues was placed into a 50 mL beaker, covered with aluminum foil, and was heated to 450 ^{o}C for 4 h in a muffle furnace (SH MF2C, Samheung Energy Inc., Sejong, Korea). After heating, 10 mL of 1 mol·L^{-1} HNO_3 was added to the plant ash and evaporated slowly on a hot plate until it dried. Then, 10 mL of 1 mol·L^{-1} HCl was added to the beaker, heated almost to boiling, and transferred to a 100 mL volumetric flask. The beaker was washed three times with small amounts of water to

minimize mineral loss, and water was added until the amount of solution in the flask reached 100 mL. The solution was filtered through Whatman No. 2 filter paper and the filtrate was analyzed by NICEM (Seoul National University, Seoul, Korea) using ICP-OES to determine the mineral composition of the plant sample.

The total amount of phenolic compounds in each plant sample was determined using the Folin-Ciocalteu assay (Slinkard & Singleton, 1977). First, 0.2 g of the fine dry powder was mixed with 12 mL of extraction solution (acetone:methanol:water:acetic acid = 40:40:20:1) in a 50 mL conical centrifuge tube for 10 s using a homogenizer (PT-MR2100, Kinematica AG, Lucerne, Switzerland). The tube was kept at 60 ^{o}C for 1 h and more extraction solution was added until the amount of solution in the tube reached 20 mL, followed by filtration. Subsequently, 1 mL of the filtrate was mixed with 1 mL of 10% Folin-Ciocalteu phenol reagent and 1 mL of 7.5% Na_2CO_3 solution, in sequence. After incubation for 2 h at room temperature, absorbance was determined using a UV visible spectrophotometer (UV-2550, Shimadzu Corp., Kyoto, Japan) at 726 nm. The same procedure was repeated with garlic acid solutions (0, 0.1, 0.2 and 0.5 mg·mL^{-1}) and a standard curve was obtained.

2.4 Statistical Analysis

SAS version 9.2 software (SAS Institute INC., Cary, NC, USA) was used for analysis of variance (ANOVA). Differences among treatment means were separated by the Fisher's least significant difference (LSD) test at $P < 0.05$.

3. Results and Discussion

3.1 Quality of Irrigation Solution

The mineral composition of nutrient solution, waste nutrient solution and tap water used for curled mallow irrigation are shown in Table 1. Waste nutrient solution generally has low EC and pH levels and high Na, Cl and SO_4 ion concentrations compared to regular nutrient solution (Kumar & Cho, 2014). In this experiment, all waste nutrient solutions applied to WNS treatment group had lower EC and pH levels and NO_3, NH_4, PO_4 and K concentration compared to nutrient solution, while Mg, Na, Cl and SO_4 concentrations were higher in waste nutrient solution. This corresponds with the results of Zekki et al. (1996) and Zhang et al. (2010), who reported Mg, Na, Cl and SO_4 concentration increased in the waste nutrient solution as the crops grew.

Table 1. The pH, EC and ion concentrations of nutrient solution, waste nutrient solution and tap water.

Treatment	pH	EC (dS·m^{-1})	Ion concentration (mg·L^{-1})								
			NO_3	NH_4	PO_4	K	Ca	Mg	Na	Cl	SO_4
Nutrient solution	5.7	2.7	1236.4	80.9	358.9	322.9	179.2	24.5	12.8	9.8	74.3
Waste nutrient solution 1	5.4	2.6	1178.6	41.3	277.8	299.3	194.2	25.9	32.6	14.1	89.1
Waste nutrient solution 2	4.6	2.0	934.5	19.6	145.2	252.8	166.3	41.6	17.0	11.2	137.8
Waste nutrient solution 3	5.0	2.0	806.8	13.7	82.7	226.9	144.6	42.9	15.5	13.1	145.0
Tap water	7.8	0.5	4.9	n.d^{z}	n.d	2.0	8.8	1.2	8.5	17.0	13.4

Note. ^{z}Not detected.

The chemical composition of nutrient solutions is determined not only based on EC and pH, but also the relative ion proportions (Steiner, 1961). In this experiment, the cation and anion composition of three waste nutrient solutions were similar to that of nutrient solution (Figure 3). The position of each point within an equilateral triangle was determined based on cation (K, Mg and Ca) and anion (NO_3, SO_4 and PO_4) ratios, and the length of the perpendiculars from the point to each side of the triangle corresponded to the proportion of each ion (De Rijck & Schrevens, 1998). The average Euclidean distance between the nutrient solution and waste nutrient solution was 0.07 for cation and anion composition, while that between nutrient solution and tap water was 0.47 and 0.63, respectively, in trilinear coordinates. This suggests that ion proportions of waste nutrient solutions from closed hydroponic spinach cultivation systems would not have negative effects on curled mallow cultivation in that the relative ion ratios of waste nutrient solutions were similar to those of 'Enshi' nutrient solution which ion balance was designed for various vegetables in all growth stages (Park & Kim, 1998).

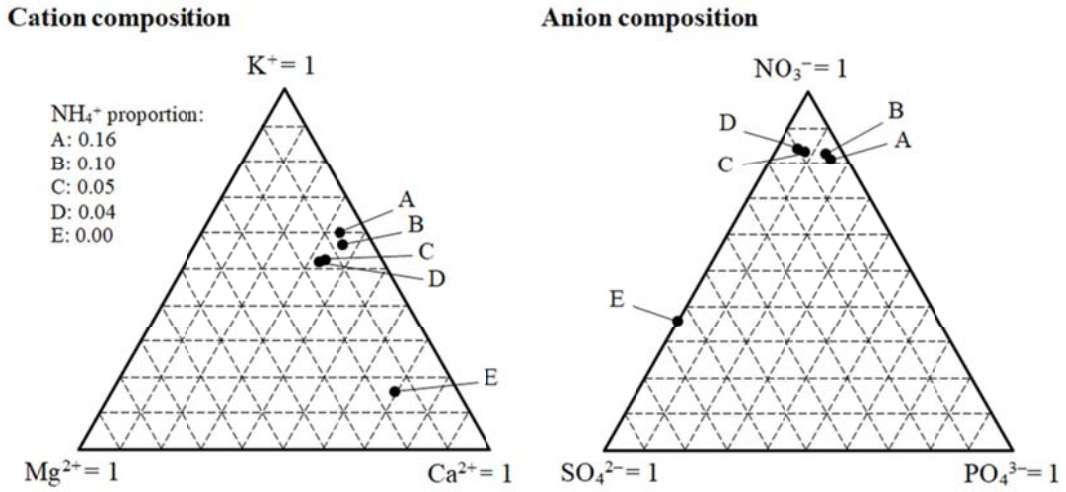

Figure 3. Cation and anion composition of the irrigation solutions. A: Nutrient solution; B: Waste nutrient solution 1; C: Waste nutrient solution 2; D: Waste nutrient solution 3; E: Tap water

According to Van Os (1999) and Tüzel et al. (2000), a closed hydroponic system provides approximately 20-40% less irrigation water and nutrients to plant than an open hydroponic system, but with a longer absorption time. Assuming that crops absorb the same amount of nutrients in the two different hydroponic systems, waste nutrient solution discharged from closed hydroponics would contain fewer minerals than open hydroponics. However, the waste nutrient solution in this experiment contained NO_3, PO_4 and K concentrations that represented 79, 47 and 80% of the concentrations in the applied nutrient solution, respectively, which seemed relatively high for effluents from closed hydroponic systems. This could be because the waste nutrient solution in this experiment was discharged from a closed hydroponic system at an early stage of the cultivation period to prevent salt accumulation, which could reduce plant growth and restrict P, K and Mg content in leaves (Savvas et al., 2005). In addition, according to Medrano et al. (2005) and Terebayashi, Takii, and Namiki (1991), every waste nutrient solution has a different mineral concentration and composition because different kinds of plants absorb different amounts of minerals throughout their growth in hydroponic systems. Grewal et al. (2011) also mentioned that the efficiency of hydroponic systems could vary depending on their design and the way the water and nutrient applications are managed.

3.2 Plant Growth

The number of leaves of curled mallows irrigated with waste nutrient solution did not vary significantly from that of nutrient solution and tap water upon first harvest (Figure 4A). Upon second harvest, the number of leaves in the WNS group (8.7 per plant) did not differ significantly compared with the NS group, but was 39.3% higher than that in the TW group (6.2 per plant) (Figure 4F).

The chlorophyll content (SPAD value) differed significantly among NS, WNS and TW groups at the first and second harvests (Figures 4B and 4G). WNS group plants had 8.4 and 6.7% lower SPAD value (44.0 and 40.5) compared to NS plants (48.0 and 43.4), while the SPAD value in WNS plants was higher by 18.4 and 22.6% compared to that in TW plants (37.1 and 33.0) upon first and second harvest, respectively. Several studies have shown that chlorophyll content positively correlated with leaf N content (Chapman & Barreto, 1997; Nageswara Rao, Talwar, & Wright, 2001) and Mg content (Ding et al., 2008). In our study, curled mallows irrigated with waste nutrient solution had higher N and Mg content compared to those irrigated with tap water, which corresponded well with previous research, but curled mallows irrigated with nutrient solution showed similar N content and lower Mg content compared to those irrigated with waste nutrient solution, although the SPAD value was higher in the NS group (Table 2). This could be due to the high salt concentration of waste nutrient solution, which could lead to salt stress in chlorophyll synthesis (Santos, 2004), but further investigation of this relationship is needed.

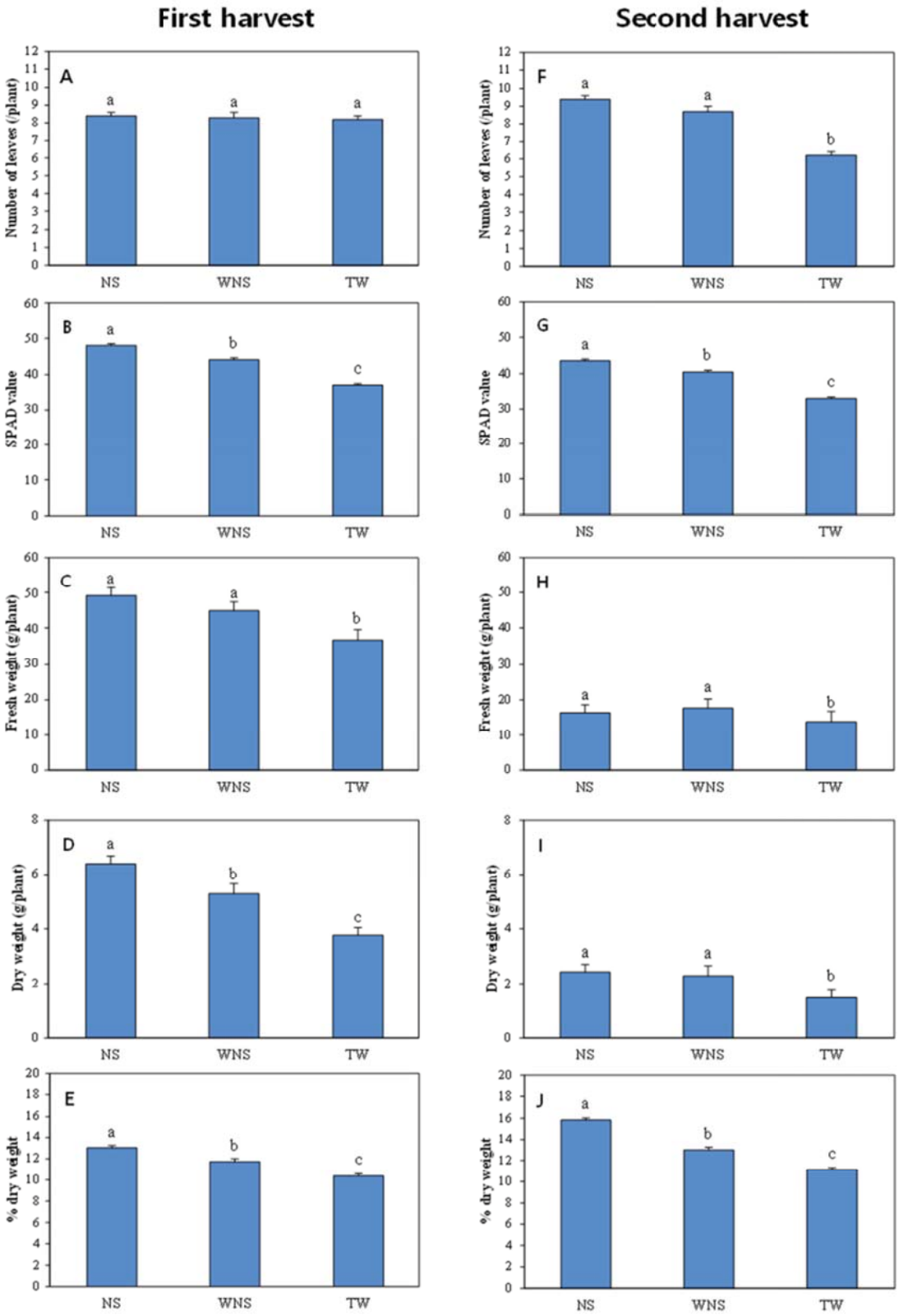

Figure 4. The number of leaves (A and F), SPAD value (B and G), fresh weight (C and H), dry weight (D and I) and percentage dry weight (E and J) of curled mallows in the NS, WNS and TW groups at the first and second harvest. NS: treatment with nutrient solution; WNS: treatment with three waste nutrient solutions; TW: treatment with tap water. Means within the same column followed by the same letter are not significantly different at the $P = 0.05$ level of probability based on Fisher's least significant difference (LSD) statistics

Table 2. Nutrient content of curled mallows irrigated with nutrient solution, waste nutrient solution and tap water at the first and second harvest

Treatment	N (%)	P ($mg \cdot g^{-1}$)	K ($mg \cdot g^{-1}$)	Ca ($mg \cdot g^{-1}$)	Mg ($mg \cdot g^{-1}$)
First harvest					
Nutrient solution	4.65 a[z]	7.80 a	64.18 a	18.56 a	2.93 b
Waste nutrient solution (1-3)	4.31 a	7.91 a	63.36 a	20.05 a	3.55 a
Tap water	3.40 b	4.64 b	62.15 a	14.41 b	3.25 ab
Second harvest					
Nutrient solution	4.15 ab	6.72 a	40.42 b	24.29 ab	4.89 b
Waste nutrient solution (1-3)	4.94 a	7.27 a	45.50 b	26.84 a	5.77 a
Tap water	3.87 b	6.65 a	59.00 a	20.37 b	4.44 b

Note. [z]Means within the same column followed by the same letter are not significantly different at the $P = 0.05$ level of probability based on Fisher's least significant difference (LSD) statistics.

The fresh weight of curled mallows irrigated with waste nutrient solution (44.8 and 17.6 g) did not differ significantly from nutrient solution (49.1 and 16.3 g), but was 22.9 and 28.7% higher compared to those irrigated with tap water (36.5 and 13.7 g) upon first and second harvest (Figures 4C and 4H). The total yield (fresh weight per unit area on first and second harvest) of curled mallows in the WNS group was 4.5% lower compared to that in the NS group, but was 24.5% higher compared to the TW group. This suggests that crops cultivated with waste nutrient solution are economically equivalent to those cultivated with nutrient solution, and economically superior to the use of tap water.

The dry weight of the WNS group (5.3 g) was 17.4% lower than that of NS (6.4 g) upon first harvest, but there was no significant difference between the two treatments upon second harvest, which implies a relative increase in the dry weight of WNS compared to NS during the period between harvest stages (Figures 4D and 4I). The dry weight indicates the amount of surplus carbohydrates from photosynthesis and respiration, and the results suggest that curled mallows irrigated with waste nutrient solution can accumulate similar amounts of carbohydrates to those irrigated with nutrient solution by second harvest. As the nutrient uptake of plants can change with plant growth stage, the nutrient requirements might decrease with increasing plant age and thus the nutrient content of WNS could meet the requirements for plant growth after the first harvest. The curled mallows in the WNS group (5.3 and 2.3 g) had 40.5 and 51.7% higher dry weight compared to that of the TW group (3.8 and 1.5 g) upon first and second harvest, respectively. Choi et al. (2011a) reported similar results for fresh and dry weights of Chinese cabbage irrigated with waste nutrient solution, which were similar or slightly lower than those irrigated with nutrient solution and similar or higher than those irrigated with groundwater 26 and 56 DAT. According to Kim et al. (2000), poinsettia irrigated with waste nutrient solution had higher fresh and dry weights of shoot compared to nutrient solution-irrigated groups. Park et al. (2005) also reported that red pepper (*Capsicum annum* L.) irrigated with hydroponic wastewater had a similar yield to those irrigated with nutrient solution and a more than 20% higher yield compared to tap water.

The percentage dry weight of curled mallows differed significantly among the three treatment groups (Figures 4E and 4J). The percentage dry weight in the WNS group (0.12) was 9.8% lower compared to the NS group (0.13) and higher by 13.2% compared to the TW group (0.1) at the first harvest. Upon second harvest, the percentage dry weight of curled mallows in the WNS group (0.13) was 13.0% lower and 15.8% higher compared to the NS (0.15) and TW groups (0.11), respectively. This suggests that the percentage dry weight is positively correlated with SPAD value (Figures 4B and 4G) which generally correlates with photosynthesis. Jacobson (1945) reported a relationship between the percentage dry weight and photosynthesis, with higher rates of photosynthesis correlating with greater percentage dry weight in green leaves when compared leaves with chlorosis.

3.3 Nutrient Content of Soil

The nutrient content of the soil for WNS plants did not vary from that of NS plants in N and P at the first harvest (Table 3). Considering that the N and P concentrations of nutrient solution were higher than those of waste nutrient solution, similar residual mineral levels in the soil of the NS and WNS groups implies that NS plants absorbed more N and P compared to WNS plants. This could be confirmed based on the total nutrient content of curled mallow shoots, which was calculated by multiplying the average plant nutrient concentration (Table 2) and dry weight (Figures 4D and 4I), with the results showing 30.8 and 19.1% higher N and P content in the NS

group compared to the WNS group, respectively. The soil nutrient content in the WNS group was 0.13%, 1.09 $g \cdot kg^{-1}$ and 0.91 $g \cdot kg^{-1}$ higher in N, P and K concentrations, respectively, compared to the TW group, but was 0.85 $g \cdot kg^{-1}$ lower in K concentration compared to the NS group on first harvest. The Ca concentration in the WNS group was lower than that in the NS group and higher than that in the TW group, while Mg concentration in the WNS group was the highest among the three treatments.

Table 3. Mean values (±standard error) of nutrient content in soil before cultivation and after the first and second harvest.

Treatment	N (%)	P ($g \cdot kg^{-1}$)	K ($g \cdot kg^{-1}$)	Ca ($g \cdot kg^{-1}$)	Mg ($g \cdot kg^{-1}$)
Commercial soil	0.51	1.21	0.90	7.14	2.14
First harvest					
Nutrient solution	0.54±0.01	2.26±0.16	2.65±0.55	9.44±1.22	1.78±0.29
Waste nutrient solution (1-3)	0.56±0.00	2.22±0.02	1.80±0.13	8.49±0.29	2.51±0.46
Tap water	0.43±0.02	1.13±0.27	0.88±0.04	6.47±0.03	1.73±0.08
Second harvest					
Nutrient solution	0.82±0.06	6.25±0.36	4.92±0.25	15.60±1.05	2.63±0.15
Waste nutrient solution (1-3)	0.57±0.03	2.72±0.48	2.49±0.09	8.81±0.78	1.95±0.17
Tap water	0.46±0.02	0.28±0.01	0.70±0.01	5.24±0.00	1.43±0.01

The soil mineral concentrations in the WNS group were lower compared to NS and higher compared to TW for all minerals analyzed upon second harvest. The N, P and K concentrations of soil in the WNS group were 0.25%, 3.53 $g \cdot kg^{-1}$ and 2.43 $g \cdot kg^{-1}$ lower compared to NS, but 0.11%, 2.44 $g \cdot kg^{-1}$ and 1.78 $g \cdot kg^{-1}$ higher compared to TW, respectively. All soil mineral concentrations in the NS and WNS groups increased upon second harvest compared to first harvest except for Mg concentration in WNS. The observed increases in N, P and K between the two harvest stages were 51.9, 176.7 and 85.6% in NS, while those in WNS were only 1.8, 22.6 and 38.4%, respectively. The difference in soil mineral concentration between the two harvest stages indicates the amount of residual soil nutrient content after mineral absorption by plants in the second cultivation period, and the above results demonstrated that more nutrients were provided to the NS group than the plants needed, causing the accumulation of excess minerals in the soil (Ju, Kou, Christie, Dou, & Zhang, 2007). The same was not seen for the WNS group. This suggests that using waste nutrient solution for irrigation instead of nutrient solution could decrease water pollution related to leaching of excess soil nutrients.

3.4 Nutrient Content of Plant Samples

Nutrient content per gram of curled mallows (leaf and stem tissues) irrigated with waste nutrient solution did not differ significantly from that of nutrient solution in N, P, K and Ca concentration upon first and second harvest (Table 2). This could be because waste nutrient solution with low EC and pH and high Na and Cl concentrations was buffered with minerals in the commercial soil. The commercial soil already contained a certain amount of minerals before irrigation and when waste nutrient solution was applied, minerals in both the solution and soil were mixed and provided to curled mallows. In addition, soil nutrients over the optimum concentration level could have affected plant nutrient content. When nutrients are provided in excess of the optimum concentration level, further mineral absorption is limited and plants cultivated within the nutrient sufficiency range contain similar mineral levels in their tissues (Campbell, 2000). In this experiment, even with a considerable excess of residual nutrients in the soil of the NS group on second harvest, curled mallows could not further absorb minerals due to having reached the optimum concentration level. Fallovo, Rouphael, Rea, Battistelli, and Colla (2009) showed similar results, in that the concentration of macronutrients in lettuce was limited even when the nutrient solution concentration exceeded a certain level.

The waste nutrient solution provided during the second cultivation period had lower EC and pH values compared to that during the first period, but nutrient content of curled mallows in the WNS and NS groups did not differ significantly between harvests. The average nutrient content in WNS upon second harvest was higher than that of NS for all analyzed ions, even those that were lower in upon first harvest. This could be due to a decrease in the optimum soil nutrient concentration between harvest stages, or by decreased nutrient uptake rate as the plants age (Warncke & Barber, 1974). Edwards and Barber (1976a, 1976b) also stated that the maximum ion influx of N and P decreased with plant age. This suggests that less minerals will be needed as curled mallows age, and that use of nutrient solution during the first cultivation period and waste nutrient solution during the second

cultivation period could be an environmentally and economically efficient approach to curled mallow cultivation, although chlorophyll content (SPAD value) and percentage dry weight may decrease compared to cultivation with nutrient solution alone (Figure 4).

Irrigation with waste nutrient solution could be an efficient way to dispose of nutrients that otherwise would leach into the environment and cause groundwater contamination. Residual soil minerals in the WNS group, which excluded minerals from commercial soil, included 80.6 and 70.0% lower N and P concentrations, respectively, compared to soil in the NS group (Table 3), while the total nutrient content of curled mallow shoots in the WNS group included 14.4 and 11.6% lower N and P concentrations, respectively, compared to the NS group during the growth period. This implies that irrigation with waste nutrient solution could result in efficient plant uptake of soil nutrients in curled mallow cultivation.

3.5 Total Phenolic Content

Phenolics, which contribute to antioxidant capacity, play an important role in the prevention of diseases and maintenance of health when consumed as food (La Vecchia, Altieri, & Tavani, 2001; Tapiero, Tew, Ba, & Mathe, 2002; P. Terry, J. Terry, & Wolk, 2001). The total phenolic compounds in curled mallow irrigated with nutrient solution varied significantly by 11.8 mg GAE g^{-1} DM (milligrams of garlic acid equivalents per gram of dry matter) from those irrigated with tap water, but did not differ significantly from those irrigated with waste nutrient solution upon first harvest (Figure 5). There were no significant differences in phenolics content among the three treatments on second harvest. This suggests that irrigation with waste nutrient solution could provide as much total phenolic compounds as nutrient solution, and indicated the potential of waste nutrient solution as an irrigation solution for production of antioxidants in curled mallow cultivation.

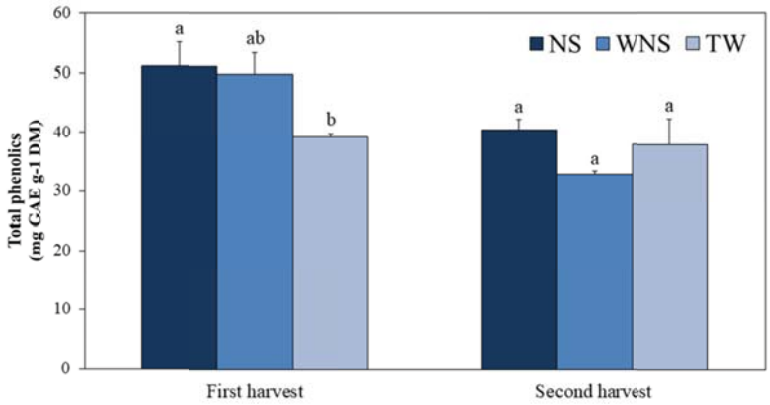

Figure 5. Content of total phenolics in curled mallows irrigated with nutrient solution, waste nutrient solution and tap water at the first and second harvest. NS: treatment with nutrient solution; WNS: treatment with waste nutrient solution; TW: treatment with tap water. Means within the same column followed by the same letter are not significantly different at the $P = 0.05$ level of probability based on Fisher's least significant difference (LSD) statistics

4. Conclusions

The waste nutrient solution used in this experiment was a promising alternative fertilizer for curled mallow cultivation. The yield and mineral content of curled mallow plants irrigated with waste nutrient solution were similar to plants irrigated with nutrient solution, and were significantly higher than plants irrigated with tap water. Total phenolic contents were similar in waste nutrient solution and nutrient solution treatments. Even though decrease in SPAD value and % dry weight needs to be considered in applying waste nutrient solution, substitution with waste nutrient solution could be economically beneficial by reducing the consumption of fertilizer without significantly decreasing yield and nutritional quality. The feasibility of reusing waste nutrient solution in curled mallow cultivation could also reduce environmental pollution by preventing leaching of excess nitrogen and phosphorus into water sources and by reducing the nutrient load of agriculture.

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Impact of Heat Damaged Corn Gluten Meal as Fertilizer on Forage Production During Winter and Summer Seasons and Soil Characteristics

K. J. Han[1] & W. D. Pitman[2]

[1] School of Plant, Environmental, and Soil Sciences, Louisiana State University Agricultural Center, Baton Rouge, Louisiana, USA

[2] Hill Farm Research Station, Louisiana State University Agricultural Center, Homer, Louisiana, USA

Correspondence: K. J. Han, 219 M. B. Sturgis Hall, School of Plant, Environmental, and Soil Sciences, Louisiana State University Agricultural Center, Baton Rouge, Louisiana 70803, USA. Tel: 1-225-578-1305. E-mail: kjhan@agcenter.lsu.edu

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Abstract

Corn gluten meal (CGM) has been used as a supplement for livestock feeding due to its high concentration of digestible nitrogen (N) compounds. Heat damaged CGM (HDCGM), which is not suitable for livestock feeding, may still have value as an organic fertilizer. Objective of the study was to evaluate the impacts of non-feed grade HDCGM on forage production from annual cool and warm season grasses and soil characteristics. Pre-plant incorporated HDCGM at 3 Mg/ha was compared with 4.2 Mg/ha poultry litter (POTL), and 160 kg/ha commercial N fertilizer (COMF), and zero fertilizer (ZERO) for production of the cool-season 'Prine' annual ryegrass (*Lolium multiflorum*), and the warm-season 'Greentreat' sorghum × sudangrass (SS) hybrid (*Sorghum bicolor*). The treatments were repeated at the same site on December 3, 2010 (planted annual ryegrass), May 26, 2011 (planted SS hybrid), October 24, 2011 (planted annual ryegrass) and May 18, 2012 (planted SS hybrid). The HDCGM had 68% more N concentration than POTL, while its P, K, Mg, and Ca were less than half in POTL. The residual N concentration in buried HDCGM and POTL increased in a similar pattern with time in soil. The HDCGM produced less dry matter (DM) of annual ryegrass and SS hybrid than POTL; however, the differences between the two treatments were not statistically significant. All treatments produced more DM in the second than first year. After two years of field test, soil receiving HDCGM contained higher soil organic matter (OM) and N than receiving POTL. Although not as beneficial as POTL for DM production, HDCGM showed potential value as a slow release fertilizer to improve DM production and soil characteristics.

Keywords: heat damaged corn gluten meal, organic fertilizer, poultry litter, forage crop, organic soil amendment

1. Introduction

Continued cultivation of annual forage crops for hay or silage can reduce soil OM and N, and eventually reduce productivity of the land. Approaches to directly supply soil OM through application of organic soil amendments such as poultry litter, which can also provide plant nutrients, have been often used to maintain soil productivity and boost crop production. Poultry litter (POTL) has been used as an alternative to commercial fertilizer, both to recycle a waste material and to provide an economical source of slow release plant nutrients.

Corn gluten meal (CGM) containing around 80 g nitrogen (N) per kg DM has conventionally been used as a protein supplement for livestock. The CGM, a byproduct produced during corn grain processing, has become an alternative to soybean (*Glycine max* (L.) Merr.) meal in livestock feeding. Extraction of starch and oil from the corn (*Zea mays* L.) grain results in the byproduct (CGM) containing a high concentration of N. Although there is variation, the average concentration of N is near 112 g/kg DM in CGM (Wu, 2001), which is higher than in poultry litter. Moreover, degradability of N in CGM is moderately high in the rumen of cattle (Firkins, Berger, Fahey, & Merchen, 1984; Titgemeyer, Merchen, & Berger, 1989), indicating timely degradation potential of CGM in soil for release of available N to forage crops when applied as a soil amendment.

Byproducts often receive less consideration than justified by their economic value, and some CGM has been improperly stored under certain circumstances. When CGM with high moisture is stored in bulk, extensive

bacterial oxidation of residual carbohydrate and protein may occur resulting in excessive heat accumulation. This condition produces an interrelated biochemical reaction resulting in an increased proportion of highly bound N, chemically identifiable as acid detergent insoluble nitrogen (ADIN) in feed materials (Van Soest & Mason, 1991). Thus increased concentrations of heat-damaged N can occur in CGM under improper storage conditions.

Since CGM utilization has been mostly focused on either as a nutritional supplement or pre-emergent herbicidal treatment, information regarding benefit of CGM as fertilizer to increase DM production and improve soil characteristics is scarce. Although not suitable for use as livestock feed, HDCGM still contains a high concentration of N and may be an effective alternative fertilizer with slow N release potential. This study was conducted to evaluate the fertilizer value of non-feed grade CGM, termed as heat damaged CGM (HDCGM), on annual DM production and soil characteristics.

2. Method

2.1 Fertilizer Treatments and Forage Crop Planting

A field plot experiment was conducted to evaluate the fertilizer value of HDCGM in comparison with POTL, a commercial fertilizer (COMF), and an unfertilized control (ZERO) at the Louisiana State University (LSU) Agricultural Center (AgCenter) Southeast Research Station at Franklinton, LA, USA. Soil at the site is a Tangi silt loam (fine-silty, mixed thermic Typic Fragiudult). The HDCGM was donated by Natural Resources Recovery Inc. (Baton Rouge, LA). Poultry litter (POTL) was collected from a broiler house at the LSU AgCenter Hill Farm Research Station. The treatments were arranged in a randomized complete block design with four replications. Plots were 1.5 by 7.6 m with six rows at 20-cm row spacing. Seedbeds were prepared using a BHT55 rotary tiller (Bush Hog® Selma, AL) with the treatments incorporated during seedbed preparation. Soil was subsequently packed using a roller. A small-plot drill planter (Kincaid Equipment Manufacturing, Haven, KS) was used to plant the grasses.

The amount of N applied in the HDCGM, POTL and COMF to each cool-season and warm-season annual forage crop was fixed at 160 kg/ha, which is an upper limit for N rate in a single application for the annual grasses (Eichhorn Jr., Redfearn, & Venuto, 2000). Since the P and K concentrations in HDCGM and POTL differed, treatments were balanced for the N amount first, and then balanced with commercial fertilizer to apply 150 kg/ha of P as superphosphate, and 150 kg/ha of K as potassium chloride. Therefore, 3 Mg HDCGM/ha and 4.2 Mg POTL/ha were applied. The N source of the commercial fertilizer was urea.

"Prine" annual ryegrass (*Lolium multiflorum* Lam.) was planted as a cool-season annual forage on December 3, 2010 and October 24, 2011, at a seeding rate of 34 kg/ha. After three harvests of annual ryegrass, 'Greentreat' sorghum-sudangrass (SS) hybrid (*Sorghum bicolor* (L.) Moench) was planted on the same plots in a double cropping approach at a 15 kg/ha seeding rate on May 26, 2011 and May 18, 2012. Same procedures were used to apply fertilizer treatments and seed forage crops for the both cool-season annual forage crops in both years. Thus grasses were established four times on the same site using the same practices, with individual plots receiving four applications of each treatment during the two years. Weather data were collected from the LSU AgCenter weather station at the Southeast Research Station.

2.2 Bioassay of HDCGM, CGM, and POTL for Degradability

Quadruplicate 0.50-g samples of HDCGM and feed grade CGM were weighed and incubated using the *in vitro* fermentation gas analysis technique (Goering & Van Soest, 1970), as a bioassay of their biological degradation potential. Samples were incubated at 39 ^{o}C in rumen fluid obtained from a cannulated Holstein cow (*Bos taurus*) and buffer solution. Fermentation gas production from each incubation bottle was recorded at 30-minute intervals using an Ankom Gas Module (Ankom Technology, Macedon, NY). Four runs of gas measurements with variation less than ±5% at 65 hr of incubation were used for the analysis. Data fitting a non-linear model were analyzed using Proc NLIN of SAS (SAS Institute, 2013), which was developed by Dr. P. J. Weimer (personal communication). Rate and extent of fermentation gas production were determined for each sample by fitting the gas production data to the single pool logistic model, modified from Schofield, Pitt, and Pell (1994):

$$V = V_F \cdot \{1 + \exp[2 + 4\mu_m/V_F \times (\lambda - t)]\}^{-1} \quad (1)$$

where, V is the amount of gas production at time t, V_F is the final gas production volume corresponding to complete substrate digestion for a fermenting pool, μ_m is the point of inflection of the gas curve, and λ is the lag time, respectively.

Residual N content dynamics of HDCGM and POTL were measured in soil using a filter bag technique. Quadruplicate 0.50-g samples of HDCGM and POTL were weighed into F57 filter bags (Ankom Technology, Macedon, NY) and buried in the control plot at a depth of 12 cm on the same day when fertilizer treatments were

incorporated. The buried filter bags were recovered at days 3, 6, 9, 12, 24, 48, 72, and 96. Recovered filter bag samples were stored in the lab freezer and processed along with the forage DM samples.

2.3 Crop Harvest and Sub-sampling

Three harvests were made during each growing season of two years. The first harvest of annual ryegrass was made in early-March and that of the SS hybrid was made in mid-June each year. The harvest intervals were approximately 45 to 55 days between the harvests. A 0.6- by 7.0-m strip of annual ryegrass was cut with a sickle bar mower (Troy-Bilt® Valley City, OH) from the center of each plot at a 5- to 7-cm stubble height. Two 7.0-m long center rows of SS hybrid were harvested using a hand sickle at a 15-cm stubble height. Fresh forage weights were recorded, grab samples were collected for DM production and herbage was removed from the plot. Afterward, the whole plot area was mowed at the same cutting height as the harvested strip. Forage samples of approximately 300 g from the fresh forage of each plot were dried at 55 ^{o}C for 72 hr to determine DM production and saved for N analysis.

2.4 Analysis of HDCGM, POTL and Forage Samples

Sub-samples of HDCGM and POTL were dried in the same method with the forage samples. Dried HDCGM, POTL, and forage samples were ground to pass a 2-mm screen using a Wiley mill (Arthur H. Thomas Company, Philadelphia, PA), and then ground to pass a 1-mm screen using a Cyclotec 1093 mill (Foss in North America, MN). Mineral concentration in HDCGM and POTL was determined by flame atomic absorption spectrophotometry (Perkin-Elmer Analyst 300, Norwalk, CT) after dry ashing at 500 ^{o}C overnight in porcelain crucibles. Ground forage samples were analyzed for Kjeldahl N in an automated colorimetric assay adapted for a flow-injection analyzer (QuickChem 8000 FIA, Lachat Instruments, Milwaukee, WI) according to AOAC (1990) procedures. Acid detergent insoluble nitrogen (ADIN) in dried HDCGM samples was determined with acid detergent fiber (ADF) as an intermediate step (Goering & Van Soest, 1970) using Kieldahl N procedure.

2.5 Soil Sampling and Soil Analysis

Three soil samples were collected per plot, using a 2-cm diameter soil sampling tube to a depth of 15 cm, the day before planting annual ryegrass (initial soil sampling) in 2010, and after SS forage harvest on Oct 18, 2012 (final soil sampling). Soil pH and macro minerals were determined according to Wang, Harrell, Henderson, and Bell (2004). Soil pH was measured from mixtures of 1:1 soil to water ratio (weight basis). Soil P was analyzed using Bray 2 (0.03 M HH_4F + 0.1 M HC) with a 1:20 soil to solution ratio (weight basis). Soil K, Ca, and Mg were extracted with 1 M NH_4AO_C using a 1:10 soil to solution ratio and then extracted with Mehlich 3 extractant (Mehlich, 1984) using a soil to solution ratio of 1:10. All elements in extracts were determined using ICP (Ciros model, Spectro Analyticsl Instruments, Inc., Fitchburg, MA 01420). Soil organic matter (SOM) was determined according to Nelson and Sommers (1996).

2.6 Statistical Analysis

Residual N of HDCGM and POTL in soil were subject to a repeated measures analysis. Due to the unequal intervals of filter bag recovery from the soil, compound symmetric structure of covariance analysis was applied for modeling of N. Coefficients of linear and quadratic functions were compared for the fixed effects (fertilizer treatment and forage type).

Data (DM production, N concentration, N production, and soil characteristics) were analyzed using the Proc Glimmix of *SAS version 9.4* (SAS Institute, 2013). Comparisons of the treatment means of forage N concentration, DM production, and N production were conducted by forage type (cool-season vs warm-season annual grasses), and year of cultivation. Harvests nested within each crop cycle were considered random effect. Pairwise comparisons of least square means of crop responses were conducted considering fertilizer treatment, forage type, year, and interactions of the independent variables as fixed effects to assess possible carryover effects of fertilizers from the first year application to the next year.

3. Results

3.1 Weather and Soil Temperature

Air temperature and soil temperature demonstrated a similar monthly pattern during two years of cool-season and warm-season annual grass cultivation (Figure 1). Soil temperature remained higher than air temperature approximately by 2 to 5 ^{o}C. Rainfall during the fall to spring period (Oct., 2010-Mar., 2011) was more variable in the first year but generally similar to than that in the second year. In contrast, rainfall in April, May, and August in the first year was much lower than that in the second year.

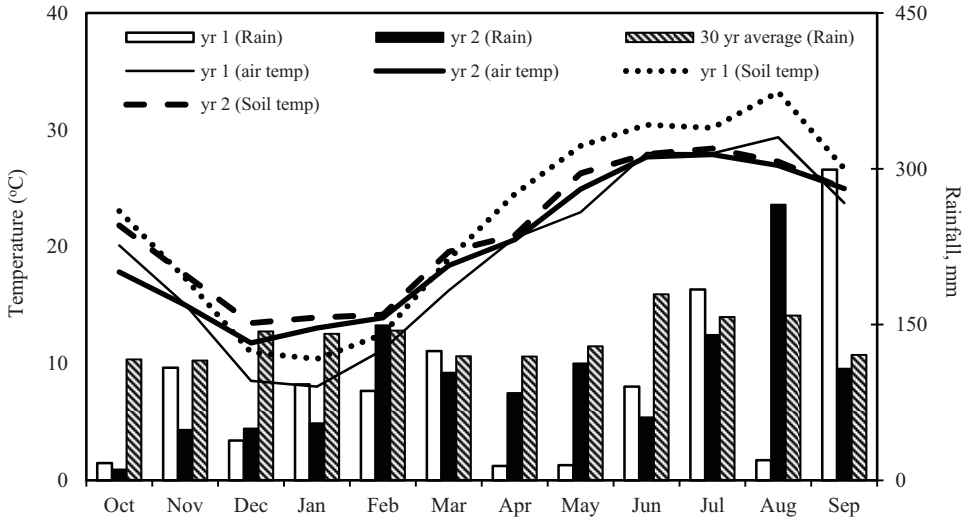

Figure 1. Monthly rainfall, air temperature, and soil temperature of yr 1 (from October 2010 to September 2011) and 2 (from October 2011 to September 2012) with 30 yr monthly rainfall average

3.2 Characteristics of CGM, HDCGM and POTL

The nutrient analysis results presented in Table 1 indicate OM comprised more than 850 g/kg DM of both POTL and HDCGM. The HDCGM had higher total N than that in POTL (by 25 g/kg DM), while the P, K, Mg, and Ca contents were much lower than those in POTL. Due to the high N concentration in HDCGM, C/N ratio of HDCGM was 6.1 units lower than that of POTL.

Comparison of the ruminal fermentation kinetics of HDCGM and feed grade CGM, using an *in vitro* incubation technique, indicated much reduced degradation of HDCGM under rumen microbial fermentation (Figure 2). Model fitting of fermentation gas curves obtained from *in vitro* ruminal incubation identified distinct difference between HDCGM and feed grade CGM. The *in vitro* ruminal fermentation parameters such as fermentation pool size, fermentation rate, and fermentation lag time of HDCGM differed from those of feed grade CGM.

Table 1. Chemical concentrations in POTL (poultry litter) and HDCGM (recycled corn gluten meal)

Nutrient	POTL	HDCGM
Moisture, g kg^{-1}	132	229
OM, g kg^{-1}	876	950
Total N, g kg^{-1} DM	36.5	61.8
C:N ratio	15.9	9.8
Ca, g kg^{-1} DM	35.6	0.7
P, g kg^{-1} DM	25.3	11.5
K, g kg^{-1} DM	38.8	16.2
Mg, g kg^{-1} DM	8.2	4.5
Zn, mg kg^{-1} DM	427	90.0

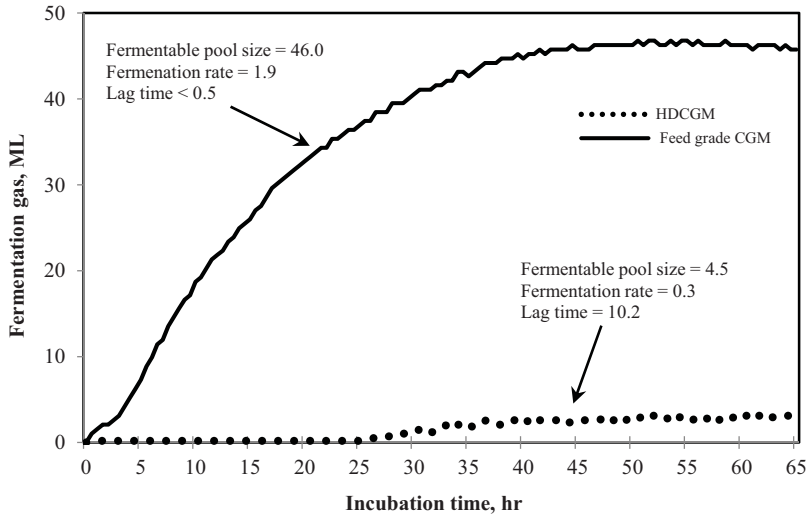

Figure 2. Comparison of the *in vitro* ruminal fermentation gas accumulation patterns of HDCGM (heat damaged corn gluten meal) and feed grade CGM (corn gluten meal)

The fermentation pool size and fermentation rate of HDCGM were one tenth and one sixth of feed grade CGM, respectively. Fermentation lag time of HDCGM was more than twenty times longer than that of feed grade CGM. The preceding indicates the highly undegradable nature of HDCGM under the ruminal bioassay conditions. Measurement of acid detergent insoluble nitrogen (ADIN) for estimation of the potentially undegradable proportion of nitrogen compounds in HDCGM indicated 93% of the total N was in ADIN form (data not presented) and thus highly undegradable.

The residual N concentration in sequentially recovered HDCGM and POTL filter bag samples, which were buried on the same days as the annual ryegrass or SS hybrid was planted, increased directly with days in soil (Figure 3). The coefficients for the intercept and both linear and quadratic day effect during cool-season and warm-season were all statistically significant (Table 2). The coefficients of linear function of day were positive but the coefficients for quadratic function of day were negative.

Table 2. Linear and quadratic coefficients of recovery day for residual N concentration in HDCGM (heat damaged corn gluten meal) and POTL (poultry litter) samples buried at the time of planting cool-season and warm-season annual forage grasses

Solution Coefficient	Cool-season		Warm-season	
	POTL	HDCGM	POTL	HDCGM
Intercept	3.22	5.95	3.31	5.94
P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Day	0.013	0.013	0.010	0.007
P-value	< 0.0001	< 0.0001	< 0.0001	0.002
Day^2	-0.00005	-0.00005	-0.00004	-0.00004
P-value	0.0006	< 0.0001	0.0043	0.0069

Note. [2], fixed effect of repeated measure analysis with HDCGM and POTL samples recovered at 0, 3, 6, 9, 12, 24, 48, 72, and 96 day from soil, averaged for the two year, during the winter growing season and summer growing season.

Based on the estimates of the coefficients of the solutions, model equations for the residual N content in HDCGM were $5.95 + 0.0133 \times day - 0.00005 \times day^2$ during the winter growing season, and $5.94 + 0.0071 \times day - 0.00004 \times day^2$ during the warm-season grass growing season. The intercepts were smaller for the POTL than HDCGM for both cool and warm growing seasons, indicating lower initial N concentration in POTL. The linear day function for HDCGM was the same as of POTL during the winter growing season, while the slopes of linear function for HDCGM were smaller than POTL during the summer growing season. The linear and quadratic day

coefficients of HDCGM and POTL during the summer growing season were smaller than those during the winter growing season.

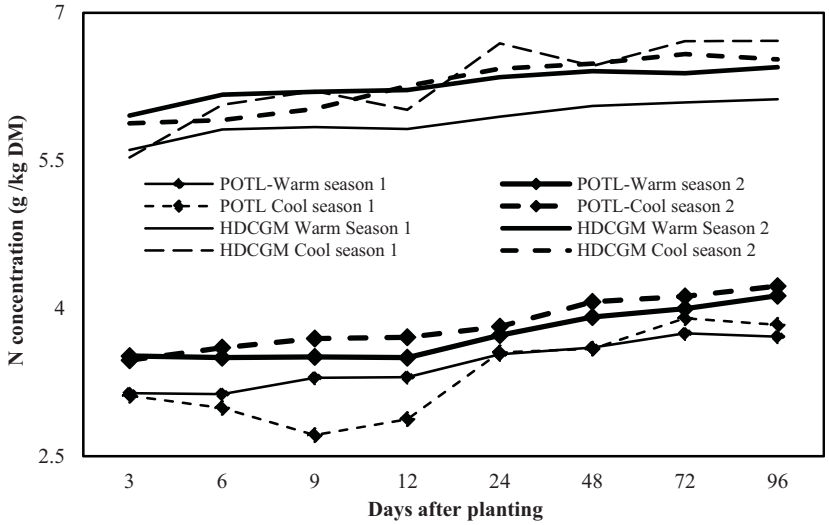

Figure 3. Mean total N content in recovered HDCGM and POTL samples, during the cool and warm seasons

3.3 Impact on N Concentration, Dry Matter Production and N Yield of Forages

The fertilizer × forage type data showed that the HDCGM, POTL and COMF consistently tended to increase (not always significantly) N concentration, DM production and N production of forages when compared to ZERO (Table 3). Significant increases over the ZERO were observed only for the SS hybrid DM production by POTL, for the ryegrass DM production by COMF, and for the ryegrass N production by POTL and COMF. Regardless of fertilizer treatment, annual ryegrass produced forage containing higher N concentration than the SS hybrid, but its DM production was less than SS hybrid. The means of forage N concentration, DM production, and N production did not show significant differences between the HDCGM, POTL and COMF treatments, except for greater N production from ryegrass with POTL than HDCGM. Since N production of forage receiving the different fertilizer treatments was estimated by the function of forage production and N concentration, N production demonstrated a different pattern from the forage production. The mean N production of annual ryegrass receiving HDCGM was lower than that of annual ryegrass receiving POTL. The N production from HDCGM was equivalent to that of the SS hybrid receiving COMF.

Fertilizer × year of cultivation data indicated that N concentration tended to be higher in the first than second year, but did not differ significantly within each fertilizer treatment, except for the ZERO treatment (Table 3). Forage DM production was significantly lower in the first year than second year for the HDCGM, POTL, and COMF, indicating nutrients carry over from the first to second year. Also the DM production was not significantly influenced by fertilizer treatments in the first year, while it was significantly greater with the HDCGM, POTL, and COMF than ZERO in the second year. The N production of the first year was lower than the second year for the POTL and COMF. Similar to the DM production, the N production did not show significant effect of the fertilizer treatments in the first year, while it was significantly greater with the HDCGM, POTL and COMF than ZERO in the second year.

Forage type × year mean of N concentration indicated annual ryegrass contained higher N than the SS hybrid, and N concentration in annual ryegrass in the first year was higher than in the second year (Table 3). Forage DM production of annual ryegrass did not differ between the years of cultivation, while the SS hybrid produced much more forage in the second than first year. The N production was similar for the second-year SS hybrid and the first-year annual ryegrass with less N production by the second-year annual ryegrass. The first-year SS hybrid produced the least amount of N compared to its second year and also ryegrass in both years.

Table 3. Averages from three harvests of SS (sorghum sudangrass) hybrid and annual ryegrass for nitrogen (N) concentration, forage dry matter (DM) production, and N production, presented as interactions of fertilizer treatment × forage type, fertilizer treatment × cultivation year, and forage type × cultivation year

		N concentration (g/kg DM)	Forage DM yield (kg/ha)	N yield (kg/ha)
Fertilizer Treatment†	Forage type	Fertilizer × forage type		
ZERO	SS hybrid	15.4b‡	2828bc	40.3c
	Annual ryegrass	26.2a	1988c	49.6bc
HDCGM	SS hybrid	17.2b	3961ab	61.0bc
	Annual ryegrass	26.8a	2537bc	65.6bc
POTL	SS hybrid	17.7b	4518a	68.3abc
	Annual ryegrass	30.2a	3370abc	94.7a
COMF	SS hybrid	16.1b	3455ab	54.5bc
	Annual ryegrass	27.7a	2920bc	71.4ab
Fertilizer Treatment	Year of cultivation	Fertilizer × year		
ZERO	1	23.4ab	1789b	45.1d
	2	18.1c	3027b	44.8d
HDCGM	1	24.3ab	1923b	51.8cd
	2	19.8bc	4575a	74.8abc
POTL	1	25.9a	2380b	66.8bcd
	2	22.0abc	5508a	96.2a
COMF	1	22.2abc	1739b	43.9d
	2	21.7abc	4636a	81.9ab
Forage type	Year of cultivation	Forage type × year		
SS hybrid	1	16.0c	1503c	21.4c
	2	17.1c	5878a	90.6a
Annual ryegrass	1	31.8a	2412b	82.4a
	2	23.7b	2995b	58.3b

Note. †, HDCGM, heat damaged corn gluten meal; POTL, poultry litter; COMF, commercial fertilizer; and ZERO, no fertilization.

‡, Mean values followed by the same letter or letters within the same column and interaction did not differ at $P > 0.05$.

3.4 Soil Chemical Properties

The SOM remained at a similar level between before annual grass cultivation and after four treatment applications (Table 4). However, the final SOM level after the HDCGM treatment was higher than that in the ZERO and COMF treatments. Soil N content after the HDCGM was higher than that of other three treatments and was the only treatment to maintain near the initial soil N level after two years of grass cultivation and treatment application.

After two years of annual grass cultivation, soil pH and Mg content for the two organic fertilizer treatments, POTL and HDCGM, were significantly greater than the ZERO and COMF treatments, and also increased from the initial pH and Mg content measured before the annual grass cultivation (Table 4). In contrast to increased soil pH and Mg with the organic soil amendments, the concentrations of P, K, and S in all treatments declined after two years of cultivation.

Table 4. Soil characteristics before cultivation and after two years of forage crop cultivation including four applications of treatments, averaged by treatment (ZERO, zero fertilization; COMF, commercial fertilizer; POTL, poultry litter; HDCGM, heat damaged corn gluten meal)

Soil characteristics	Before cultivation†	After cultivation with fertilizer treatment			
		ZERO	COMF	POTL	HDCGM
Organic matter, g/kg	34.1	32.0b‡	31.1b	33.7ab	37.1a
Nitrogen, g/kg	1.59	0.91b	0.82b	0.88b	1.46a
pH (1:1 Water)	5.9	5.9b	5.9b	6.2a	6.2a
P, mg kg^{-1}	44.2	20.1c	31b	38.4a	26.7b
K, mg kg^{-1}	66.2	40.4b	55a	47ab	55.5a
S, mg kg^{-1}	13.5	9.5a	8.8b	9.9a	9.2a
Mg, mg kg^{-1}	199.5	189.1b	192.2b	261.1a	250.6a
Zn, mg kg^{-1}	1.5	1.0a	0.9a	2.0a	1.2a

Note. †, Soil characteristics of before cultivation were measure with the random samples of whole plot area.

‡, Numbers within a row followed by the same letter(s) did not differ at $P < 0.05$.

Due to high P concentration in the POTL, soil P level after grass cultivation was significantly higher with POTL than other treatments, and it was also near the initial P level. Compared with COMF, the S and Mg in soils receiving POTL and HDCGM were higher after the two years of treatment.

4. Discussion

Release of N from organic materials such as HDCGM and POTL is a function of soil microorganisms, soil moisture, aeration, and soil temperature (Sistani, Adeli, McGowen, Tewolde, & Brink, 2008). The differences in forage DM production and N production between HDCGM and POTL during cool-season probably reflect differences in their N mineralization rates under low temperatures. Similar forage production for the two treatments during the warm season indicates that the labile pool of N in soil treated with HDCGM increased to a similar level to that of the POTL and COMF treatments, perhaps due to a combination of the warmer soil temperature in summer and the accumulated N from the combined spring and fall applications of HDCGM. Such a summer temperature benefit agrees with Adair et al. (2008) who reported improved performance of SOM decomposition models based on first order kinetics with increasing soil moisture and temperature. Such a temperature effect can even occur with unusually warm winter weather (Lehrsch, Brown, Lentz, Johnson-Maynard, & Leytem, 2016).

Although more rapid mineralization of HDCGM and POTL was expected in the summer soil environment than during the winter due to high soil temperature and perhaps periodically higher soil moisture, the residual N dynamics did not reflect the seasonal DM production difference. Samples of HDCGM and POTL buried in filter bags indicated similar N dynamics during the two seasons (Figure 3 and Table 2). The filter bag samples buried at the 12-cm depth in soil during the summer growing season were probably in drier soil conditions than those in the winter growing season, due to low precipitation and high evaporation rate of soil moisture under the hot and dry summer weather. Especially in the summer growing season of the first year (between April and June 2011), southeastern LA received much less rainfall than the 30 year long-term average (Figure 1). In contrast to the summer growing season, mild winter weather and less evaporation rate of soil moisture may have provided a more suitable environment for decomposition of SOM at 12 cm soil depth. However, modeling mineralization of N with soil temperature and soil moisture is more challenging than for soil C. Leiros, Trasar-Cepeda, and Seoane (1999) reported difference of model performance between evolvement for N and C. Their attempt could only explain around 30% of variance for N, while the same attempt for evolvement of C could explain 80% of the variance. This difference in model performance between N and C was explained with a relatively complicated N cycle in soils and involvement of specific soil microbes in N mineralization (Leiros et al., 1999). There would also be interaction between existing SOM and added OM from the HDCGM and POTL. Complexity of N mineralization from organic soil amendments with multiple interacting effects and the need for additional research to allow estimation of net N mineralization responses has been assessed by Cabrera, Kissel, and Vigil (2005). In the summer growing season, residue of cool-season grass in the soil may have been more readily degraded because of the relatively low C-N ratio for the forage from annual ryegrass (due to higher N concentration) compared to that of the warm-season SS hybrid, as indicated by their N concentrations presented

in Table 3. With a high-N soybean residue, approximately 26% of incorporated N from the soybean residue was recovered by a following crop (Norman & Werkman, 1943).

Coefficients of quadratic function of day for the residual N concentration were all negative and significant for both POTL and HDCGM during both the winter and summer growing seasons (Table 2), because the residual N concentration in the sequentially recovered filter bag samples increased in a linear pattern. However, with subsequent days in soil, the N concentration increased at a lower rate (Figure 3). Even with the higher N concentration (Table 1) and lower C-N ratio of HDCGM than that of POTL, because of its high proportion of bound N, the N release from the HDCGM would not be expected to be as fast as that in POTL. However, the coefficients of day function during cool season were same for the HDCGM and POTL, while POTL had higher coefficient of day than HDCGM during warm season (Table 2). Differences in N release from HDCGM and POTL during warm season were similar to those found between composted and non-composted poultry litter (Preusch, Adler, Sikora, & Tworkoski, 2002). The bound N in HDCGM should be highly resistant to microbial degradation in soil as demonstrated by the *in vitro* fermentation analysis in this study (Figure 2). The lower ruminal degradation of HDCGM than that of feed grade CGM indicates recalcitrance of HDCGM. Bound N in HDCGM as quantified using ADIN analysis (Licitra, Hernandez, & Van Soest, 1996) averaged around 86% in this study (data not presented). This high proportion of ADIN in the total N apparently contributed to the slower and reduced contribution of HDCGM to forage production compared to POTL, and also contributed to higher OM and N in the soil after 2 years (Table 4).

Since the three harvests in each growing season were nested in year and considered as a random effect in the statistical analysis of these data, the forage DM production by harvest number are presented as an appendix. The three cool-season harvests presented a different forage DM production pattern for COMF and POTL than those of HDCGM. The COMF and POTL treatments demonstrated a sharp increase of forage production at the second harvest among the three harvests and then sharp reduction to the third harvest (Appendix A), while HDCGM produced more uniformly distributed DM production throughout the growing season. Kowaljow, Mazzarino, Satti, and Jimenez-Rodriquez (2010) reported that application of inorganic fertilizer increased DM production primarily through an initial pulse of available N, while organic fertilizers should have more positive sustained impact on soil chemical and biological properties. This is supported by the much slower N release from HDCGM and perhaps POTL than that of COMF. Overall, the two organic fertilizer treatments in this study, POTL and HDCGM, produced linearly increasing forage DM production throughout the three cool-season harvests, while COMF produced the most forage in the second harvest, indicating a fast and early peak of the available N pool in the soil following application of COMF.

Forage DM production in the second year was greater by 69 to 167% than in the first year (Table 3). The largest increase in forage production was with COMF (167%), while the increase with HDCGM and POTL was around 135% and with ZERO control by 69%. Thus, forage production indicated more favorable growing conditions in second than first year. The second-year DM production demonstrated advantage of the N credit from the applied N in first year, as indicated by larger DM production increase in second year over first year by the HDCGM, POTL and COMF treatments than the ZERO, which would not be expected from the often volatile urea in the commercial fertilizer. Similar pattern was also shown by the N production data. Slower release of N from the organic materials is expected to provide potential for greater second-year benefits from the HDCGM and POTL as N sources than from COMF. This was not, however, the result obtained. Earlier, Gill (2019) observed N from urea was carried over to benefit crop in next year, when the growing season was dry and higher than recommended N rate was applied. Our results are consistent with those of Lehrsch, Brown, Lentz, Johnson-Maynard, and Leytem (2017) who demonstrated such second-year N benefits from organic N sources only when the amount of N applied exceeded the N demand of the crop grown in the initial year. Higher SOM levels after two years of HDCGM and POTL than COMF and ZERO indicate that some of the N from the applied HDCGM and POTL was not mineralized during the two years, and may benefit crops in following years.

Although cool-season forage DM production was less than that during the warm season, the high N concentration of the cool-season forage (ryegrass mean N concentration = 27.7 g/kg DM) resulted in greater N production than the warm-season forage (SS hybrid mean N concentration = 16.6 g/kg DM) (Table 3). The difference in N production between the 2 years was mostly a function of increased forage production in the second year, despite lower N concentration in forage.

5. Conclusions

With higher N concentration than poultry litter, HDCGM has value as an alternative organic fertilizer producing an amount of annual grass DM comparable to that of a commercial fertilizer with more uniform forage

distribution. The highly bound N in HDCGM was not as effective as that of POTL to boost annual forage crop production during the cool season. However, HDCGM was as effective as POTL for warm-season grass production. The season of use should therefore be a consideration with HDCGM application as a source of N. Application of HDCGM is less likely to result in N leaching than commercial fertilizer sources and also contributes to increased soil N and SOM without a substantial negative impact on establishment of cool-season or warm-season annual forage grasses.

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Appendix A

Averages Forage Dry Matter Production Presented by Harvest Number × Fertilizer Treatment × Forage Type

Harvest number	Treatment			
	ZERO	COMF	POTL	HDCGM
Cool season ryegrass harvests				
1	1757	2150	2575	2206
2	2336	4590	4975	2988
3	2133	2018	2560	2417
Warm season SS hybrid harvests				
1	1787	2281	2119	2340
2	2189	3606	4651	3614
3	4509	4478	6785	5930

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Effect of Agricultural Credit Access on Rice Productivity: Evidence from the Irrigated Area of Anambe Basin, Senegal

Mouhamadou Foula Diallo[1], Jiajun Zhou[1], Hamidullah Elham[1] & De Zhou[1,2]

[1] College of Economics and Management, Nanjing Agricultural University, Nanjing, China

[2] China Center for Food Security Studies, Nanjing Agricultural University, Nanjing, China

Correspondence: De Zhou, College of Economics and Management, Nanjing Agricultural University, No. 1, Weigang, Xuanwu District, Nanjing 210095, China. Tel: 86-182-6007-5456. E-mail: zhoude@njau.edu.cn

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Abstract

Rice is an important staple food in many developing countries, especially in Senegal. However, rice production in Senegal only meet 20% of the domestic demand largely due to the poor performance of rice farmers and low productivity. Access to agricultural credit has strong impacts on the technical efficiency of farmers and would promote inputs and new technology adoption. But that is not clear enough in previous studies. This study investigates the impact of agricultural credit access on rice productivity and technical efficiency with 260 random sampled rice farmers from Anambe basin in Senegal. The Stochastic Frontier Analysis (SFA) was adopted to estimate the technical efficiency. The results indicate that the inputs of rice production, including labor, pesticide, herbicides and fertilizer, have significant impacts on rice productivity. Furthermore, the results present that the average efficiency is of 0.813 and the inefficiency estimation model reveals that the influences of agricultural credit access, gender, education, ethnicity, use of improved seed and land tenure system on technical inefficiency of rice production are significant. Particularly, for the access to agricultural credit, rice farmers without agricultural credit would get 3.8% higher production inefficiency. The farmers with access to credit yield 37.32% higher rice production than their counterparts. Therefore, our study provides strong empirical evidence to promote agricultural credit in rice production.

Keywords: agricultural credit, rice productivity, technical efficiency, stochastic frontier analysis, irrigated area, Senegal

1. Introduction

Agriculture plays an important role in the economy of most developing countries. According to Otsuka (2013), agriculture provides 70% of full-time employment, 40% export earnings and also generates 35% national income in Africa. In Senegal, agriculture is a privileged sector which employs half of the labor forces, yet contributes 14.8% of the GDP (Seck, 2019). This poor performance of agriculture sector in Senegal is partially caused by the lack of financial supports rendered to farmers and inadequate incentive programs dedicated to the productivity improvement.

Rice is a cash crop and an important staple food in Senegal. In 2015, the average consumption was reported to be 72.29 kg/person/year by FAO (2015). However, the Senegalese rice production only meets 20% of the domestic demand. This leads a large volume of rice imports which piles up from 650000 to 850000 tons and the bulk cost of 165 billion XOF on average (PNAR, 2014). To improve the rice production performance and achieve self-sufficiency, the Senegalese government carried out an economic policy in 2014 to boost annual paddy rice production to the level of 1600000 tons. The government focused on the cultivation of irrigated rice in the Senegal river valley and Anambe basin. That areas account for 5% of the total arable land in Senegal but yield 83% of the total rice production (PRACAS, 2014). Though the implementation of the policy came up with some positive results, such as increasing the national rice production from 436153 to 1007277 tons over the period of 2014-2017 (PNAR, 2018), the country is still far from achieving rice self-sufficiency.

The performance of rice farmers is urgent to be enhanced to promote the productivity and rice yield. According to the existing literature, credit access is an important factor for farmers' performances in Senegal (Fall, 2008). Access to financial resources enables farmers to get enough inputs and likely reach high productivity. However,

up to the present, credit access is one of the major weaknesses that inhibits agricultural development in Senegal. The available credit system lags behinds the rapid increasing demand of farming households. Particularly, rice production in Anambe basin is plagued with limited access to credit. Eventually, that makes it difficult for farmers to access adequate inputs and adopt modern agricultural innovations. Therefore, this study intends to shed light on the impact of access to agricultural credit on rice productivity in Senegal with the data collected from the irrigated area of Anambe basin.

There are some researches on the impacts of credit access on productivity. However, the impacts of credit access on agriculture productivity and technical efficiency in Senegal are not clear enough in present literature. Scholars have abstractly and empirically examined how credit access is vital for producers' performances in the agricultural sector. Iqbal et al. (2003), Rahman et al. (2014), and Owusu (2017) provided empirical evidences for the existence of positive effects of credit on agricultural production. Also, Seck (2018) applied endogenous switching regression model to examine the heterogeneous credit constraints and small farming holders' productivity in Senegal. The results indicated that credit constraints hinder farmers' production performance. Sjah et al. (2003) and Wicaksono (2014) also got the similar conclusions and pointed that agricultural credit has stronger influences on the intensified farming. Enyim et al. (2013), applied the unit-root cointegration to investigate the relationship between banking sector credits and agricultural activities in Nigeria. Their results suggested that credits supplied to farmers have positive relationship with their productivity. Agunuwa et al. (2015) applied time series to study the influence of commercial bank credit on agricultural productivity and pointed out that there is a positive impact of commercial bank credit on agricultural productivity. Moreover, findings in present studies suggest that there are more constraints, such as market imperfections, break-down of fertilizer supply and weaknesses of credit system in the process of investigation the access to credit. And Rezitis et al. (2003) highlighted that, besides agricultural credit, other factors, such as better use resources, information and better management should be adopted in order to improve technical efficiencies. Those researches provide the useful covariates in our models.

However, the results from Reyes et al. (2012) and Mghenyi (2015) show that the access to credit does not improve the agricultural productivity as credit is allocated to inputs that are already sufficient. Despite the existence of myriad studies on agricultural credit and its effect on productivity, the topic remains largely unexplored in Senegal. Furthermore, the literature on rice production with respect to credit access is scarce and this would hinder the complement of cogent policies in rice production.

To fulfill this research gap, this study tries to link rice production with agricultural credit access and scrutinize the impacts of agricultural credit access on rice farmers' productivity and technical efficiencies in the Anambe basin with the use of SFA model. The results of present study are highly essential for policymakers as they provide strong empirical evidence for the policy formulation and implementation to warrant the increase of rice productivity. The remainder of this paper is structured as follows: Section 2 presents the material and method, Section 3 displays the results and presents the discussion, and Section 4 shows the conclusion and recommendation of the study.

2. Material and Method

2.1 Data and Variable Definition

The data used in the study come from a household survey that was conducted in Anambe basin of Senegal in June and July of 2019. Anambe locates in Upper Casamance in the Kolda region which covers an area of 110,000 ha (watershed) with nearly 55,000 ha arable land suitable for irrigated crops. And there are 5 rural communes (Kandia, Saré Coly Sallé, Bonconto, Sinthiang Koundara, Ouassadou, Médina Chérif) and two communes (Kounkané and Diaobé-Kabéndou). A two-stage sampling procedure was adopted in the survey. In the first stage, Anambe villages were conveniently selected given it is one of the famous rice production areas with prevalence of agricultural credit access. In the second stage, 260 farmers were randomly selected using household lists obtained from the Society for Agricultural and Industrial Development in Senegal (SODAGRI). It was a door to door survey using a participatory research approach, and the pretested questionnaires were administered to households by well-trained enumerators. The data are composed of the quantity of rice production, amount of land used, amount of chemical fertilizers, quantity of seeds, labor availability, hired machine, a dummy variable of agricultural credit receipt, and some important socioeconomic and demographic variables. A summary of variables description is presented in Table 1. Stata 15 and Excel were used to analyse the data.

Table 1. Variables description

Variable	Definition
Yield	Quantity of rice produced in tons per hectare
Seed	Amount of seed used in tons per hectare
Labor	Amount of labor used includes own labor involved (hours per hectare)
Pesticide	Amount of pesticide used in liters per hectare
Herbicide	Amount of herbicide applied in liters per hectare
Hired_machine	Amount of machine used in hours per hectare, including both hired and farmer's machinery
Fertilizer	Amount of fertilizer (Urea and NPK) used in tons per hectare
Credit_received	Dummy for credit access, 0 = no credit received and 1 = credit received
Age	Age of the respondent: number of years
Gender	Dummy variable, 0 = female and 1 = male
Education	Dummy for educational status, 0 = illiterate and 1 = literate
Training	Dummy for training in rice cultivation, 0 = no training received and 1 = received training
Marital_status	Marital status of the respondent, 0 = single and 1 = married
Ethnicity	Ethnics of the farmer, 0 = Fulani and 1 = others
Farming_experience	Number of years in rice cultivation
Organisation_member	Organization membership, 0 = no and 1 = yes
Family_size	Number of people in the household
Irrigation_cost	Cost of irrigation in XOF per hectare
Rice_variety	Seed quality, 0 = non-improved seed and 1 = improved seed
Tenure_syst	Land tenure system, 0 = own land and 1 = rented land

2.2 Model Specification

Stochastic Frontier Analysis (SFA) was used to assess rice productivity and examine the effect of access to agricultural credit on the efficiency of rice production in Anambe basin (Senegal). SFA is a parametric technique that uses standard production functions, such as Cobb-Douglas production function and Translog production function, and explicitly considers the maximum feasible output level for a given set of inputs. It is used in modeling functional relationships with theoretical bounds such as: 1) modeling cost functions and analyzing cost efficiency, 2) modeling production functions and analyzing production efficiency, 3) modeling revenue functions and analyzing revenue efficiency, etc. That analysis tool was proposed by Aigner et al. (1977) and Meeusen et al. (1977) which involved a production function with an error term. The error term is consisted of two components—one is random effect (measurement errors and other random factors such as weather, strike, luck, etc.) and the other is technical inefficiency. It has been used in a vast number of empirical applications and extended in a number of ways. Following Battese et al. (1995), the SFA production function is as follows:

$$y_i = x_i\beta + v_i - u_i\,;\, i = 1, 2, \ldots N \tag{1}$$

Where, y_i is logarithm of output for farmer i^{th}, x_i is $k \times 1$ vector of logarithms of inputs for farmer i^{th}, β is a vector of unknown parameters, v_i is random variable assumed to be an iid $N(0,\sigma_v^2)$ and u_i is inefficiency error term which is a non-negative random variable associated with technical inefficiency of production and assumed to be independently distributed, such u_i is obtained by truncation (at zero) of the normal distribution with mean $z_i\delta$ and variance σ_u^2, z_i is a $p \times 1$ vector of variables that are assumed to have influences the technical efficiency and δ is an $1 \times p$ vector of parameters to be estimated. The technical inefficiency effect u_i can be modeled as:

$$u_i = z_i\delta \tag{2}$$

The variables which explain the extent to which the production of i^{th} farmer fall short of the corresponding stochastic frontier production value $(x_i\beta + v_i)$ are included in the inefficiency model. With the production function, technical efficiency of farmer i^{th} can be estimated as the ratio of observed output to the potential output defined by the frontier function. Formally, technical efficiency of farmer i^{th} is:

$$TE_i = \frac{y_i}{\exp(x_i\beta + v_i)} = \frac{\exp(x_i\beta + v_i - u_i)}{\exp(x_i\beta + v_i)} = \exp(-u_i) \tag{3}$$

Taking the input variables into consideration, the specified empirical SFA production function is as follows:

$$\begin{aligned} ln(Yield) = {} & \beta_0 + \beta_1 ln(Seed) + \beta_2 ln(Labor) + \beta_3 ln(Pesticide) + \beta_4 ln(Herbicide) \\ & + \beta_5 ln(Hired\_machine) + \beta_6 ln(Fertilizer) + (v_i - u_i) \end{aligned} \tag{4}$$

All the coefficients β are expected to have a positive sign (except for β_0 whose sign cannot be expected a-priori) which means a positive relationship between the quantity of inputs and the output.

The empirical regression on technical inefficiency component u_i is as follows:

$$u_i = \delta_0 + \delta_1(Credit\_received) + \delta_2(Gender) + \delta_3(Age) + \delta_4(Edu) + \delta_5(Train) + \delta_6(Marit\_status) + \delta_7(Ethnicity) + \delta_8(Farming\_exper) + \delta_9(Org\_member) + \delta_{10}(Family\_size) + \delta_{11}(Irrig\_cost) + \delta_{12}(Rice\_variety) + \delta_{13}(Tenure\_syst) \quad (5)$$

The SFA model was estimated by using the FRONTIER 4.1 software which is based on the Three Step Estimation Methodology proposed by Coelli et al. (1996): (1) Ordinary Least Squares (OLS) estimation of the function are obtained; (2) a two-phase grid search for $\gamma = \sigma_u^2/(\sigma_u^2 + \sigma_v^2)$ which ranges from zero to one is conducted with the β parameters setted to OLS values (except β_0) and the $\beta_0 \& \sigma^2$ parameters are adjusted according to Corrected OLS presented in Coelli et al. (1996); (3) the values selected in the grid search are used as starting values in an iterative procedure (using the Davidon-Fletcher-Powell Quasi-Newton Method) to obtain final MLE estimates.

3. Results and Discussion

3.1 Descriptive Statistics

Prior to the interpretation of the model estimation, the descriptive analysis of factors, including gender, age, ethnicity, rice variety used, marital status, organization membership, education level, training and farmers' financial status etc., were presented in Table 2.

Table 2. Summary statistics

Table 2A

Variable	Obs.	Mean	Std. Dev.	Min	Max
Age	260	40	8.36	25	62
Family_size	260	12	5.36	3	34
Farming_experience	260	12	8.17	2	40
Farm_size	260	1.82	3.76	0.25	50
Loan obtained	140	399809	321291.80	80000	3400000
Loan demand	140	460405	485161.20	100000	4500000

Table 2B

Variable	Obs.	Mean	Variable	Obs.	Mean
Gender			*Tenure_syst*		
Female (%)	85	32.69	Owned (%)	51	19.62
Male (%)	175	67.31	Affected (%)	209	80.38
Education			*Rice_variety*		
Literate (%)	120	46.15	Improved seed (%)	231	88.85
Illiterate (%)	140	53.85	Non improved (%)	29	11.15
Training			*Credit_received*		
No (%)	24	9.23	No (%)	76	29.23
Yes (%)	236	90.77	Yes (%)	184	70.76
Marital_status			*Sufficient_among_credit*		
Married (%)	238	91.54	No (%)	64	34.78
Single (%)	22	8.46	Yes (%)	120	65.21
Ethnicity			*Obtain_loan*		
Fulani (%)	215	82.69	Difficult (%)	83	45.10
Other (%)	45	17.31	Easy (%)	94	51.08
Organization_member			Very difficult (%)	7	3.80
No (%)	97	37.31			
Yes (%)	163	62.69			

Note. Loan demanded and Loan obtained by farmers are measured in Senegalese currency (XOF).

The average age of farmers in the study is 40 years, with the range of 25-62 which covers most of the active farming age. Thus, given the focus of the investigation, the sample comprises of an ideal group. In terms of gender, most surveyed farmers are males (67.31%) while female farmers account for 32.69%. This finding is consistent with the typical setups in rural Africa where most households are dominated by males. Interestingly, 62.69% of Anambe farmers in our survey belong to a cooperative as cooperatives serve them cardinal information and knowledge related to farming activities. Regarding seed adoption, 88.85% of farmers use improved rice seeds, while 11.15% resort to non- improved seeds. The finding presents remarkable progress for rice farmers and the country as improved seeds hold a great potential to increase productivity. Since Anambe basin comprises of small-scale farmers (average of farm size is 1.82 ha, with the range of 0.25 to 50 ha), use of improved seed is possible without credit access. However, for the large-scale farmers, the cost of improved seeds becomes a challenge in adoption.

According to the data, the proportion of married farmers is higher. Generally, the married households are large size households, there are 12 persons per household on average. It provides additional labor for households in farming and ultimately saves labor costs. More revelations indicate that 90.77% of famers are trained in rice cultivation. The proportion of illiterate farmers is of 53.85%, while those having formal education account for 46.15%. Thus, the high proportion of illiteracy might deter training and production performance.

Finally, 70.76% of the farmers received credit from Agricultural Credit Bank of Senegal (CNCAS). 51.08% revealed that it is easy to obtain loan from the bank while the rest claimed that it is difficult. On the other hand, 34.78% of farmers who received credit reported that they did not obtain enough credit as they demanded. Figure 1 shows a gap between farmers' financial demand and agricultural credit supplied by CNCAS (Agricultural Credit Bank of Senegal). Also, it has important implications for policymakers to find other avenues that would make credit access in agriculture sufficient.

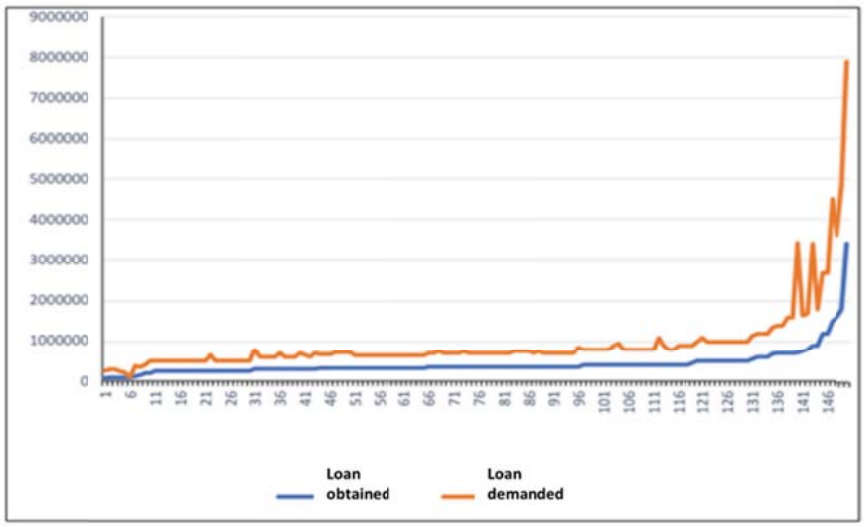

Figure 1. Loan demanded and money obtained by farmers in Anambe basin of Senegal

Note. The figures are based on the data collected during the household survey.

3.2 Technical Efficiency in Rice Production in Anambe Basin

The results of the stochastic frontier model are presented in Table 3. The results indicate that labor, pesticide, herbicides and fertilizer positively and significantly affect the rice productivity in the Anambe basin. Particularly, the coefficient of labor is significant at 1% with a positive value of 0.23. This implies that 1% increase of labor would increase the farmers yield by 0.23%. This result corroborates the findings from Nosiru (2010) that farmers with larger household size were more productive than those with small household size. In fact, large size households have more labor inputs. Similarly, use of pesticide was significant at 1%, with a positive value of 0.27. This result indicates that the use of pesticide is paramount in rice production in the study area. This result is also consistent with the findings from Sjah et al. (2003) that the use of pesticide contributes to intensification and improvement of farmers' production. Also, the use of herbicide is crucial in helping rice farmers to keep satisfactory performances. Its coefficient is statistically significant at 1% with a positive value of 0.04. Lastly,

fertilizer significantly affects the rice productivity in the Anambe basin. Its corresponding coefficient is significant at 1% with a positive value of 0.21. This signifies that the use of 1% more fertilizer by farmers would increase the rice output by 0.21%. This is also consistent with the research conclusions of Jiang et al. (2017) who provided evidence that the use of fertilizers in the rice production significantly yields positive outcomes.

On the other hand, hired machine is negatively correlated with the rice productivity but not significant. The use of machine in the rice production might be a poor substitution of manpower in the Anambe basin. The coefficient of seed is also not significant in the model.

Table 3. Maximum likelihood estimates of the stochastic frontier function

Variables	Parameters	Coef.	Std. Err.	P-Value
Cons	β_0	-0.880	0.282	0.002***
lnSeed	β_1	-0.014	0.040	0.722
lnlabor	β_2	0.231	0.064	0.000***
lnPesticide	β_3	0.271	0.038	0.000***
lnHerbicide	β_4	0.047	0.014	0.001***
lnHired_machine	β_5	-0.002	0.025	0.951
lnFertilizer	β_6	0.220	0.030	0.000***

Note. *** Significance at 1%; ** Significance at 5%; * Significance at 10%.

The inefficiency model was estimated with main influential variables and the results are listed in Table 4. There are some important technical efficiency determinants, including agricultural credit, gender, education, ethnicity, irrigation cost, rice variety, and land tenure system, which the Senegalese government should focus on to boost rice productivity.

The access to credit for farmers has a substantial effect on rice production inefficiency. The coefficient of credit is -0.038 and significant at 10% level. This suggests that agricultural credit access would decrease rice production inefficiency. This result is consistent with the results from Sjah et al. (2003) and Wicaksono (2014). Ethnicity is significant at 10% that means Fulani are more inefficient in rice cultivation than other ethnics in Anambe area.

On the other hand, the coefficient of gender is of 0.031 and significant at 5%, indicating that male farmers are less efficient in rice production. Fall (2008) and Diagne (2002) also found the rice production of female farmers is higher than that of male farmers. Similarly, the rice farmers' literacy has significant impacts on inefficiency reduction. In fact, educated farmers are more efficient than those who were not (Akyina et al., 2015). Tenure system represents another important determinant of rice production inefficiency. Its coefficient is negative and significant, namely, farmers who own the land are less efficient in rice cultivation probably due to weak awareness of land cost.

Lastly, the seed quality is an essential factor in the technical inefficiency model. The use of non-improved seeds has significant adverse effects on the rice production and would reduce rice production efficiency. This result is consistent with the findings of Sjah et al. (2003) who pointed out that the use of improved seeds is a necessary input to boost the rice production through intensification.

Table 4. The results of inefficiency estimation model

Variables	Parameters	Coef.	Std.Err.	P-Value
Cons	δ_0	0.197	0.293	0.502
Credit_received	δ_1	-0.038	0.022	0.082*
Gender	δ_2	0.031	0.015	0.048**
Age	δ_3	-0.001	0.001	0.615
Education	δ_4	-0.086	0.009	0.000***
Training	δ_5	-0.0011	0.024	0.964
Marital_status	δ_6	-0.007	0.025	0.764
Ethnicity	δ_7	0.033	0.019	0.079*
Farming_experience	δ_8	0.001	0.001	0.661
Organisation_member	δ_9	0.010	0.019	0.61
Family_size	δ_{10}	-0.001	0.002	0.675
Irrigation_cost	δ_{11}	0.0002	0.0006	0.473
Rice_variety	δ_{12}	0.040	0.023	0.087*
Tenure_syst	δ_{13}	-0.035	0.010	0.000***

Note. ***significance at 1%; **Significance at 5%; *Significance at 10%.

3.2 Agricultural Credit Access, Technical Efficiency and Rice Yield

To shed light on the detail impacts of access to agricultural credit on technical efficiency of rice production, we also show the distribution of technical efficiency for both farmers with access to agricultural credit and farmers without credit access. Table 5 reveals a sharp difference between the two groups and provides a hint of the importance of access to credit. In fact, farmers with access to credit have efficiency scores above 0.5 and majority of them are distributed in the range of 0.9-1 which is obviously higher than their counterpart. Such evidence suggests policymakers should pay more attention to credit for the efficiency improvement in rice production.

Table 5. Technical efficiency distribution across different groups

TE Category	Percentage		
	Credit Access	Non-Credit Access	Pooled
< 0.5		11.84	3.46
0.5-0.59	3.26	14.47	6.54
0.6-0.69	10.33	23.68	14.23
0.7-0.79	15.76	9.21	13.85
0.8-0.89	20.65	31.58	23.85
0.9-1	50.00	9.21	38.08

We also conducted t-tests to validate whether the differences of technical efficiency and rice yields between different agricultural credit access groups (Table 6). The technical efficiency for beneficiaries is 0.856 on average (ranging 0.517-0.988), while non-beneficiaries get an average efficiency of 0.711 (ranging 0.305-0.966). This implies that there is a 0.145 technical efficiency gap in favor of beneficiaries, and the difference between two groups are statistically significant. The pattern is also similar in rice yield where credit beneficiaries have 37% higher yields than their counterparts and it is significant at 1% level. These results suggest that agricultural credit may be positively associated with both technical efficiency and rice yields.

Table 6. Differences in technical efficiency and rice yield between farmers with different credit access

Item	Agricultural credit access status	N	Mean (Std.Err.)	Difference	% Change
TE	Access	184	0.856 (0.008)	0.145 (0.018)***	20.39
	Non-access	76	0.711 (0.020)		
Rice Yield	Access	184	3.742 (0.045)	1.017 (0.075)***	37.32
	Non-access	76	2.725 (0.046)		

Note. *** Significance at 1%; ** Significance at 5%; * Significance at 10%.

4. Conclusion and Recommendations

As many African countries, Senegal has high level of rice consumption. However, the national rice production level is far from meeting the domestic demand largely due to the poor performance of farmers in rice production and productivity. Even the Senegalese government has undertaken the national rice self-sufficiency program to address the issue, the country still falls behind rice self-sufficiency. According to the existing literature, credit access is an important factor for farmers' performances in Senegal, but the picture of the impacts of credit access on rice production is still not clear enough. Therefore, the present study investigated the effect of agricultural credit on rice farmers' productivity and efficiency with the data collected from the irrigated area of Anambe basin.

The results indicate that labor, pesticide, herbicides and fertilizer positively and significantly affect the rice productivity in the Anambe basin. The model also indicates that there are some important technical efficiency determinants such as agricultural credit, gender, education, ethnicity, irrigation cost, rice variety, and land tenure system have strong influences on the technical efficiency of rice production in Senegal. Particularly, the coefficient of credit is -0.038 and significant at 10% level, implying the agricultural credit access would decrease rice production inefficiency. The technical efficiency for credit beneficiaries is 0.856 on average which is higher than the average efficiency of 0.711 for non-beneficiaries. Furthermore, credit beneficiaries have 37% higher rice yields than their counterparts.

The policy implications of these findings are that agricultural credit allows farmers to decrease their technical inefficiency in rice cultivation. The government could set up policies to improve the technical efficiency of rice production by supporting better access to credit. More education programs for rice farmers and empowering women farmers would also be helpful for the production improvement. It is also necessary to provide farmers improved seed in order to increase their efficiency.

There are some limitations in present study. Firstly, only TE of farmers in the irrigated area of Anambe basin was evaluated. Secondly, the use of cross-sectional data does not support the assessment of the impact of agricultural credit over time. However, given the importance of staple rice to food security in Senegal, both farmers and government should take measures to improve the rice production performance and achieve rice self-sufficiency finally.

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Quantification of Bioactive Molecules, Minerals and Bromatological Analysis in Carao (*Cassia grandis*)

Jhunior Abrahan Marcía Fuentes[1,2], Ismael Montero Fernández[3], Héctor Zumbado Fernández[4], Jesús Lozano Sánchez[5], Ricardo Santos Alemán[6], Miguel Navarro-Alarcon[5], Isabel Borrás-Linares[7] & Selvin Antonio Saravia Maldonado[8]

[1] Faculty of Technological Sciences, Universidad Nacional de Agricultura, Catacamas, Olancho, Honduras

[2] Faculty of Pharmacy and Food, University of Havana, Cuba

[3] Department of Organic and Inorganic Chemistry, University of Extremadura, Cáceres, Spain

[4] Faculty of Health Sciences, Universidad Técnica de Manabí, Ecuador

[5] Department of Food Science and Nutrition, University of Granada, Granada, Spain

[6] Department of Food Sciencie, Louisiana State University, USA

[7] Functional Food Research and Development Centre, Granada, Spain

[8] Faculty of Earth Sciences and Conservation, Universidad Nacional de Agricultura, Catacamas, Honduras

Correspondence: Jhunior Abrahan Marcia Fuentes, Faculty of Technological Sciences, Universidad Nacional de Agricultura Road to Dulce Nombre de Culmí, Km 215, Barrio El Espino, Catacamas, Olancho, Honduras. E-mail: juniorabrahamm@yahoo.com

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Abstract

Medicinal plants have various beneficial conditions for humanity, one of them is its medicinal contribution due to the presence of phytochemicals and antioxidants, characterized by these bioactive compounds as the main source of nutraceuticals. The fruit of *Cassia grandis*, traditionally known as *carao*, is a plant that is attributed antimicrobial and medicinal properties. The objective of this work was to determine the bromatological, mineralogical composition and bioactive molecules of *carao* in the department of Choluteca (Honduras). Total phenolic compounds determined by the Folin-Ciocateau method resulted in higher concentrations in the seeds 11.1±0.3 mg EAG 100 g^{-1}. The antioxidant activity was also found to be higher in *carao* seeds, with concentrations of 7.31±0.11 μg g^{-1} of DPPH and total carotenoids showed higher concentration in the pulp with a concentration of 4.12±0.11 μg mL^{-1}. Among the macro minerals, high concentrations of magnesium and calcium stand out in the seed with a concentration of 18.27±0.14 mg 100 g^{-1} and 7.31±0.23 mg 100 g^{-1} respectively. Among the microminerals, iron stands out in higher concentrations than in the rest of the microminerals being higher in the shell with concentrations of 1.71±0.23 mg 100 g^{-1} followed by manganese in concentrations of 0.51±0.12 mg 100 g^{-1}.

Keywords: iron, carotenoids, biotechnology, DPPH

1. Introduction

The *carao*, whose scientific name is *Cassia grandis*, belongs to the legume family and is a sub family of the *Cesalpinaceae*, it is commonly known by different names such as *caña*, *fistula*, *cañandonga* and *carao* (Lagarto & Guerra, 2005; Carvalho, 2006; Lafourcade et al., 2014). There are approximately more than 500 *Cassia* species worldwide, represented as herbs, shrubs and trees (Sadiq et al., 2012; Korlam et al., 2016). This species is found in the wild mainly in India, China, East Africa, South Africa and in some countries of the American continent, such as Brazil, Colombia, Mexico, Cuba, El Salvador, Nicaragua, Costa Rica and Honduras (Lafourcade et al., 2014; Ramos et al., 2014; Marcía et al., 2017; Bomfim Gois et al., 2018). The *carao* fruits is rigid and can only be opened by applying mechanical forces, inside it many lobes are seen separated by very thin transverse partitions of yellowish color. A dark red flesh, thick consistency, strong odor, sweet taste and easily soluble in water (Jaime et al., 2012).

The *carao* is a tree that grows from 15 to 30 m high, with a width of 45 to 100 cm with a cylindrical shaft that branches from the middle, round crown with about 8 m in diameter, its bark is smooth, of brown gray color, 30 mm thick, the leaves are composed and alternate with 15 to 20 pairs of opposite leaflets, 2 to 5 cm long and 1 to 1.5 cm wide, rounded base and green , the inflorescences have 15 or more flowers, an intense pink color, the seeds are 2 to 4 cm long and 1.5 to 2.5 cm wide and the pod that contains it can reach up to 75 cm in length (Figure 1) (Ramos et al., 2014).

Figure 1. Carao (*Cassia grandis*)

Currently there are studies that validate the medicinal potential of *Cassia grandis*, due to its phytochemical composition, its antioxidant capacity and its high bioavailability. Romero et al. (2018) affirm that the seed of the fruit can be used as an anti-diabetic potential, due to its inhibitory effect of trypsin. Fruit pulp from in vivo models showed a reduction in blood glucose levels (Lafourcade et al., 2018; Lodha et al., 2019). Its nanodispersion exerts a hypoglycemic effect with a potent inhibition of alpha-glucosidase and pancreatic lipase (Lafourcade et al., 2019). In addition, the fruit of *Cassia grandis* has shown its anti-anemic potential from in vivo studies, due to its inorganic iron content and good bioavailability (Tillán et al., 2004; Lafourcade et al., 2014; Lafourcade et al., 2016). From studies of phytochemical characterization in carao fruit, it was determined that it contains good iron content, presence of saponins, porphyrins, flavonoids, tannins, phenols, essential oils and a high antioxidant capacity (Deshpande & Bhalsing, 2011; Jaime et al., 2012; Ramos et al., 2014; Kabila et al., 2017).

Other investigations on carao extracts (*Cassia grandis*) determined the presence of the galactomannan biopolymer and, due to its rheological characteristics, can be used as a substitute for gums in the food industry (Harsha & Kapoor, 2003). The objective of this work was to carry out a bromatological characterization of the different parts of the *carao* fruit, mineral analysis as well as composition of total phenolic compounds, antioxidant activity and carotenoids in the different parts of the fruit.

2. Material and Methods

2.1 Sample Collection and Preparation

The samples were collected in the department of Choluteca (Honduras) between February and March 2019, and were subsequently taken to the Biotechnology Laboratory of the National University of Agriculture, Catacamas (Honduras) where they were separated in different parts of the fruit and dried in an air circulation oven for 48 hours to maintain constant weight. Subsequently they were ground, sieved and stored in suitable containers until the time of the analysis.

2.2 Bromatological Analysis

The bromatological parameters analyzed to later determine the total energy value in the different parts of the fruit were the humidity, quantity of ashes carried out in a muffle at 600 °C and the percentage of ashes calculated by mass difference. Total proteins were determined by the Kjeldahl distillation method with previous sulfuric digestion. The percentage of total lipids was determined by Soxhlet type extractor with hexane and the percentage of carbohydrates was calculated by difference using Equation 1, according to the methodology described by IAL (2008).

$$\text{Energetic value (kcal 100 g}^{-1}) = (P*4) + (L*9) + (C*4) \quad (1)$$

Where, P = protein value (%), L = lipid value (%), C = carbohydrate value (%), 4 = kcal conversion factor determined in calorimetric pump for proteins and carbohydrates and 9 = kcal conversion factor determined in calorimetric pump for lipids.

2.3 Mineral Analysis

For the determination of minerals, the samples were first subjected to perchloric nitric digestion (3:1), the following elements being determined: Ca (λ = 422.70 nm), Mg (λ = 285.21 nm), Fe (λ = 248.33 nm), Zn (λ = 213.80 nm), Mn (λ = 279.48 nm), Cu (λ = 324.75 nm) by FAAS, Na and K by EAS and by molecular spectrophotometry Uv-visible phosphorus (λ = 660 nm) and sulfur (λ = 420 nm) according to the methodology described by EMBRAPA (2008).

2.4 Phenolic Compounds and Antioxidant Activity

Total phenolic compounds was determined using the Folin Ciocateau method with formation of a blue complex using gallic acid as a reference standard, the absorbance readings being performed on a Uv-Visible spectrophotometer at 765 nm according with the methodology described by Wolfre et al. (2013). To determine the antioxidant activity, the method of the radical 1,1-diphenyl-2-picrilhydrazil (DDPH) was used and the iron reduction method was used. In the first method, the absorbance reading at 515 nm was performed (Miranda & Fraga, 2006), the calibration being made from dilutions of a 60 mM DPPH solution in methanol. The second method of determining the antioxidant activity was the method based on the reduction of Fe^{3+} for Fe^{2+} according to the methodology proposed by Sanchez Moreno et al. (2008), with the readings in Uv-Visible molecular absorption spectrophotometry a 690 nm.

2.5 Total Carotenoids

For the quantification of total carotenoids, one g. of sample was extracted with 18 mL of acetone, the readings were made in UV-Visible molecular absorption spectrophotometer at 470 nm, 661 nm and 664 nm respectively, being calculated using Equations 2-4 described by Lichtenthaler and Buschmann (2001) (Equations 2-4),

$$C\ \text{carotenoids}\ (\text{mg mL}^{-1}) = (1000\ A_{470} - 1.90\ Ca - 63.14\ Cb)/214 \quad (2)$$

$$Ca\ (\text{mg mL}^{-1}) = 11.24\ A_{661} - 2.04\ A_{644} \quad (3)$$

$$Cb\ (\text{mg mL}^{-1}) = 20.13\ A_{664} - 4.19\ A_{661} \quad (4)$$

2.6 Statistical Analysis

The data was analyzed in the SPSS software, version 25.0, using, tukey test at ($P < 0.05$) to identify significant differences.

3. Results and Discussion

3.1 Bromatological Analysis

In Table 1, the values of the nutritional composition and total energy value for the different parts of the fruit studied are presented.

Table 1. Bormatological composition and total energy value in *carao*

Fruit parts (%)	**Humidity**	**Ashes**	**Lipids**	**Carbohydrates**	**Proteins**	**Energetic Value** (Kcal 100 g^{-1})
Pulps	26.72a	2.80b	0.21b	61.93c	8.34b	**282.97c**
Shell	8.19c	2.42c	0.14c	88.01a	1.24c	**358.26a**
Seeds	9.58b	3.74a	1.17a	75.40b	10.11a	**352.57b**
Whole fruits	17.31	3.14	0.74	71.4	7.41	**321.9**

Note. * Means with different letters in the same column indicate statistical differences ($P \leq 0.05$) with Tukey test.

The highest humidity values for the different parts of the fruit studied are in the pulp, with values of 26.72% and the part that presented less moisture was the crust with only 8.19%. The ash content in *carao* is one of the lowest bromatological parameters, with the seeds having the highest mineral value with 3.78%. Among the parameters that contribute to the energy value of the fruit are lipids, carbohydrates and proteins, the amount of lipids is very low, reaching the value at 1.17% for the seeds. Again, the amount of protein is higher for seeds with 10.11%.

Carbohydrates, including fibers, are the major constituents of *carao*, with the peel presenting the highest percentage of carbohydrates with 88.01%.

Among the parameters that contribute to the energy value of the fruit are lipids, carbohydrates and proteins. The amount of lipids is very low, reaching the value at 1.17% for the seeds. Again, the amount of protein is higher for seeds with 10.11%. Carbohydrates, including fibers, are the major constituents of *carao*, with the peel presenting the highest percentage of carbohydrates with 88.01%. Given the high percentage of carbohydrates found in legumes, and especially in *carao*, they can be used as an unconventional food source. The carbohydrate content in this legume is higher than that found in other tropical legumes such as Inga, whose percentage reaches 27.62% (Mendoza et al., 2016). As for the energy value, the part of the fruit that has an important contribution is the shell with 358.26 kcal 100 g^{-1}. The daily energy recommendations of legumes are around 2,000 kcal 100 g^{-1} in accordance with the specifications of the European Economic Community 90/496/EEC of September 24, 1990.

3.2 Mineralogical Analysis

Table 2 shows the values of the different minerals analyzed for the different parts of the fruit studied, as well as for the whole fruit of *carao*. Among the macro minerals, magnesium stands out as the majority, being its highest concentration for seeds with values of 18.27±0.14 mg 100 g^{-1}. This element is of great importance for the body as it is involved in numerous metabolic reactions (Wolfe & Cittadini, 2003). The recommendations of this element according to DRI (2011) are 420 mg day^{-1} for men and 320 mg day^{-1} for women. The next element in importance within the macro minerals is calcium which, like magnesium, this element is in higher concentrations in seeds with a concentration of 7.31±0.21 mg 100 g^{-1}. This element is essential for the mineralization of bones and teeth (França & Martini, 2014) being the recommendations of 1000 mg day^{-1} for both sexes, according to the recommendations of the DRI (2011). Sodium and potassium are also two important elements to maintain the electrolyte balance in the cell such as the sodium potassium pump (Cuppari & Bazanelli, 2010). In *carao* fruit, potassium is found in concentrations higher than sodium, reaching values of 8.23±0.18 mg 100 g^{-1} for seeds and instead sodium is found in higher concentrations in the pulp, in concentrations of 2.56±0.13 100 mg g^{-1}.

Table 2. Shows the macro and micromineral values in the different parts of the *carao*, as well as in the whole fruit

Concentration (mg $100g^{-1}$)	**Seeds**	**Pulps**	**Shells**	**Whole fruits**
Ca	7.31±0.21a	5.67±0.12b	4.67±0.17c	6.21±0.12
Mg	18.27±0.14a	14.31±0.12b	11.21±0.07c	15.46±0.07
K	8.23±0.18a	3.45±0.07b	2.43±0.14c	4.31±0.08
Na	0.85±0.07c	2.56±0.13a	1.31±0.07b	1.47±0.31
Fe	1.71±0.23a	1.54±0.12b	0.81±0.07c	1.14±0.21
Cu	0.21±0.08b	0.14±0.03c	0.71±0.12a	0.44±0.11
Zn	0.46±0.09a	0.34±0.11b	0.21±0.07c	0.27±0.13
Mn	0.51±0.12a	0.25±0.07b	0.21±0.07c	0.38±0.08
P	0.47±0.07a	0.21±0.02b	0.14±0.07c	0.26±0.08
S	0.08±0.01c	0.17±0.04a	0.11±0.03b	0.04±0.01

Note. * Means with different letters in the same line indicate statistical differences ($P \leq 0.05$) with Tukey test.

The daily recommendations for this element in adulthood are 8 mg day^{-1} for men and for women aged 19-50 years. The recommended concentrations are 18 mg day^{-1} and from 50 years of 8 mg day^{-1} according to the DRI (2011). Manganese is another of the micronutrients found in carao in significant concentrations, the highest concentration being for seeds with a concentration of 0.51±0.12 mg 100 g^{-1}. Manganese is the second micronutrient after iron of interest to plants (Malavolta, 2006), but at the same time it plays an antagonistic role with iron in the body, since in the diet, excess manganese can cause reduced absorption of iron causing anemia in addition to affecting the central nervous system (Roels et al., 1997). Zinc has different physiological functions in the cell, such as the hepatic mobilization of vitamin A, in sexual maturation, fertility and reproduction, phagocytic, cellular and humoral immune function (Manganaro, 2008) the concentration in *carao* seeds being 0.46±0.09 mg 100 g^{-1}. Copper is another essential nutrient not synthesized by the body, being found in fruits in concentrations between 0.02-0.66 mg 100 g^{-1} according to Amancio (2017).

In *carao*, the copper concentrations found are very low, being in greater concentration in the bark of this fruit in a concentration of 0.71±0.12 mg 100 g^{-1}. Two other elements analyzed in this fruit were phosphorus and sulfur. The highest phosphorus concentration was found in the seed with a concentration of 0.47±0.07 mg 100 g^{-1} acting in the energy metabolism of ATP, involved in carbohydrate metabolism and present at the same time in the synthesis of phosphated sugars, nucleic acids and coenzymes (Epstein & Bloom, 2006). Sulfur was found in low concentrations in *carao* with concentrations of 0.17±0.04 mg 100 g^{-1}, being an element that is also part of the structure of biomolecules such as proteins and found in the body in concentrations of up to 140 grams (Lisbon, 2015).

3.3 Phenolic Compounds, Antioxidant Activity and Total Carotenoids

Table 3 shows the values of phenolic compounds, antioxidant activity and total carotenoids in the different parts of the *carao* fruits.

Table 3. Phenolic compounds, antioxidant activity and total carotenoids in different parts of *carao*

Parts	**Total Phenolic Compounds** (mg EAG 100 g^{-1})	**Antioxidant Activity**		**Total carotenoids** ($\mu g\ mL^{-1}$)
		DPPH ($\mu g\ g^{-1}$)	**Iron reduction** (mg g^{-1})	
Pulp	5.6±0.2b	6.07±0.02b	0.21±0.01b	4.12±0.11a
Shells	2.3±0.1c	5.12±0.04c	0.18±0.02c	2.21±0.07c
Seeds	11.1±0.3a	7.31±0.11a	0.41±0.02a	3.76±0.03b
Fuit total	6.3±0.1	6.48±0.07	0.34±0.04	2.56±0.04

Note. * Means with different letters in the same column indicate statistical differences ($P \leq 0.05$) with Tukey test.

The total phenolic compounds determined in the different parts of the *carao* samples as well as in the whole fruit varied between 2.3±0.1 mg EAG 100 g^{-1} for the shell to concentrations of 11.1±0.3 mg EAG 100 g^{-1} for the seeds, this part being the one with the highest concentration of phenolic compounds. In comparison with other legumes such as *Vicia faba*, these values are within those determined by Valente et al. (2018), reaching values of 13±0.1 mg EAG 100 g^{-1}. The antioxidant activity, was carried out by two methods: by means of the DPPH technique and on the other hand by means of the iron reduction method, being again the *carao* seed who has the highest antioxidant activity with antioxidant activity values of 7.31±0.11 μg g^{-1} by the DPPH method and 0.34±0.04 mg g^{-1} by the iron reduction method. Godevac et al. (2008) study the antioxidant activity of nine species of *Fabaceae*, obtaining values higher than those obtained for *carao*. Other authors such as Pirela et al. (2011), study the antioxidant activity in the *Genisteae* also belonging to the *Fabaceae* family, obtaining values of 0.15 to 0.50 mg mL^{-1}, being lower than those determined in this work. The last group of molecules studied in this work are the carotenoids that give the compound a certain added biotechnological potential, since they are precursors of vitamin A, they have antioxidant, anti-inflammatory and anti-tumor properties (Rehman, 2020). The concentrations of this group of substances in the study species varied between 2.21±0.07 μg mL^{-1} for the cortex of the *carao*, reaching values of 4.12±0.11 μg mL^{-1} for the pulp, being in this part of the fruit where the highest amount of carotenoids is found.

4. Conclusions

Although *carao* has been traditionally used in countries of Central America as a nutritional alternative, specifically to meet the needs of iron in blood, there is not much data regarding its chemical composition, so this work serves to highlight the energy, mineralogical and Bioactive molecules that this fruit has to be used with biotechnological potential in neutraceutical foods.

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Poultry Farming Practices Affect the Chemical Composition of Poultry Manure and Its C and N Mineralization in a Ferric Acrisol

Delwendé Innocent Kiba[1], Nongma Armel Zongo[1], Ouakoltio Youssouf Abidine Traoré[1], Mohamed Louré[1], Harouna Barry[1], Sogo Sanon Bassirou[1], Gnankambary Zacharia[1], Ouandaogo Noufou[1], François Lompo[1] & Michel Papaoba Sedogo[1]

[1] Département Gestion des Ressources Naturelles et Systèmes de Production, Institut de l'Environnement et de Recherches Agricoles, Ouagadougou, Burkina Faso

Correspondence : Delwendé Innocent Kiba, Département Gestion des Ressources Naturelles et Systèmes de Production, Centre National de la Recherche Scientifique et Technologique, Institut de l'Environnement et de Recherches Agricoles, BP 476 Ouagadougou 01, Burkina Faso. Tel: 226-7076-2747. E-mail: innokiba@yahoo.fr

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Abstract

Industrial poultry farming is a booming sector in Africa. This activity generates a significant amount of manure that could be used to improve crop yields on low-productivity soils. We wanted to characterize the variability in the chemical composition of poultry manure and its ability to release mineral nitrogen when applied to soils compared to other organic sources of nutrients such as cattle manure and human feces. We conducted a survey in 79 poultry farms to characterize their practices such as the type of poultry raised, the type of feed and the bedding litter. Poultry manure, cattle manure and human feces samples were collected and analyzed to determine their chemical composition. An incubation study was conducted with all three types of organic resources for 91 days to measure mineral nitrogen release. We found that agricultural practices explain more than 60% of the chemical composition of poultry manure. Wood chips were the most common bedding litter (77% of cases) and about 70% of farms use industrial poultry feed. Broiler manure contains more C and N than laying hens that contain more Ca. Poultry manure releases nitrogen faster than cattle manure when applied to the soil. A combination of broiler chicken manure and laying hen manure could be more beneficial to the crops.

Keywords: poultry manure, Ferric Acrisol, incubation, mineral nitrogen release

1. Introduction

In addition to poor weather conditions and low fertility soils, agriculture in sub-Saharan Africa suffers from low mineral fertilizer inputs (Yasuhiro et al., 2019) resulting in low yields. Indeed, the quantities of mineral fertilizers applied are well below the quantities declared for other regions of the world. For example, the amount of nitrogen per hectare of cultivated land is less than 15 kg ha^{-1} in Africa compared to more than 100 kg per hectare in Asia (Ciceri et al., 2019). Several reasons are often cited to explain this low level of mineral fertilizer use, including low farmer income, lack of subsidies, inappropriate agricultural policies, and poor market access (Mugwe et al., 2019).

In such a context of farmers' inability to purchase mineral fertilizers, local organic resources remain the main source of nutrients for soil fertility management and agricultural production. The use of organic resources in agriculture is particularly relevant for the majority of soils in sub-Saharan Africa such as Ferric Acrisol, given their low aggregate stability, low organic matter content and low cation exchange capacity (Traoré et al., 2016). Therefore, the functioning of these soils and their ability to produce a good yield rely strongly on the quantity and quality of the organic resources applied.

While organic resources are important to improve the productivity of Ferric Acrisol, their availability and quality according to their origin remains a major challenge. Crop residues, due to the high demand for animal fodder, energy and other domestic uses, are exported from the fields, leading to nutrient mining when there is no return to the soil. The use of manure from small and large ruminants is limited by their low availability, especially when the animals are not in housing, which is the most common case in small farms. Composting has long been promoted as an option to produce good quality organic matter and also to solve the problem of lack of organic resources by combining different sources of animal and plant origin. For example, in Burkina Faso, the

government launched the operation of compost pits in 2001 to promote the use of organic resources on farms (Lompo et al., 2009). However, the lack of water, labour for the transport of organic resources and regular turning of the compost limit the adoption of this technology by farmers.

Among the organic resources, poultry manure from industrial farms is a potential available source. Indeed, on the outskirts of African cities, the poultry industry has grown so rapidly over the past decade due to the high food demand associated with rapid population growth. The development of this sector has created an important source of organic resources that can be used to improve soil productivity, particularly in peri-urban and semi-rural areas close to these organic resources. In Burkina Faso, the number of poultry heads is estimated at about 36.5 million (MRA, 2009). Coulibaly et al. (2018) showed in the Bobo-Dioulasso region, the second largest city in Burkina Faso, that extensive poultry farming, estimated at about 70% of farms, produces about 5 kg of manure per head of poultry per year, whereas for intensive livestock production this production can reach 19 kg. Considering the poultry population estimate by MRA (2009) and by Coulibaly et al. (2018), we can expect about 300,000 tons of poultry fertilizer per year. According to Coulibaly et al. (2018), the amount of poultry manure is estimated at about 35% of the total organic resources produced on farms.

Various materials are used as bedding in poultry farming. The quality of these materials such as their absorption capacity have an impact on poultry productivity (Garcia et al., 2012; Munir et al., 2019). Differences in poultry moisture have been observed with different bedding materials (Shepherd et al., 2017). Miles et al. (2011) have shown that the higher the humidity in the litter, the greater the loss of nitrogen by volatilization.

While much work has been done specifically on the impact of bedding materials on poultry productivity and nitrogen losses, little has been done on the overall practices of poultry farmers and their impact on the agronomic value of poultry manure (Dexter et al., 2019). In this study, we hypothesized that: i) the diversity of farming practices explains a large part of the variability of the chemical composition of the poultry manure, ii) among the farming practices, the type of bedding material is an important factor determining the chemical composition of the poultry manure and iii) the type of bedding determine the C:N ratio of the poultry manure leading to differences in nitrogen mineralization.

2. Material and Methods

2.1 Survey and Sampling

The study was conducted at the poultry farms located at the vicinity of the city of Ouagadougou in Burkina Faso (12°30′ and 12°25′ North latitude and 1°27′ and 1°35′ West longitude) at the communes of Komsilga, Pabré, Loumbila, Saaba, Koubri, Tanghin Dassouri, Sourgoubila, and Komki-Ipala. A survey was conducted in 79 poultry farms with an average of about 8 farm per commune. For a given commune a first farm was identified as a starting point of the survey and then the next farm was identified with the help of the previous farmer. Each farmer was questioned about the livestock practices namely the size of the farm, the type of poultry raised, the type of feed, the type of bedding litter used, the frequency of bedding litter change, the use of vaccine, the management strategies of the manures.

At each poultry farm, the manures were sampled at three points in the barns along the diagonal and mixed to yield one composite sample. For manures outside the barns and stored in pits or piles, samples were taken at different depth and points and a composite sample was also made for each type.

2.2 Incubation Study

The incubation study was conducted in laboratory at the research station of INERA Kamboinsé. A Ferric Acrisol from the long term field trial of Saria receiving 5 t ha^{-1} of manure every second year was used. This soil according to Kiba (2012) has a pH of about 5.8 and contains 3.73 g kg^{-1} of total C and 320, 149 and 32 mg kg^{-1} of total N, P and available Bray I P, respectively. The following treatments were considered: Control: non amended soil; LCNrD: soil + manures with low C:N ratio (17 to 21); HCNrD: soil + manures with high C:N ratio (41 to 82); CAM: soil+ cattle manure; HMF: soil+ human feces. The cattle manure was collected from a farm located at the vicinity of Ouagadougou and the human feces collected from popularized latrines within the ecological sanitation project (ECOSAN). For each sample, a carbon content of 2 g C from the substrate/kg dry soil was targeted when applying the organic substrates (AFNOR, 2009). According to this method, 35 mg N per kg of dry soil in the form of KNO_3 was also applied at the beginning of the incubation to ensure that the decomposition of the organic substrates will not be nitrogen limited.

After the application of the organic substrates and the KNO_3 the samples were moistened at 45% water holding capacity. For each sample of organic substrate 3 replicates were considered. An amount of 25 g of each treated soil was then placed in hermetically sealed jars. Two beakers, one containing 20 ml of NAOH 0.1 N to trap the

CO_2 released and another one containing 20 ml of water to maintain the moisture were also placed in each jar. The jars were then placed in an incubator at 25 °C. The CO_2 emission was measured at 1, 3, 7, 14 and 21 days after incubation. For each sample the mineral nitrogen (NO_3^- and NH_4^+) was also extracted at 0, 7, 14, 28, 49, 70 and 91 days of incubation and measured.

2.3 Chemical Analyses

The pH in water of the different substrates was measured with a glass electrode pH meter and direct reading in a substrate/solution ratio of 1/2.5. The total C of the organic substrates was determined by weight loss after a calcination at 550 °C during 2 hours using an electrical furnace CARBOLITE. The total elements N and P contents of the composts were determined by an automatic colorimeter SKALAR (Skalar SANplus Segmented flow analyzer, Model 4000-02, Breda, Holland) after a wet digestion using the Kjedhal method adapted by Novozansky et al. (1983). The total potassium was determined in the digested samples by a JENCONS flame emission spectrophotometer using the method proposed by Walinga et al. (1989) while the total Ca, Fe, Mg, Zn, Mn were measured by atomic absorption spectrometry using the method proposed by Pinta (1973). The mineral nitrogen of the incubated samples was extracted using a potassium chloride solution at a ratio of 10g treated soil in 100 ml of KCl and measured in colorimetry with the SKALAR as described above.

2.4 Statistical Analyses

A non-parametric Kruskall-Wallis test was performed to compare the chemical composition of the poultry manure according to the poultry farming practices using GenStat 9.2. In the incubation study, one way analyze of variance was performed to compare the mean chemical composition of the two types of poultry manures, the cattle manure and the human feces using also GenStat 9.2. Principal Component Analysis was performed to study the variability of chemical composition of the poultry manures across the farms using CANOCO 5.1. A redundancy analysis using CANOCO 5.1 was performed to summarize the variability of the chemical composition and moisture of the poultry manures explained by poultry farming practices. The qualitative information was encoded as dummy variables using 1 for the presence and 0 for the absence.

3. Results

3.1 Poultry Farming Practices

The Table 1 shows that two types of poultry manures were encountered. These were manures not mixed with litter accounting for about 6% and manures mixed with various types of litter accounting for about 94%. The types of litter used for bedding were wood chips, rice bran, rice husk and peanut shells encountered in 77.5%, 4.5% 16.9% and 1.1% of cases, respectively. The raising of laying hen was the most important practice (55.7%) followed by broiler chicken (27.8%). The majority of the farms feed the animals with industrial food (70.9%) while about 20.3% of them produce the food locally.

Table 1. Poultry farming practices in percentage of cases in the peri urban areas of Ouagadougou, Burkina Faso

Manures purity		**Type of litter**		**Type of poultry**		**Type of feeding**	
Mixed with litter	93.7%	Wood chips	77.5%	Laying hen	55.7%	Local food	20.3%
		Rice bran	4.5%	Broiler chicken	27.8%	Industrial food	70.9%
Not mixed	6.3%	Rice husk	16.9%				
		Groundnut shell	1.1%	Other	16.5%	Both	8.9%

3.2 Variability of Farms According to the Chemical Composition of the Poultry Manures

The diversity of the farms is explained by about 50% of the chemical composition of their poultry manures (Figure 1). The horizontal axis explaining about 26% of the variability was correlated with the major nutrients N, P and K while the vertical axis explaining about 22% of the variability was correlated by the total carbon, pH, secondary and micro-nutrients.

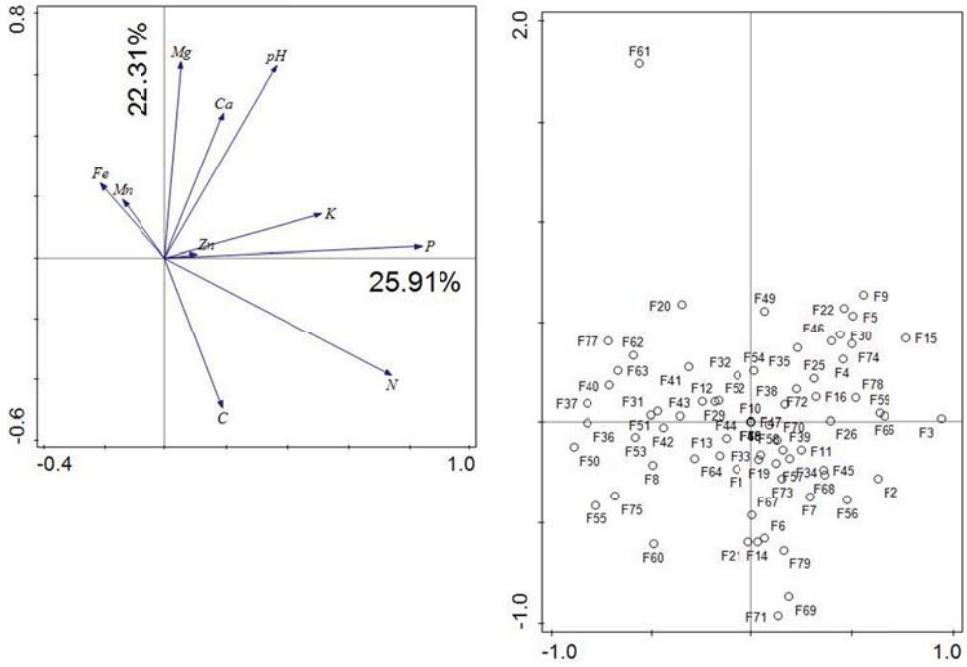

Figure 1. Principal component analysis showing the variability of chemical composition of the poultry manures across the farms at the periphery of Ouagadougou, Burkina Faso. The arrows indicate the chemical composition; the circles indicate the farms

3.3 Variability of the Poultry Manures as Affected by Livestock Practices

Livestock practices explained about 60% of the variability in moisture and chemical composition of poultry manures (Figure 2). The horizontal axis explaining about 34% of the variability was correlated with turkey, guinea fowl farming and the use of rice straw as litter. The vertical axis explaining about 26% of the variability was correlated with the rearing of laying hens and local chickens. The Zn content of poultry manures was positively correlated with the absence of litter and the rearing of quail. The C content was positively correlated with the use of rice husk and wood chips as bedding. Ca content and pH were positively correlated with the rearing of laying hens. The Fe content was positively correlated with guinea fowl rearing. The content of N, P, K, Mg in poultry manure were positively correlated with the use of the vaccine and negatively correlated with the rearing of local chickens and mixed breed chicken. The type of food, whether produced locally or by industry, had little influence on the variability of the chemical composition of the manures.

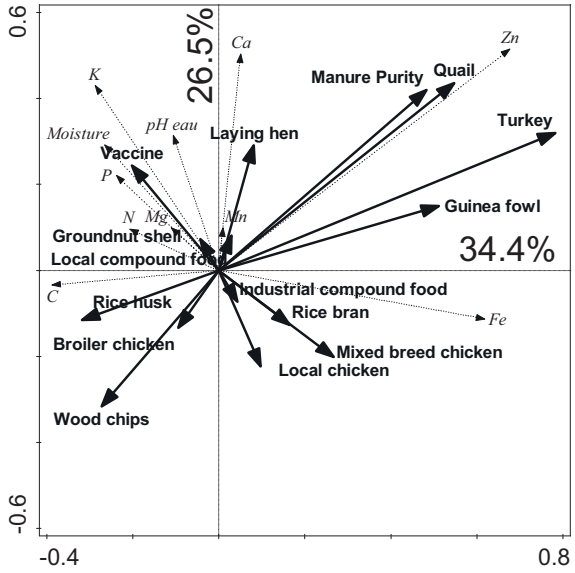

Figure 2. Redundancy analysis summarizing the variability of the chemical composition and moisture of the poultry manures explained by poultry farming practices at the periphery of Ouagadougou, Burkina Faso. The dotted arrows indicate the chemical composition; the bold arrows indicate the livestock practices

3.4 Chemical Composition of the Poultry Manures

The chemical composition of the poultry manures was not affected by most of the livestock practices (Table 2). Significant differences were only observed for the total C, N and Ca contents between the manures from laying hen and those from the broiler chickens. Indeed, the manures from broiler chickens showed the highest C and N contents while the manures from laying hen showed the highest Ca content.

The comparison between selected high and low C:N ratios manures, cattle manure and human feces (Table 3) showed significant differences for the contents of C, N, P, Fe, Mg, Zn, pH, C:N ratio and no differences for K, Ca and Mn contents. Human feces had the highest pH, the highest Mg, Zn contents, the lowest C content and the lowest C:N ratio. The lowest N content was measured in the high C:N ratio poultry manures and the human feces while the lowest P content was measured in the high C:N ratio poultry manures and the cattle manure. Excluding Fe, all measured elements had low levels in poultry manure with a high C:N ratio compared to poultry manure with a low C:N ratio.

Table 2. Chemical composition of poultry manures as affected by the livestock practices. LHM: laying hen manures; BCM: Broiler chicken manures

Poultry farming practices	**C**	**N**	**P**	**K**	**Ca**	**Fe**	**Mg**	**Zn**	**Mn**	**pH**	**C:N**
	---------- gkg^{-1} ----------							----- mgkg^{-1} -----			
Type of poultry											
LHM	385	14.6	7.2	19.5	14.2	7.6	5.1	214	298	7.7	30.3
BCM	452	18.2	8.6	21.0	9.0	6.9	6.1	145	193	7.6	30.0
p value[1]	**	*	*NS*	*NS*	***	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
Barn litter type											
Wood ships	405	15.7	7.5	19.3	11.6	8.7	5.4	144.2	262.7	7.6	30.2
Other litter	351	15.3	7.1	21.2	15.2	10.7	5.1	426.6	271.3	7.6	24.6
p value[1]	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
Type of feed											
Industrial	407	17.8	7.4	21.4	13.9	8.2	6.1	184.8	397.5	7.7	30.3
Local	394	15.0	7.4	*19.0*	11.6	9.2	5.2	183.0	225.7	7.6	26.3
p value[1]	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*

Note. [1]Kruskal-Wallis non parametric test Chi-square probability.

Table 3. Chemical composition of poultry manures, cattle manure and human feces used in soil incubation study. LCNrPM: Low C:N ratio poultry manures; HCNrPM: High C:N ratio poultry manures, CAM: Cattle manure; HMF: Human feces; Pro: Probability; LSD: Least significant difference

		LCNrPM	HCNrPM	CAM	HMF	*Pro*	*LSD*
C		420	386	446	75.1	<0.001	82
N		20.2	5.42	13.5	8.80	<0.001	5.21
P		13.1	2.76	1.75	8.61	<0.001	2.24
K	gkg^{-1}	22.8	19.8	19.5	25.9	0.402	9.31
Ca		14.8	8.62	5.22	10.7	0.220	9.65
Fe		7.30	14.1	4.36	4.27	0.003	4.52
Mg		5.81	3.99	3.28	15.0	<0.001	4.03
Zn	$mgkg^{-1}$	157	176	126	857	<0.001	139
Mn		405	255	224	378	0.450	294
pH		8.0	7.8	8.3	9.3	<0.001	0.5
C:N		21.2	75.4	34.3	8.7	0.002	25.4

3.5 Mineralization of Poultry Manures

The incubation study (Figure 3A) showed a decrease in CO_2 emission from day 1 to day 3 in the control treatment, cattle manure and human excreta, while an increase was observed with poultry manures with high and low C:N ratios. Cattle manure and control treatments reached the CO_2 emission peak at 7 days of incubation while for other treatments, the peak was reached later at 14 days of incubation. The treatments were distributed as follows with regard to the CO_2 emissions Human feces < cattle manure < Low C:N poultry manure < High C:N poultry manure ratio.

The highest nitrogen release during the incubation period (Figure 3B) was observed with human feces up to 14 days, from which time the release increased with poultry manures with low and high C:N ratios. A decrease in nitrogen release was observed with cattle manure, which led to the lowest release, even below the control treatment, between 0 and 7 days of incubation before it started to increase.

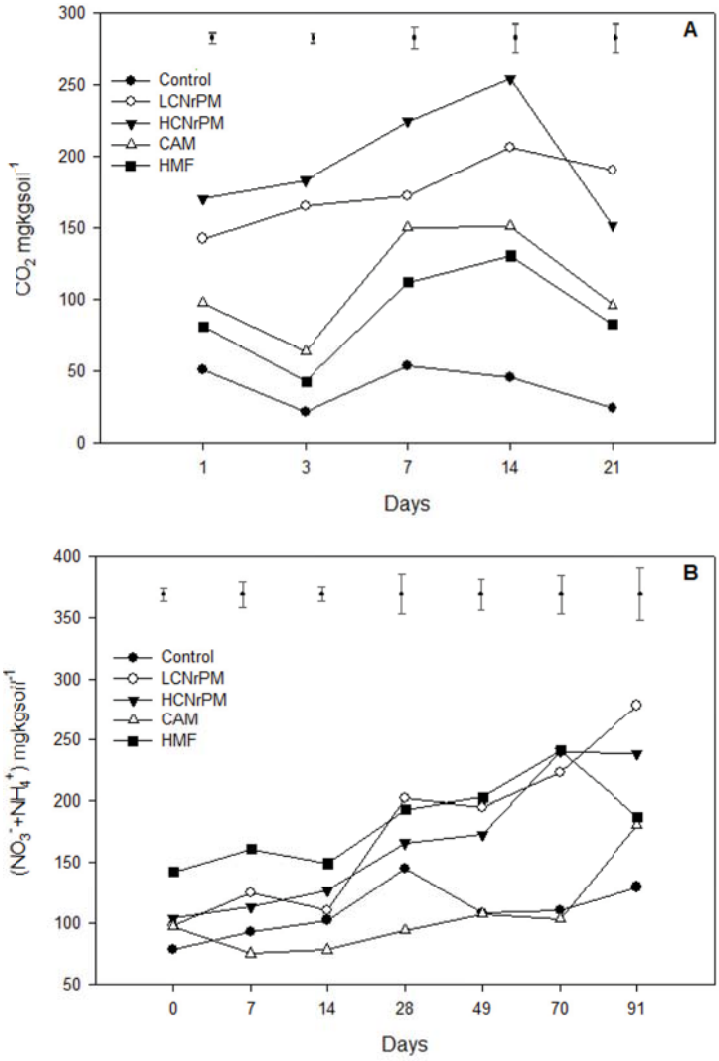

Figure 3. CO_2 (A) and mineral nitrogen (B) release in soil amended with poultry manures, cattle manure and human feces during a soil incubation. LCNrPM: Low C:N ratio manures; HCNrPM: High C:N ratio manures; CAM: cattle manure; HMF: human feces; Error bars are LSD:5

4. Discussion

4.1 Diversity of Poultry Farming Practices

The choice of litter type depends on many factors such as cost, availability, ease of handling, reusability, absorption capacity (Garcia et al., 2012). In our case, wood chips are an available material that can be collected from wood industries. Rice husks are available ressources at farm level probably because they are difficult to compost given their high C:N ratio and also because this biomass is not recommended to feed animals due to their low cellulose and sugar contents (Thiyageshwari et al., 2018). The study of Garcia et al. (2012) also showed that the use of wood chips and rice husk as bedding materials was the best option for Brazilian poultry farmers who base their judgment mainly on the possibility of reuse, followed by ease of handling and cost. It is interesting to notice that farmers' criteria for selecting litters are based on their ability to improve poultry productivity rather than obtaining good poultry manure. One of the main differences between laying hens (55.7% of cases) and broilers (27.8% of cases) is the length of time they are kept, which can influence the quality of manure. In most cases, broiler chicken rearing lasts about 45 days, whereas with laying hens, it can take up to 24 months.

4.2 Effects of Poultry Farming Practices on Poultry Manure Chemical Composition

The highest level of micronutrients such as Zn in pure manure, mainly quail manure, is due to the fact that the contents are not diluted by the addition of bedding materials. With the poultry farming practices studied, we were able to explain about 60% of the variability in the chemical composition and moisture content of poultry manure with the two canonical axes suggesting that the production of good poultry manure for agronomic purposes should be achieved through better chickens breeding practice. Rice husks and wood chips as bedding, due to their high carbon content, have increased the C content in poultry manure. The fact that litter influences the chemical composition of poultry manure is known (Bolan et al., 2010). Poultry farmers to obtain solid eggs enrich chicken feed with certain products such as bone meal and egg shells. Waheed et al. (2019) have shown that egg shell waste is an important source of Ca that can be used for food fortification and the production of calcium-rich food sources. This explains the high Ca content of laying hen manure compared to broiler chicken. Our results show that, whether it is the use of industrial or local food, the chemical composition of poultry manure does not change significantly. This suggests that farmers have a certain level of knowledge to produce good quality food locally assuming that industrial and homemade feed have the same quality. It is logical to believe that healthy poultry release the best excreta and therefore to understand the positive relationship between vaccine use and manure N, P, K and Mg content. Clearly, these vaccines have been used more for purebred chicken than for mixed and local chicken. The high C and N content of broiler manure compared to laying hen manure is due to the short breeding time of broiler chicken, which does not allow a long period of manure mineralization. Poultry manure with a high C:N ratio probably received more litter than that with a low C:N ratio, which explains the low nutrient levels due to a dilution effect. High nutrient levels in poultry manure with a low C:N ratio compared to cattle manure support the conclusions of Saha et al. (2007). The higher P content in poultry manure compared to cattle manure is due to the fact that cereals known to have a high P (phytate) reserve are an important part of poultry feed.

4.4 Nitrogen Mineralization of Poultry Manure

Poultry manure with a low C:N (21) or high C:N (75) ratio is better mineralized than cattle manure, as evidenced by the higher release of CO_2 and mineral nitrogen. Previous studies have also shown the high mineralization potential of poultry manure applied to the soil (Shah et al., 2013; Abbasi & Khaliq, 2016). In addition to the C:N ratio, the C form is also an important factor determining the mineralization potential of the organic amendment. Cattle manure is a combination of cattle excrement and the rest of cereal straw, which can contain a large amount of lignin known to contain component difficult to mineralize and is therefore an important factor in the decomposition of litter (Yue et al., 2016). Abbasi and Khaliq (2016) reported lignin levels 2 times higher in wheat straw than in poultry manure, respectively. These recalcitrant components may have created mineral nitrogen immobilization from day 7 to day 50, as our results show. Such immobilization induced by the application of C from dairy cow manure has also been demonstrated by Griffin et al. (2005). Human feces due to their low C:N ratio and probably the absence of recalcitrant components are easily mineralized as evidenced by the high release of mineral nitrogen during the incubation period. Our results suggest that poultry manure and human feces are potential and better sources of nitrogen for plants than cattle manure.

5. Conclusion

Poultry manure is an important source of nutrients that can be used to improve the productivity of Ferric Acrisol. These sources release mineral nitrogen faster than cattle manure when applied to the soil. The quality of poultry manure is influenced by the farming practices, which account for more than 60% of the variability in their chemical composition. It was found that the manure of broiler chicken contained more C and N than that of laying hens, which contained more Ca. These results suggest that a combination of these two types of poultry manure could be more beneficial to crops.

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Cassava Root Necrosis Disease (CRND): A New Crop Disease Spreading in Western Democratic Republic of Congo and in Some Central African Countries

Bakelana Zeyimo[1,5,6], Laura M. Boykin[3], Monica Kehoe[7], Justin Pita[5], Monde Godefroid[5,6], Mahungu Nzola[2], Lema Munseki[6], Tshilenge Kanana[6] & Kalonji Mbuyi[6]

[1] National Institute for Agricultural Research Studies (INERA), Democratic Republic of Congo

[2] International Institute of Tropical Agriculture (IITA), Democratic Republic of Congo

[3] School of Molecular Sciences and Australian Research Council Centre of Excellence in Plant Energy Biology, University of Western Australia, Crawley, Perth, WA, Australia

[4] Institute Faculty of Agricultural Science, Yangambi, Democratic Republic of Congo

[5] West African Virus Epidemiology (WAVE), Abidjan, Ivory Coast

[6] Agriculture Faculty, Kinshasa University, Democratic Republic of Congo

[7] Department of Primary Industries and Regional Development Diagnostic and Laboratory Services, South Perth, WA, Australia

Correspondence: Bakelana Zeyimo, National Institute for Agricultural Research Studies (INERA), Democratic Republic of Congo. Tel: 243-81-190-8783. E-mail: bakelanatony@hotmail.com

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Abstract

Cassava is consumed in the Democratic Republic of Congo (DRC) as a staple food for the majority of the Congolese population. This crop is used in several forms: as *fufu*, *chikwangue* and *pondu*; cassava leaves are the most consumed vegetable in the country.

In 2002, cassava root symptoms similar to cassava brown streak disease (CBSD) were reported for the first time in western DRC. PCR assays, using primers specific to *Cassava brown streak virus* (CBSV), failed to detect or identify any viral pathogens in diseased cassava samples from western DRC. Therefore, next generation sequencing (NGS) techniques were used as they are able to sequence full organism genomes and are widely used for the identification of pathogens responsible for new diseases. The main objective of this study was to identify the pathogens causing root necrosis in western DRC.

Whatman®FTA™ cards were used to collect 12 cassava leaf samples from plants with symptoms indicative of very severe root necrosis, as well as two asymptomatic samples. These 12 samples were sent to Australia at the University of Western Australia in Perth for next generation sequencing (NGS) using the Illumina HiSeq platform.

Additional bioinformatics tools included Geneious, CLC workbench, ParaKraken and Kaijou software for short DNA sequences. No viruses (including CBSV) were found in any of the DRC samples. These preliminary results confirm all the previous negative results obtained using PCR and CBSV primers. However, NGS analyses did reveal the presence of a number of bacterial and fungal taxa. These will require further investigation and tests such as the Koch Postulates, to establish their specific pathogenic role in cassava.

This is the first scientific evidence that no currently known virus is responsible for the disease which had been referred to previously as 'CBSD-like disease'. Consequently, the disease found in DRC cassava samples has been designated 'Cassava Root Necrosis Disease' or CRND.

Keywords: NGS, PCR, Illumina HiSeq, CBSD-like, CRND

1. Introduction

Cassava (*Manihot esculenta* Crantz, family Euphorbiaceae) produces carbohydrate-rich storage roots, which are a staple food crop for approximately 800 million people worldwide (Food and Agriculture Organization, 2013). In Africa, cassava is the second most important food staple in terms of *per capita* calories consumed (Nweke, 2004).

Cassava (*Manihot esculenta*) production is important to the economy of the Democratic Republic of Congo (DRC). It is one of the country's principal crops, with per capita consumption of 353 kg per year, which is the highest in the world (Mbago & Lotombe, 2017). Zaire, now DRC, was the world's largest consumer of cassava with Republic of Congo ranked second in 1996 (Dufour et al., 1996).

Storage roots are used as a fresh source of carbohydrates and the flour derived from the processed roots is consumed as an everyday-food, sold in local markets or used in several industrial food products (Hillocks & Thresh, 2002). Recent research has suggested that, in comparison to other staple food crops, cassava may be highly resilient to climate change and could provide food security opportunities for Africa (Jarvis et al., 2012).

Cassava production of in East and Central Africa is severely constrained by two viral diseases, cassava mosaic disease (CMD) and cassava brown streak disease (CBSD). Together, it is estimated that these diseases cause annual losses of US$1 billion (IITA, 2014b) and adversely affect food security in the region (Patil et al., 2015). In 2004, CBSD, which had been thought to be confined to coastal lowlands, was found at altitudes above 1000m above sea level (Alicai et al., 2007). Infections of cassava plants showing CBSD symptoms at higher altitudes in Uganda were confirmed by RT-PCR (Alicai et al., 2007). There have since been additional CBSD reports from Burundi (Bigirimana et al., 2011), Rwanda (FAO, 2011), eastern DRC (Mulimbi et al., 2012), South Sudan (Alicai et al., 2017) and Mayotte Island (Roux-Cuvelier et al., 2014).

CBSD is caused by a single-stranded RNA virus, family Potyviridae; genus *Ipomovirus* (Mongeret al., 2001a). Two genetically-distinct strains of CBSV were recognized in East Africa (Mbanzibwa et al., 2009). These were shown to be two distinct species, namely *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV) (Monger et al., 2010).

Both CBSV and UCBSV are transmitted by the whitefly species complex *Bemisia tabaci* Gennadius in the field (Mware et al, 2019) and through the propagation of infected cuttings used for planting. CBSD was known to be endemic in coastal East Africa and in parts of Malawi until recently when outbreaks were reported in Uganda, western Tanzania and Kenya. Other countries where CBSD has been reported include Mozambique, Rwanda, Burundi and in isolated parts of the DRC (Ndunguru et al., 2015). The strains of *Cassava brown streak virus* cause economic losses of up to $100 million USD annually (Ndunguru et al., 2015).

In the early 2000s, cassava root necrosis (Figure 1) similar to that caused by CBSD was first reported in the western provinces of DRC (Kinshasa and Kongo Central) by Mahungu et al. (2003).To date, diagnosis through PCR has been unsuccessful in detecting any known potential causal agent for the observed symptoms. Thus, up to now, the disease has been referred to as 'CBSD-like disease' (Bakelana et al., 2019a).

Figure 1. Typical root necrosis of CRND observed in western DRC

In November 2018, in Kinshasa during the drafting meeting of the DRC cassava viral disease response plan, one of the recommendations was that the term 'CBSD-like' should no longer be used, but that the term Cassava Root Necrosis Disease (CRND) should be used instead.

This recommendation was also reminded during the scientific day on diseases and pests of cultivated plants in DRC. This scientific day was organized on August 3rd by the Plant Clinic of Kinshasa.

This name change was based on the results of NGS analysis undertaken in this study. This analysis did not find evidence of any virus (including known CBSD viruses) in our symptomatic cassava samples.

Several attempts have been undertaken since 2004 to identify the causative agent for CRND in western DRC, using cassava leaf samples (including those from plants showing very severe symptoms)with no success to date (Bakelana et al., 2019a).

Molecular diagnosis results from five different laboratories, using PCR primers specific for the two known CBSVs (CBSV and UCBSV), produced negative results. This suggested that the causal agent of the CBSD-like disease in western DR Congo might be different from those known at present (Bakelana et al., 2019a). Conventional molecular methods have their limitations—as indicated by Adams et al. (2009) who indicated that real-time PCR may be too specific or not broad enough to successfully detect all the known variability within virus species and thus resulting in false negatives. With the advent of next generation, high-throughput, sequencing platforms (NGS), the metagenomic sequencing of diseased cassava plants to identify plant viruses has now become a widely-used method (Adams et al., 2009; Kreuze et al., 2019).

Despite the absence of typical CBSD foliar and stem symptoms and a failure of existing test methods to identify potential causal viral agents in diseased plants in western DRC, the project considered that it was still likely that CBSD viruses were spreading from East to Central Africa and causing this disease. It seemed feasible that other strains of CBSD—unidentified to date—might be responsible for these disease symptoms. Therefore, the aim of this study was to search for the causal agent using a broad diagnostic tool—that is, one not designed against specific or known targets.

In this paper, we report the first use of next generation sequencing to analyze the symptomatic cassava leaf samples from DRC. Based on the NGS results, we also propose that non-viral causal agents may be responsible for the symptoms exhibited in our cassava plant samples. Consequently, we are using the name ‘Cassava root necrosis disease’ and its acronym ‘CRND’ within this manuscript to refer to this disease.

2. Materials and Methods

2.1 Field Sample Collection for NGS Analysis

Figure 2. Cassava root of plants used for leaf sampling on FTA™ cards

Cassava fields (with plants more than 16 months old) in the 2 localities considered hotspots (ref) in western DRC were surveyed. Leaf material from plant with root necrosis was collected (Figures 2) on FTA™ cards.

A total of 12 leaf samples (Table 1) were collected from plants. They were crushed onto FTA™ cards according to the manufacturers’ protocol in order to extract their DNA.

Cassava leaf samples were collected at Mvuazi research center and Lukuakua village on 29 and 30 November 2016.

Leaves samples were sampled according to Rwegasira et al. (2011) who found that the most suitable tissue samples for CBSV-detection were young tender leaves, youngest symptomatic leaves and the non-necrotic storage root tissue. The CBSD viruses were not detected from root necrotic tissues.

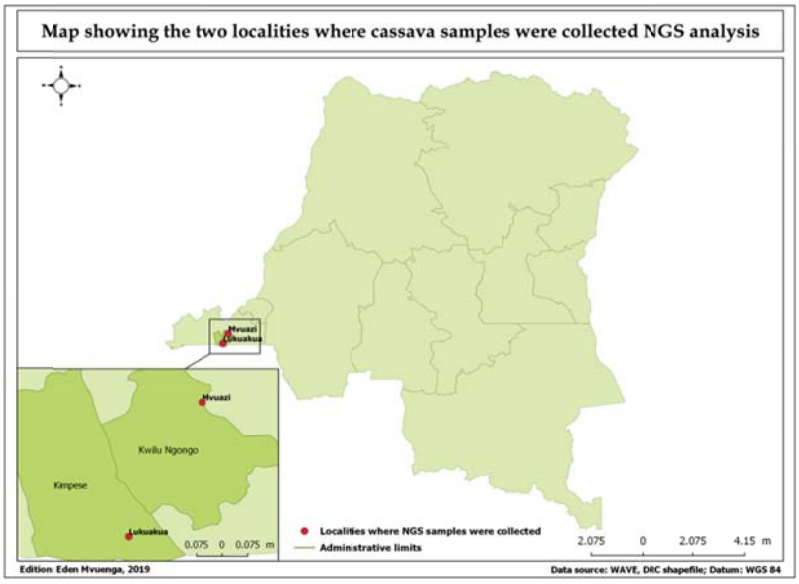

Figure 3. Hotspot disease locations where samples were collected

Table 1. Leaf samples details

Sample ID	Genotype	Location	Status
1	Mputa	Mvuazi	Diseased plants
2	Mputa	Mvuazi	Diseased plants
3	Mputa	Mvuazi	Diseased plants
4	Mputa	Mvuazi	Diseased plants
5	Mputa	Mvuazi	Diseased plants
6	Mvuazi	Lukuakua	Diseased plants
7	Mvuazi	Lukuakua	Diseased plants
8	RAV	Lukuakua	Diseased plants
9	RAV	Lukuakua	Diseased plants
10	Mputa	Lukuakua	Diseased plants
11	RAV	Lukuakua	Apparently healthy plants
12	TME 419	Lukuakua	Apparently healthy plants

The 12 FTA™ sample cards, previously labeled, were shipped to the University of Western Australia (UWA) in Perth to complete the DNA extraction and for subsequent NGS processing.

2.2 Symptom Assessment

A symptom severity score was then recorded for the root of each plant sampled, using the 1-to-5 scoring method described in Hillocks and Thresh (2000). Root necrosis severity was assessed as follows: 1 = apparently healthy root; 2 = less than 2% necrotic tissue; 3 = 2-5% necrotic tissue; 4 = 5-50% necrotic tissue; 5 = more than 50% necrotic tissue.

2.3 RNA Extraction (Ndunguru et al., 2015)

RNA was extracted from approximately 100 mg of cassava leaf using the CTAB (cetyltrimethyl ammonium bromide). The leaves were ground in a mortar containing 1 ml extraction buffer (2.0% (w/v) CTAB, 2.0 M NaCl, 2.0% PVP, 0.5M EDTA, 1 M Tris-HCl and 0.2% β-mer-captoethanol (added immediately before use)). Then 750 µl of the extract was transferred into a 1.5 ml micro-centrifuge tube and incubated at 65 °C for 15 min while shaking vigorously several times. The extract was then mixed with an equal volume (750 µl) of chloroform: isoamyl alcohol (24:1); vortexed briefly and centrifuged (Hettich Centrifugen, D-78532, Germany) at 12,000 rpm for 10 min at 4 °C. The top aqueous solution (500 µl) was transferred into new micro-centrifuge tubes to which 0.6 vol (300 µl) cold isopropanol was added. The content was then incubated at -20 for at least 10 min followed by centrifugation (Hettich Centrifugen, D-78532, Germany) at 13,000 rpm for 10 min at 4 °C and the supernatant was discarded.

The RNA pellet was then washed in 700 ml of 70% ethanol and the tubes vortexed briefly before being incubated at -20 °C for at least 10 min. The tubes were then centrifuged for 5 min at 13,000 rpm. The ethanol was then removed and the pellet was air-dried. Finally the dried RNApellet were re-suspended in 100 µl 1XTE/sterilized double distilled H20 on ice for about 30 min and stored at -20 °C before use.

2.4 cDNA Library Preparation and Illumina Sequencing (Ndunguru et al., 2015)

Total RNA extracts that presented 260/280 and 260/230 purity indices equal to or greater than 2.0 and integral RNA in electrophoresis and Bioanalyzer measurements (RIN > 8) were selected. The cDNA libraries were prepared from 1 µg of total RNA using the IlluminaTruSeq Stranded Total RNA Sample Preparation kit with Ribo-Zero^{TM} Plant according to the manufacturer's instructions (Illumina, San Diego, California). Briefly, after rRNA depletion and RNA fragmentation, first and second strand cDNA was synthesized, adapters were ligated to the 50 and 30 ends of the fragments and the fragments enriched by PCR. cDNA libraries final size and concentration of each library was estimated using a Bioanalyzer (Agilent, Santa Clara, CA, USA) and the Qubit (Invitrogen, Carlsbad, CA, USA), respectively. Ten nM library pools were prepared by mixing the libraries to achieve an equal molar concentration of each. Libraries were normalized, pooled and sequenced using a 2 × 300 cycle PE V3 Illumina kit. Paired end reads were generated using the Illumina MiSeq System at the Biosciences Eastern and Central Africa-International Livestock Research Institute (BECA-ILRI) Hub in Nairobi, Kenya.

2.5 De Novo Sequence Assembly and Mapping (Ndunguru et al., 2015)

For each sample, reads were first trimmed using CLC Genomics Workbench 6.5 (CLCGW) (CLC Bio) with the quality scores limit set to 0.01, maximum number of ambiguities to two and removing any reads with < 30 nucleotides (nt). Contigs were assembled using the de novo assembly function of CLCGW with automatic word size, automatic bubble size, minimum contig length 500, mismatch cost two, insertion cost three, deletion cost three, length fraction 0.5 and similarity fraction 0.9. Contigs were sorted by length and the longest subjected to a BLAST search (blastn and blastx). In addition, reads were also imported into Geneious 6.1.6 (Biomatters) and provided with reference sequences obtained from Genbank.

2.6 Library Preparation and Illumina Sequencing

Total RNA and DNA extractions was carried out in the UWA from FTA samples and were sent to the Australian Genome Research Facility of the UWA for library preparation and sequencing on an Illumina HiSeq 2500.

2.7 Sequences Analysis

For each sample, reads were first trimmed using CLC Genomics Workbench 6.5 (CLCGW) (CLC Bio) with the following parameters: quality scores limit set to 0.01, maximum number of ambiguities set to twoand removal of any reads with < 30 nucleotides. Contigs were assembled using the *de novo* assembly function of CLCGW with automatic word size, automatic bubble size, minimum contig length 500, mismatch cost two, insertion cost three, deletion cost three, length fraction 0.5 and similarity fraction 0.9. Contigs were sorted by length and the longest subjected to a BLAST search (blastn and blastx) (Altschul et al., 1990). In addition, reads were also imported into Geneious 6.1.6 (Drummond et al., 2010) (Biomatters) and provided with reference sequences obtained from Genbank (NC012698 for CBSV, GQ329864 for CBSV-T and NC014791 for UCBSV). These methods have been used previously for the successful recovery of whole CBSV and UCBSV genome sequences (Ndunguru et al., 2015; Alicai et al., 2016; Ateka et al., 2017).

Mapping was performed using Kaiju software with minimum overlap 10%, minimum overlap identity 80%, allow gaps 10% and fine tuning set to iterate up to 10 times.

While recent taxonomic classification programs achieve high speed by comparing genomic k-mers, they often lack sensitivity for overcoming evolutionary divergence; these results in large fractions of the metagenomic reads remaining unclassified. Kaiju is a novel metagenome classifier, which finds maximum (in-) exact matches on the protein level using the Burrows-Wheeler transform (Menzel et al., 2016).

It has been shown that that Kaiju classifies reads with higher sensitivity and similar precision compared with current k-mer-based classifiers, especially in genera that are under-represented in reference databases. It has also been demonstrated that Kaiju classifies up to 10 times more reads in real metagenomes. Kaiju can also process millions of reads per minute and can run on a standard PC (Menzel et al., 2016).

3. Preliminary Results and Discussion

After trimming and assembling NGS data outputs using CLC workbench and Geneious software, sequences were processed using the Kaiju and outputs are presented in Figures 4 and 5 below. The bioinformatic processes and

analyses did not find evidence of any virus (including known CBSD viruses) in our symptomatic cassava samples. However, a number of bacterial and fungal taxa were recorded.

Samples 1-10, which were collected on diseased plants, presented fungi and bacteria while samples 11 and 12, which were collected on apparently asymptomatic plants presented only bacteria. No fungi were found in asymptomatic plants.

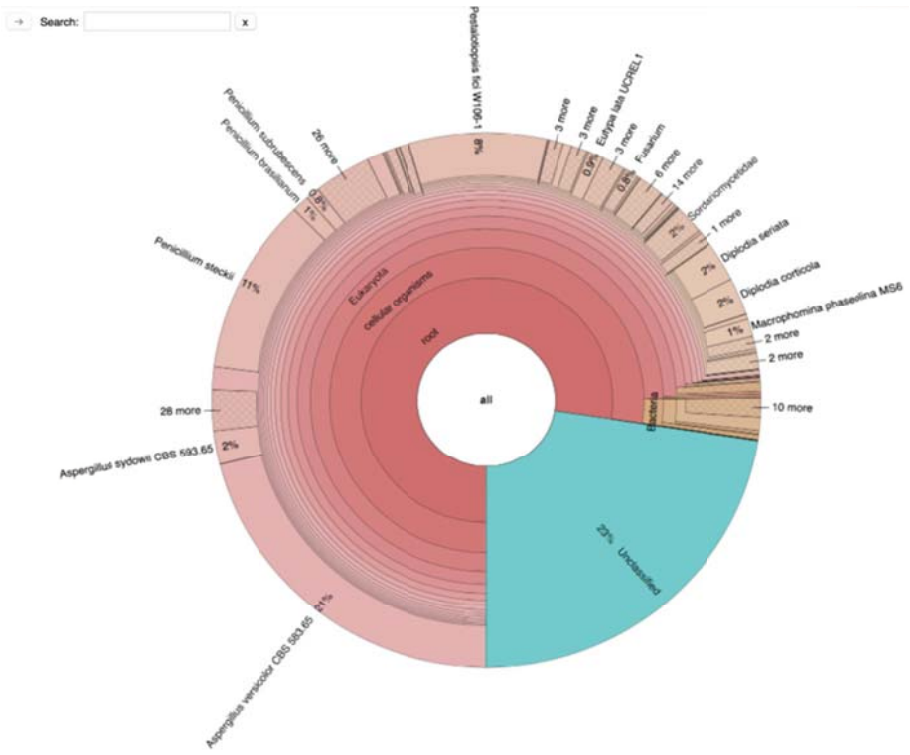

Figure 4. Example of a sample results showing list of microorganisms (bacteria and fungi) identified using Kaiju software

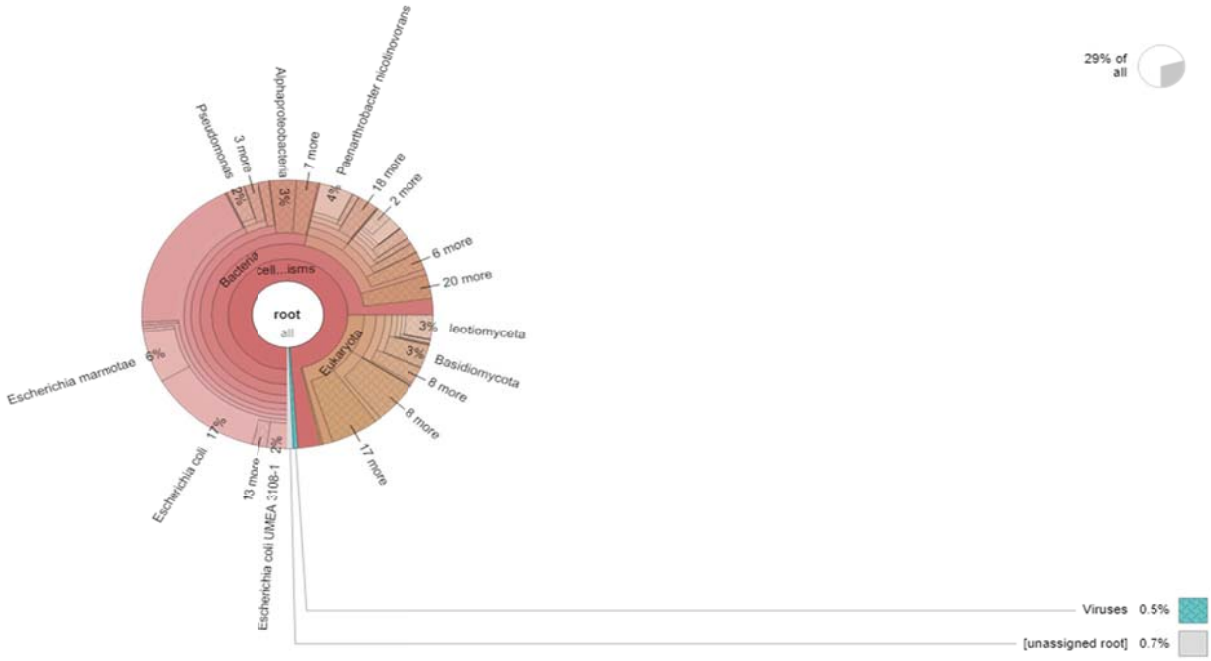

Figure 5. Lack of viruses in all tested samples (Kayju software)

The figure 5 shows that viral sequences were quantified at 0.5%.

The list of all microorganisms identified in all 12 samples and those suspected to play a pathogenic role in plant diseases according to the literature are presented in Tables 2 and 3 below.

Table 2. Bacteria and fungi identified through NGS in all 12 samples

Microorganisms identified	Classification
Acremonium chrysogenum	Fungus
Aspergillus niger	Fungus
Aspergillus sp.	Fungus
Aspergillus sydowii	Fungus
Aspergillus versicolor	Fungus
Diaporthehelianthi	Fungus
Diaportheampelina	Fungus
Diaporthehelianthi	Fungus
Dickeya zeae	Fungus
Diplodia sp.	Fungus
Diplodia orticola	Fungus
Diplodia serata	Fungus
Erwinia sp.	Fungus
Fusarium sp.	Fungus
Macrophomina parvum	Fungus
Macrophominaphaseolina	Fungus
Neofusicoccum parvum	Fungus
Pseudomonas fluorenscens	Bacteria
Pseudomonas libanensis	Bacteria
Pseudomonas aeruginosa	Bacteria
Pseudomonas tolaasii	Bacteria
Penicillium brasiliarum	Fungus
Penicillium chrysogenum	Fungus
Penicillium decumbens	Fungus
Penicillium digitatum	Fungus
Penicillium expansum	Fungus
Penicillium marneffei	Fungus
Penicillium steckii	Fungus
Pestalotiopsis sp.	Fungus
Pestalotiopsisfici	Fungus
Pseudomonas aeroginosa	Bacteria
Pseudomonas brassicacearum	Bacteria
Pseudomonas dioxanivorans	Bacteria
Pseudomonas fluorecens	Bacteria
Pseudomonas fuscovaginae	Bacteria
Pseudomonas mendocina	Bacteria
Pseudomonas pseudoalcaligenes	Bacteria
Pseudomonas syringae	Bacteria
Pseudomonas tolaasii	Bacteria
Pseudoxanthomonas sp.	Bacteria
Pseudoxanthomonas spadix	Bacteria
Sordariomycetidae	Bacteria
Xanthomonas sp.	Bacteria
Xanthomonas citri	Bacteria
Xanthomonas euvesicatoria	Bacteria
Xanthomonas phaseoli	Bacteria
Xanthomonas sacchari	Bacteria

Table 3. Plant pathogenic microorganisms among list of bacteria and fungi identified through NGS—according to literature review

Microorganisms
Diplodiaseriata
Diplodiacorticola
Macrophominaphaseolina
Neofusicoccum parvum
Diaporthehelianthi
Diaportheampelina
Pestalotiopsis

Neofusicoccum parvum is the predominant species within the *Botryosphaeriaceae.* Several *Botryosphaeriacea* species are important grapevine pathogens causing dieback and decline worldwide, and in recent years they have been recognized as causing serious problems in New Zealand vineyards (Baskarathevan et al., 2012).

Diplodia corticola A.J.L. Philips, Alves et Luque is a well-known canker pathogen of oak (*Quercus* spp.) that is contributing to the decline of oaks in the Mediterranean region (Alves et al., 2004). Recently, the pathogen has been affecting *Quercus* spp. in California, *Vitis vinifera* in California and Texas (Lynch et al., 2010; Úrbez-Torres et al., 2009; Úrbez-Torres et al., 2010), and live oak (*Q. virginiana* Mill.) in Florida (Dreaden et al., 2011).

Diplodia seriata (= *Botryosphaeriaceaeobtusa*) and *Neofusicoccum parvum* (Pennycook & Samuels) Crous, are the most common pathogens associated with grapevine dieback worldwide (Auger et al., 2004; Larignon et al., 2001; Phillips, 2002, Taylor et al., 2005; Úrbez-Torres et al., 2006; Úrbez-Torres et al., 2006; Van Niekerk et al., 2004).

Species of Diaporthe and their Phomopsis asexual states have broad host ranges and are widely distributed, occurring as plant pathogens, endophytes or saprobes, but also as pathogens of humans and other mammals (Webber & Gibbs, 1984; Carroll, 1986; Boddy & Griffith, 1989; Rehner & Uecker, 1994; Garcia-Reyne et al., 2011; Udayanga et al., 2011).

Diaporthe sp. are responsible for diseases on a wide range of plants hosts, some of which are economically important worldwide, causing root and fruit rots, dieback, cankers, leaf spots, blights, decay and wilt (Uecker, 1988; Mostert et al., 2001a; van Rensburg et al., 2006; Santos et al., 2011; Thompson et al., 2011).

More researches are currently ongoing and each suspected microorganisms above needs to be confirmed by the Koch Postulates assays as causative pathogen(s) of CRND in western DRC.

Isolations of bacteria and fungi are currently ongoing with the partnership of the Plant Clinic of Kinshasa. Microorganisms that will be isolated from cassava roots necrotic tissues will be genetically characterized and sequenced.

Koch Postulates trials will be done with the involvement of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) in Germany.

It is possible that the CRND root necrosis disease could be caused by the action of a bacterium-fungus complex.

The disease could be initiated by an initial attack of bacteria and root necrotic symptoms externalized by a secondary attack of fungi. Further studies are required to confirm or refute this hypothesis.

4. Conclusion and Perspectives

This study points to the apparent absence of CBSV in western region of DRC and suggests that CRND could be caused by other microorganisms such as bacteria, fungi or a combination of both. There appear to be two distinct diseases, namely CRND and CBSD which have similar root symptoms but different stem and foliar symptoms.

Since 2004, CBSD has been spreading from East Africa to Central Africa and was confirmed in 2012 in eastern DRC; it is expected to spread to western DRC and on to West Africa. At the same time, CRND is spreading from western DRC towards West Africa and eastern DRC.

If no control measures (quarantine, etc.) are put in place, there is a strong possibility that both diseases will spread to West Africa. Should this event cause cases of infections of both diseases, the results are likely to mean devastating cassava root crop losses and significant economic impacts on farmers' livelihoods. Ultimately, this has serious implications for food security in Central Africa.

We consider that further research on CRND pathogens identification is paramount. Koch's Postulates on isolated microorganisms from diseased plants and other biological assays will help to elucidate the causal pathogens of this disease. Information on disease etiology will allow for future disease epidemiology and genetic disease resistance research.

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RNA-Seq Reveals Differentially Expressed Genes and Pathways Affecting Intramuscular Fat Metabolism in Huangshan Black Chicken Population

S. H. Yang[1,†], C. S. He[1,†], C. H. Li[1] & G. Q. Liu[1]

[1] College of Food and Biological Engineering, Hefei University of Technology, Hefei, China

Correspondence: Guoqing Liu, College of Food and Biological Engineering, Hefei University of Technology, Hefei, Anhui, China. Tel: 86-551-65785485. E-mail: 13675512000@163.com

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† *These authors contributed equally to this work.*

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Abstract

Intramuscular fat (IMF) plays an important role in meat quality due to its positive correlation with juiciness, tenderness, and flavor. However, for chickens, the molecular mechanisms underlying IMF deposition in thigh muscle have not yet been determined. Here, to identify candidate genes and signaling pathways related to IMF deposition, we deeply explored the chicken transcriptome from thigh muscles of Huangshan Black Chickens with extremely high and low phenotypic values for intramuscular fat content. A total of 128 genes differentially expressed genes (DEGs) were detected, of which 94 were up-regulated and 34 were down-regulated. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways revealed these DEGs (including *FABP4*, *G0S2*, *PLIN1*, *SCD1*, *LFABP*, *SLC1A6*, *SLC45A3*, *ACSBG1*, *LY86*, *ST8SIA5*, *SNAI2*, *HPGD*, *EDN2*, and *THRSP*) were significantly enriched in lipid biosynthetic process, steroid biosynthetic and metabolic process, fatty acid metabolic process, and regulation of unsaturated fatty acid metabolic pathways. Additionally, we concluded an interaction network related to lipid metabolism, which might be contributed to the IMF deposition in chicken. Overall, we proposed some new candidate genes and interaction networks that can be associated with IMF deposition and used as biomarkers in meat quality improvement.

Keywords: meat quality, IMF deposition, thigh muscle, chicken transcriptome, interaction networks

1. Introduction

During the past decades, the breeding of meat type poultry has been predominantly focused on increasing growth rate and yields of breast and thigh meat. Although the impressive progress made in these meat quality traits, there were some poor performances, such as larger fiber diameters, lower intramuscular fat, and higher proportion glycolytic fibers, which seriously decreased sensory acceptability for consumers (Du et al., 2010). It is an ongoing challenge to maintain growth rate meanwhile improve meat quality. As a main determinant of meat quality, the deposition of intramuscular fat (IMF) plays an important role in flavor of meat and can dramatically promote tenderness of meat.

Previous studies have discovered some important quantitative trait loci (QTL) associated with chicken IMF, which are mainly located on chromosomes 1, 2, 5, 23 (Zhao et al., 2007; Sarsenbek et al., 2013; Zhang et al., 2015). Additionally, a number of genes including *CD36*, *ACC*, and *DGAT2* (Jeong et al., 2012), *FABP* (Serao et al., 2011), *DGAT1* (Li et al., 2013), *LPL* (Zhang et al., 2015), and *SLC27A1* (Qiu et al., 2017) were also recognized as candidate genes for IMF in chickens. However, the metabolic pathways underlying IMF deposition is very complicated, the molecular mechanisms affecting IMF remains poorly understood.

With the development of high-throughput sequencing technologies, especially RNA-Seq has been widely utilized to explore potential candidate genes which affect important economic traits in animals. Although, previous studies have analyzed the transcriptome of chicken breast muscle (Cui et al., 2012), skeletal muscle (Ye

et al., 2014) and thigh muscle (Cui et al., 2018) utilizing microarrays, and identified some potential candidate genes that influence IMF deposition, no further transcriptome studies in chickens have been taken in identical breed with distinct IMF levels.

As an indigenous breed in China, the Huangshan Black Chicken has a distinct appearance and quality in meat products. The difference in IMF content of thigh muscles makes them as great materials to understand the molecular mechanism of IMF deposition in chickens. In the present study, we used RNA-Seq technology to examine differentially expressed genes (DEGs) in thigh muscle tissues between two groups of Huangshan Black Chickens with extremely high and low phenotypic values of IMF content. We then proposed some new candidate genes and a gene network that can be related to IMF deposition by conducting integrated analysis. Thus, the elucidation of the precise molecular mechanisms underlying IMF traits in Huangshan Black chickens will have both economic and biological consequences.

2. Method and Methods

2.1 Ethics Statement

All animal procedures were authorized by the Institutional Animal Care and Use Committee (IACUC) of Hefei University of Technology (Permit Number: DK838). In the present study, animals were sacrificed as necessary to ameliorate suffering.

2.2 Animals and Sample Collection

Huangshan Black Chickens (Anhui conservation farm for Huangshan Black Chicken, Huangshan, China) with the same genetic background were maintained in free-ranging flocks in a standardized farm, using a diet as: maize 64.0%, wheat bran 16.0%, full-fat soybean 10.0%, fish meal 5.0%, feed yeast powder 2.0%, bone meal 1.5%, inorganic additives 0.7%, Lysine 0.3%, salt 0.3%, Methionine 0.2%. Ten male chickens with an average weight of 1.82 kg at 120 days old were selected randomly according to our detection. To keep the environment factors identical, feed and water were provided ad libitum during the experiment.

All the chickens were fasted for 12 h, and weighed before being killed by stunning and exsanguination. The thigh muscle samples from the left leg of the chickens were collected within 30 min after slaughter. The samples for each chicken were were snap-frozen and stored at -80 °C before analysis. Meanwhile, sufficient samples were minced and kept at -20 °C for IMF analysis.

2.3 IMF Measurement

IMF content of thigh muscle was determined by Soxhlet extraction according to previous studies (Folch and Lees, 1957) and expressed as percentages of the muscle, on the basis of the dry weight.

2.4 RNA Isolation and Validation

Total RNA of the thigh muscle samples was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After the quality verification on gel electrophoresis, the concentration and purity of the RNA samples were assessed on a NanoPhotometer® spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The integrity of RNA was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

2.5 RNA Sequencing

With a final 2.0 μg/μl concentration, RNA from each sample was pooled based on the IMF content. A total of 3μg RNA from per pooled sample was used as the input material for RNA sample preparations. Based on the manufacturer's instructions, the transcriptome library was constructed using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA). Furthermore, TruSeq PE Cluster Kit v3-cBot-HS (Illumina) was used to cluster the index-coded samples on a cBot Cluster Generation System. After cluster generation, the library preparations were sequenced using an Illumina HiSeq 2000 platform, which was followed by FASTQ file generation and the failed reads elimination by CASAVA ver.1.8.2 (Illumina).

2.6 Sequencing Data Analysis

Using CASAVA ver.1.8.2 (Illumina), the sequencing-derived raw images were transformed into raw reads by base calling. After obtained the raw reads, we removed reads containing low quality reads, adapter and reads containing ploy-N to get clean data through in-house perl scripts. Additionally, the description statistics for the clean data, such as Q20, Q30, and GC-content were calculated for high-quality downstream analysis. The clean data with high quality were used for the downstream analyses.

2.7 Reads Mapping

Based on the reference genome, only these reads with a perfect match or one mismatch were further analyzed and annotated. The clean reads were mapped to the reference genome of chicken (version UMD 4.1) using Tophat2 software (version 2.1.0). The index of the reference genome was built using Bowtie v2.2.3 and paired-end clean reads for each individual chicken were aligned to the reference genome using TopHat v2.0.12. In addition, HTSeq v0.6.1 was used to count the reads numbers mapped to each gene.

2.8 Differential Expression Analysis

Differential expression analysis of different groups (the high and low groups with phenotypic values for IMF content) was identified using the DESeq R package (1.10.1) based on the negative binomial distribution. Furthermore, the Hochberg and Benjamini method was used to adjust the p-values for controlling the false discovery rate (Benjamini and Hochberg, 1995). Genes with a FDR value < 0.05 and jlog2-fold changej > 2 were assigned as differentially expressed.

2.9 Functional Enrichment Analysis

GO and KEGG pathway enrichment analyses of the DEGs were implemented by the Database for Annotation, Visualization and Integrated Discovery (DAVID) website (Huang et al., 2007). GO terms and KEGG pathways with a hypergeometric test from the R package ($P < 0.1$, FDR-adjusted) were considered significantly enriched among the DEGs. Pathways with fewer than three known chicken genes were discarded.

To validate the repeatability and reproducibility of the sequencing results, qRT-PCR was carried out to detect 10 randomly selected DEGs. Primers were designed via Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm) and are shown in Supplementary Table S1. qRT-PCR was carried out in triplicate with the LightCycler® 480 SYBR Green I Master Kit (Roche Applied Science, Penzberg, Germany) in a 15 μ L reaction on a ABI7500 (Applied Biosystems Inc., USA), using the following program: 95 °C for 10 min; 40 cycles of 95 °C for 10 s, 60 °C for 34 s, and 72 °C for 10 s; 72 °C for 6 min. The mRNA levels of the DEGs were normalized by the housekeeping genes GAPDH and β-actin, and the relative gene expression values were calculated using the $2^{-\Delta\Delta Ct}$ method. Finally, the correlations between RNA-Seq for 10 genes and the mRNA expression level from qRT-PCR were estimated using R (V3.2).

3. Results

3.1 Differences in IMF Content

The IMF content of 20 samples in thigh tissues was detected using soxhlet extraction method and the data were shown in Table 1, respectively. Of these, according to the value of the IMF content, the pooled RNA of sample 1-3 and sample 4-6 were selected as IMFL1 and IMFL2 while sample 7-9 and sample 10-12 as IMFH1 and IMFH2 to explore the chicken transcriptome by paired-end RNA sequencing.

Table 1. Analysis of IMFs in thigh muscle of Huangshan Black chickens

Sample	IMF content (%)	Group	Mean±SD (%)
sample1	2.72		
sample 2	2.63	IMFL1	2.69±0.05
sample 3	2.72		
sample 4	2.28		
sample 5	2.18	IMFL2	2.22±0.05
sample 6	2.20		
sample 7	3.80		
sample 8	3.88	IMFH1	3.86±0.06
sample 9	3.90		
sample 10	3.90		
sample 11	3.94	IMFH2	3.93±0.03
sample 12	3.95		
sample 13	2.93		
sample 14	3.43		
sample 15	3.71		
sample 16	3.54		
sample 17	2.86		
sample 18	3.15		
sample 19	2.99		
sample 20	3.22		

Note. IMFH1and IMFH2 means samples with extremely high phenotypic values for intramuscular fat content; IMFL1and IMFL2 means extremely low phenotypic values, respectively.

3.2 RNA Sequencing of Thigh Muscle Tissue

We acquired a total of 240.02 million clean reads with an average of 60.01 million (range, 57.85 to 62.40 million) for each sample (Table 2). Alignment of the sequence reads against the chicken reference genome UMD 4.1 yielded 71.39-72.61% of uniquely aligned reads across the four samples, of which 76.1-76.8% fell in annotated exons, 5.9-7.7% were located in introns, and 16.1-17.2% fell in intergenic regions (Supplementary Figure S1). The data sets analyzed are available in the NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank) and the BioProject ID is PRJNA471361. Furthermore, the correlation coefficient (R^2) between the four individuals within the IMFH and IMFL groups was calculated based on the FRPM value of each sample and was shown to be 0.937 and 0.964, respectively, indicating that the similarity of the two biological samples within each group was sufficiently high (Supplementary Figure S2).

Table 2. Basic sequencing data for each sample

Sample_name	IMFH1	IMFH2	IMFL1	IMFL2
Total reads	60063910	62401086	57857950	59716094
Total mapped	42879675 (71.39%)	45311164 (72.61%)	41473305 (71.68%)	43052414 (72.1%)
Multiple mapped	1021027 (1.7%)	1029884 (1.65%)	1013360 (1.75%)	1190835 (1.99%)
Uniquely mapped	41858648 (69.69%)	44281280 (70.96%)	40459945 (69.93%)	41861579 (70.1%)
Non-splice reads	24719076 (41.15%)	26119518 (41.86%)	23288965 (40.25%)	25891090 (43.36%)
Splice reads	17139572 (28.54%)	18161762 (29.1%)	17170980 (29.68%)	15970489 (26.74%)

Note. IMFH1and IMFH2 means samples with extremely high phenotypic values for intramuscular fat content; IMFL1and IMFL2 means extremely low phenotypic values, respectively.

3.3 The Identification of DEGs Related to IMF Metabolism

Using the RPKM method, the differential gene expression profile between IMFH and IMFL was examined. In total, 128 genes were detected significantly different between IMFH and IMFL groups. Of these, 34 genes were down regulated while 94 genes were up regulated. Additionally, the volcanic plot of the two comparison groups was displayed in Figure 1. Furthermore, using integrated analysis of RNA-Seq and gene function, the top 20

genes with the highest absolute value of expression in the thigh muscle tissue between IMFH and IMFL are shown in Table 3. Strikingly, the fat associated genes *SCD1*, *FABP4*, and *LFABP* accounted for a significant proportion.

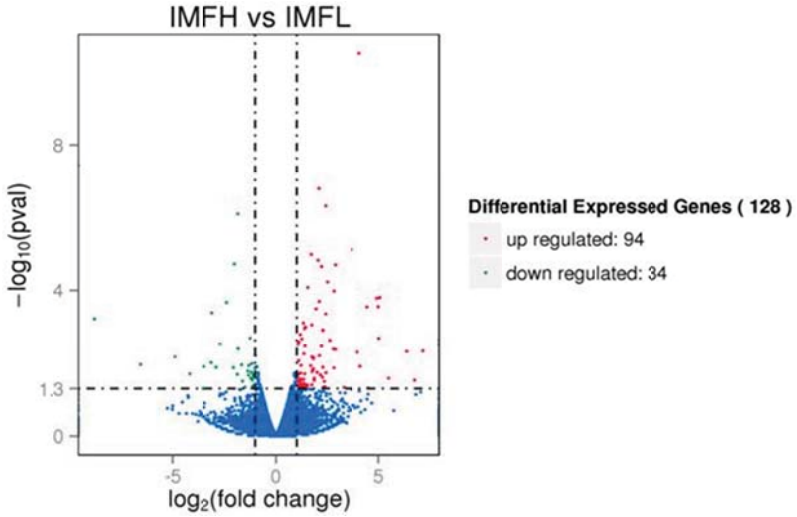

Figure 1. Volcano plot displaying DEGs within two different comparison groups

Note. Significantly DEGs were expressed in red (up-regulated) and green (down-regulated), with no significant difference in genes expressed in blue dots; abscissa representing gene expression fold changes in different samples; and ordinate representing genes statistical significance of differences in expression changes.

Table 3. Top 20 differentially expressed genes between high and low IMF content in thigh muscle tissue

Symbol	Gene name	CHR	Log_2 fold change	P-value	Gene function
SCD1	stearoyl-CoA desaturase	6	1.3369	3.18E-04	involved in fatty acid biosynthesis
FABP4	fatty acid binding protein 4	2	1.5286	4.06E-03	involved in fatty acid uptake, transport, and metabolism
LFABP	fatty acid binding protein 1	4	-1.9926	4.59E-03	metabolism and transport of long-chain fatty acids
SLC1A6	solute carrier family 1, member 6	28	2.0888	3.50E-08	involved in the rapid removal of released glutamate
SLC45A3	solute carrier family 45 member 3	26	1.1902	8.26E-07	related with Glycosaminoglycan metabolism and Metabolism
ACSBG1	acyl-CoA synthetase bubblegum family member 1	10	-1.8164	1.04E-04	plays a central role in brain very long-chain fatty acids metabolism
NALCN	sodium leak channel, non-selective	1	1.3774	6.18E-03	maintenance of substantia nigra pars reticulata
THRSP	thyroid hormone responsive	1	2.3941	6.67E-03	controlling tumor lipid metabolism
PLIN1	perilipin 1	10	2.757	3.06E-03	the inhibition of lipolysis
G0S2	G0/G1 switch 2	26	1.0932	1.15E-02	Regulation of lipid metabolism
SLCO4C1	solute carrier organic anion transporter family member 4C1	Z	1.3165	1.37E-02	the regulation of membrane transport of ouabain
CIDEC	cell death inducing DFFA like effector	11	2.2232	1.52E-02	plays important roles in apoptosis
SS2R	somatostatin receptor 2	18	2.315	2.01E-04	inhibits cell growth
ADORA3	adenosine A3 receptor	26	1.8249	1.91E-05	mediates cell proliferation and cell death
ST8SIA5	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 5	Z	1.8304	2.02E-05	involved in the synthesis of gangliosides
LY86	lymphocyte antigen 86	2	1.6957	2.57E-03	mediates the innate immune response
EDN2	endothelin 2	23	-1.0282	5.01E-06	involved in hypertension and ovulation
SNAI2	snail family transcriptional repressor 2	2	1.0259	3.98E-03	involved in the generation of neural crest cells
ST8SIA2	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2	10	-1.0012	1.09E-03	involved in the production of polysialic acid
HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	4	-1.0833	2.12E-03	involved in the metabolism of prostaglandins

3.4 Validation of DEGs

Ten random DEGs (*SCD1*, *SLC1A6*, *FLT3*, *EDN2*, *CCL4*, *RBP7*, *DBX2*, *LY86*, *MSMB*, and *RRP36*) were selected for qRT-PCR to validate the RNA-Seq results and the result showed that the correlations between the mRNA expression level of qRT-PCR and RNA-Seq were all consistent (Figure 2). Thus, the reproducibility and repeatability of gene expression data in this study are reliable.

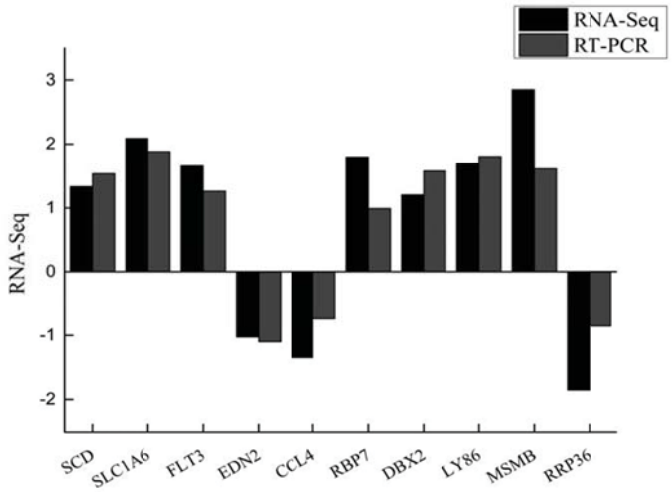

Figure 2. Correlations of mRNA expression level of 10 randomly DEGs between high and low intramuscular fat percentage using RNA-Seq and qRT-PCR

Note. The x-axis represents the gene name, the y-axis represents the log2 (ratio of mRNA levels) measured by RNA-seq and columns of different colors represent data from RT-PCR or RNA-Seq.

3.5 Functional Enrichment Analysis of DEGs

To gain insight into the biological relationships of genes that differentially expressed in thigh muscle tissue between high and low IMF content, we performed GO and KEGG pathway enrichment analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource. The results showed that 12 GO biological process terms related to metabolic process of lipid were significantly enriched ($P < 0.05$), which included steroid biosynthetic process (GO:0006694) and metabolic process (GO:0008202), lipid biosynthetic process (GO:0008610), unsaturated fatty acid metabolic process (GO:0033559), stearoyl-CoA 9-desaturase activity (GO:0004768), acyl-CoA desaturase activity (GO:0016215), unsaturated fatty acid biosynthetic process(GO:0006636), fatty acid metabolic process (GO:0006631), positive regulation of fat cell differentiation (GO:0045600), long-chain fatty acid-CoA ligase activity (GO:0004467), fatty acid derivative metabolic process (GO:1901568) and lipid metabolic process (GO:0006629) (Table 4).

Table 4. Summary of the GO analysis of DEGs

GO accession	Description	No. of DEGs	P-value
GO:0006694	steroid biosynthetic process	4	7.38E-04
GO:0008202	steroid metabolic process	5	9.60E-04
GO:0008610	lipid biosynthetic process	7	3.60E-03
GO:0033559	unsaturated fatty acid metabolic process	3	2.13E-03
GO:0004768	stearoyl-CoA 9-desaturase activity	1	6.27E-03
GO:0016215	acyl-CoA desaturase activity	1	5.31E-03
GO:0006636	unsaturated fatty acid biosynthetic process	2	6.44E-03
GO:0006631	fatty acid metabolic process	4	8.57E-03
GO:0045600	positive regulation of fat cell differentiation	2	2.08E-02
GO:0004467	long-chain fatty acid-CoA ligase activity	1	2.48E-02
GO:1901568	fatty acid derivative metabolic process	2	2.53E-02
GO:0006629	lipid metabolic process	9	3.01E-02

Four KEGG pathways were significantly enriched ($P < 0.05$), including PPAR signaling pathway (gga03320), glycine, serine and threonine metabolism (gga00260), fatty acid metabolism (gga01212) and fatty acid biosynthesis signaling pathway (gga00061).

3.6 Candidate Genes

Integrated analysis of DEGs, GO and KEGG results, QTL mappings and gene function allows us to suggest *FABP4*, *G0S2*, *PLIN1*, *SCD1*, *LFABP*, *SLC1A6*, *SLC45A3*, *ACSBG1*, *LY86*, *ST8SIA5*, *SNAI2*, *HPGD*, *EDN2*, and *THRSP* as the 14 candidate genes for affecting IMF content. Of these, *SNAI2* and *HPGD* involved in steroid biosynthetic and metabolic process, 9 differentially expressed genes (*SCD1*, *ST8SIA2*, *EDN2*, *ACSBG1*, *FABP4*, *G0S2*, *PLIN1*, *THRSP*, and *SLC1A6*) involved in lipid biosynthetic process, unsaturated fatty acid metabolic process and lipid metabolic process. Additionally, two genes, *LFABP* and *SLC45A3*, are important for transport of long-chain fatty acids. Meanwhile, *LY86* positively regulated lipopolysaccharide-mediated signaling pathway. The details of these candidate genes are shown in Table 3. Taken together, the proposed molecular regulatory network affecting IMF metabolism during chicken development is presented in Figure 3. These findings provide new clues for revealing the molecular mechanisms underlying IMF metabolism in chickens.

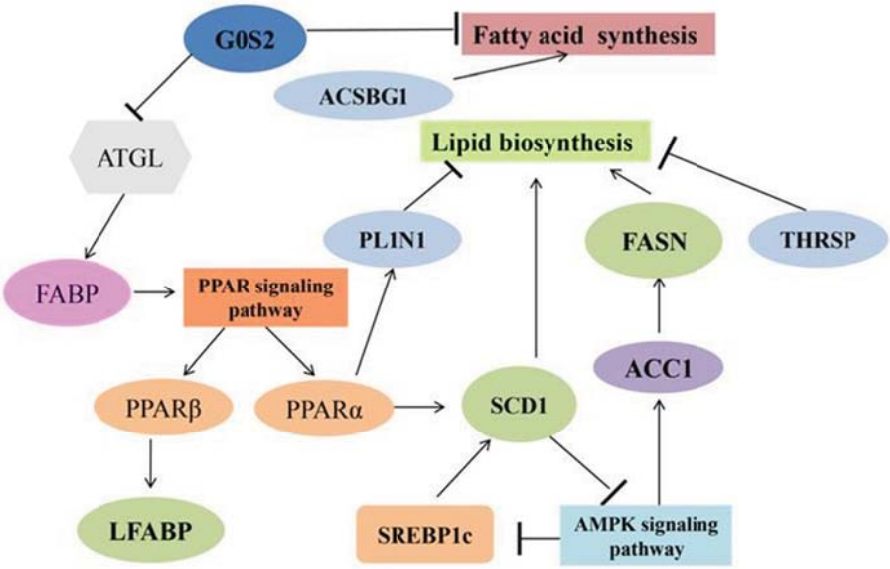

Figure 3. The interaction network of the DEGs between high and low intramuscular fat content.

Note. → represents activate, ⊣ represents inhabit, respectively.

4. Discussion

Fat deposition is highly correlated with meat quality, growth and reproductive performance, and immunity of animals. In chickens, deposited lipids include mainly abdominal fat, subcutaneous fat and IMF. Of these, IMF is an important sensory aspect of meat quality and directly affects the flavor of the meat. Until now, some studies have systematically analyzed the gene expression profiles and regulatory mechanism associated with IMF in breast and thigh tissues of chickens by cDNA microarray. However, few studies have analyzed the thigh tissue transcriptome of chickens using RNA-sequencing. Compared with AA broiler, the Huangshan Black Chicken displayed higher polyunsaturated IMF content in performance traits. Nonetheless, the precise mechanisms of Huangshan Black Chicken contributing to IMF composition remain unclear. Thus, the present study is the first to systematically explore gene expression profiles in thigh tissues using RNA-sequencing to identify global genes and pathways affecting chicken IMF metabolism.

Compared with traditional cDNA microarray technologies, RNA-Seq has many advantages, such as greater dynamic range, removed bias, lower false positives, and higher reproducibility (Cui et al., 2014; Li et al., 2016). Moreover, the correlations between RNA-Seq and the mRNA expression level from qRT-PCR were relatively high (Mao et al., 2015). Obviously, a pooling strategy can dramatically improve accuracy (Kendziorski et al., 2005). In our study, the pooled RNA samples (n = 3 birds) were used for each group and potential candidate DEGs associated with IMF deposition were rigorously defined, with their expression to differ across all

comparisons (IMFH1 vs IMFL1, IMFH1 vs IMFL2, IMFH2 vs IMFL1 and IMFH2 vs IMFL1). To confirm results from the RNA-seq, qRT-PCR was conducted and fold-changes in gene expression between the two methods were correlated ($r^2 = 0.98$) in Huangshan black chickens. Thus, these results showed that RNA-Seq are still recommended to facilitate the accurate detection.

According to integrated analysis on basis of 128 common known DEGs, 14 DEGs (*FABP4*, *G0S2*, *PLIN1*, *SCD1*, *LFABP*, *SLC1A6*, *SLC45A3*, *ACSBG1*, *LY86*, *ST8SIA5*, *SNAI2*, *HPGD*, *EDN2*, and *THRSP*) related to IMF metabolism were detected in this study. Among them, Fatty acid binding protein 4 (*FABP4*) had significantly up-regulated in this study ($P < 0.01$), which was in accordance with that observed in previous reports (Cui et al., 2018). *FABP4* plays an important role in systemic metabolic homeostasis and lipid-mediated biological processes through the regulation of diverse lipid signals (Bag et al., 2015; Floresta et al., 2017). As a lipid chaperon, *FABP4* is responsible for the transportation and metabolism of free fatty acid in adipocyte. Correspondingly, our study revealed that *FABP4* was near to the peak positions of two QTLs for fat traits. These results strongly supported the view of up-regulation of *FABP4* in thigh tissue, revealing that thigh tissue had the stronger lipid biosynthesis.

Metabolic regulation is essential for all biological functions. As a multifaceted regulator, the G0/G1 switch gene 2 (*G0S2*) is abundantly expressed in metabolically active tissues and involved in a variety of cellular functions including proliferation, metabolism, apoptosis and inflammation (Zagani et al., 2015). Particularly, recent studies revealed that *G0S2* acts as a molecular brake on triglyceride (TG) catabolism by selectively inhibiting the activity of rate-limiting lipase adipose triglyceride lipase (ATGL) (Yim et al., 2016; EI-Assaad et al., 2015; Zhang et al., 2017). Similarly, our study revealed that the expression levels of *G0S2* had significantly up-regulated in thigh tissue. In addition, *G0S2* was near to the peak positions of two QTLs for fat traits. In summary, we therefore speculated that *G0S2* may be a promising candidate gene for intramuscular fat percentage in chickens.

As a central regulator of fatty acid metabolism, stearoyl-coenzyme A desaturase 1 (*SCD1*) catalyzes the synthesis of monounsaturated fatty acids (MUFAs), mainly palmitoleate and oleate, which are important in the regulation of lipid and glucose metabolism in metabolic tissues. In addition, *SCD1* is mainly regulated by sterol responsive element binding protein (SREBP)-1c, cyclic AMP response element binding protein 1 (CREB1) and peroxisomal proliferator-activated receptors (PPARs) at the transcriptional level, which were regulatory factors inducing the expression of *SCD1* along with enzymes of denovo fatty acid biosynthesis (ALJohani et al., 2017). Likewise, nucleotide variants of *SCD1* were able to produce significant effects on fatty acid composition, such as milk fat, physicochemical composition, and the quality characteristics in animals (Wen et al., 2018). Among others, the expression levels of *SCD1* had significantly up-regulated in our recent study. Hence, *SCD1* was considered as a major gene affecting fat traits.

Similarly, the expression levels of *THRSP*, *PLIN1*, *SLC1A6*, *SLC45A3*, *ST8SIA5*, *SNAI2*, and *LY86* had significantly up-regulated in thigh tissue ($P < 0.01$). As the previous reported, thyroid hormone responsive (*THRSP*) gene encodes a small acidic protein involved in control of lipogenic enzymes (Liaw & Towle, 1984), perilipin 1 (*PLIN1*) is a lipid droplet-associated protein and has the important function in the regulation of adipocyte lipolysis and lipid storage (Zhou et al., 2016), solute carrier family 1 member 6 (*SLC1A6*) and solute carrier family 1 member 6 (*SLC45A3)* are purported to transport sugars, thereby playing an important potential role in maintaining intracellular glucose levels and the synthesis of long-chain fatty acids (Deng et al., 2007; Shin et al., 2012). ST8 Alpha-N-Acetyl-Neuraminide Alpha-2, 8-Sialyltransferase 5 (*ST8SIA5*) is involved in metabolism and transport of proteins for subsequent modification. However, no previous studies have linked *SNAI2* or *LY86* with lipid differentiation and further study of these genes seems to be warranted.

On the other hand, the expression levels of *LFABP*, *ACSBG1*, *HPGD*, and *EDN2* had significantly down-regulated in our study. Liver-type fatty acid-binding protein (*LFABP*), also frequently known as fatty acid-binding protein 1 (*FABP1*), is involved in intracellular lipid transport from cell membrane to the intracellular sites of fatty acid utilization (Rodriguez et al., 2017). Acyl-CoA Synthetase Bubblegum Family Member 1 (*ACSBG1*) is an acyl-CoA synthetase mediating the activation of long chain fatty acids for the synthesis of cellular lipids, and degradation via beta-oxidation. Additionally, the protein encoded by this gene possesses long-chain acyl-CoA synthetase activity. However, the precise biological functions of *HPGD* and *EDN2* are not known and further research is required to understand the molecular mechanisms of these genes on lipid metabolism in chickens.

Meanwhile, the regulatory network underlying chicken IMF deposition was explored by KEGG pathway analysis. As expected, the well-known PPAR pathway was found and 5 DEGs (novel gene, *PLIN1*, *ACSBG1*,

SCD1, and *LFABP*) involved in PPAR signaling pathway here were screened, which have been proven to be functional in lipid metabolism, such as *PLIN1*, *ACSBG1*, *SCD1*, and *LFABP*. Of special interest, three pathways (fatty acid metabolism, fatty acid biosynthesis and glycine, serine and threonine metabolism) also were enriched, and it was revealed that these three pathways may be the points for the interaction. These findings provide new clues for revealing the molecular mechanisms underlying IMF metabolism in chickens. This novel speculation and its detailed mechanism through pathways related to lipid metabolism identified here needs further validated.

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Evaluating Growth and Yield Parameters of Five Quinoa (*Chenopodium quinoa* W.) Genotypes Under Different Salt Stress Conditions

Asad Sarwar Qureshi[1] & Ashenafi Worku Daba[2]

[1] International Center for Biosaline Agriculture, Dubai, UAE

[2] Ethiopian Institute of Agricultural Research, Ethiopia

Correspondence: Asad Sarwar Qureshi, International Center for Biosaline Agriculture (ICBA), P.O. Box 14660, Dubai, UAE. Tel: 971-56-174-7731. E-mail: busyperson65@gmail.com

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Abstract

Soil salinization is a global problem which restricts the choice of crop for cultivation. Management and reclamation of salinity using costly techniques may not be affordable by subsistence farmers. Therefore, it is important to look for new alternate crops like "quinoa" which are more salt-tolerant. As crops vary in their tolerance to salinity, they need to be evaluated for different salinity conditions. This study was conducted to evaluate five quinoa (*Chenopodium quinoa* W.) genotypes (ICBA-Q1, ICBA-Q2), ICBA-Q3, ICBA-Q4 and ICBA-Q5) for salinity tolerance under four artificially induced salinity (5, 10, 15, 20 dS m^{-1}) levels. The pot trials were conducted in a greenhouse, using 6 kg of Fluvisol soil in each pot. For comparison, trials were also conducted under field conditions. The parameters studied were rate of seed germination, plant height, fresh and dry biomass, chlorophyll content and grain yield. As expected, salinity had generally an inhibitory effect on all parameters. Out of the five quinoa varieties (ICBA-Q1 to ICBA-Q5), ICBA-Q3 and ICBA-Q4 proved to be more salt-tolerant. Therefore these two genotypes are recommended to farmers for large-scale adaptation.

Keywords: chlorophyll, germination, grain yield, quinoa, soil salinity, Ethiopia

1. Introduction

Increasing soil salinization has raised serious concerns of food security for the growing population of the world, which is expected to reach to 9 billion by 2050 (FAO, 2015). FAO estimated that over 1,000 million ha (mha) are globally affected by salinity and sodicity problems. Out of this, about 400 mha are saline, 450 mha are sodic and the remaining saline-sodic in nature (FAO, 2015). Currently, about 20% (62 mha) of the global irrigated land (over 300 mha) is affected with salinity whereas an additional 2,000 ha are added to this menace annually (Qadir et al., 2014). The growing existence of saline soils is reducing natural biodiversity as well as farm and livestock productivity. It is also threatening the sustainability of irrigated agriculture, which produces more than 80% of the total grains. Therefore there is a strong need to control spread of soil salinization. The possible solutions include using physical practices such as improved soil-water-crop management or adopt biological practices such as introducing salt-tolerant species (Ashenafi & Bob, 2016).

Ethiopia stands first in Africa in the extent of salt-affected soils with an estimated 11 mha of land exposed to salinity (Ashenafi & Bob, 2016; Frew et al., 2015). This relates to 13% of the total irrigated area of the country (Birhane, 2017). The saline soils are mainly located in the Rift Valley, Wabi Shebelle River Basin, the Denakil Plains and other lowlands of the country, where about 10% of the population lives (Sileshi, 2016). The problems of soil salinity are expected to increase in future due to the establishment of large-scale irrigation schemes without the provision of adequate drainage facilities (Birhane, 2017). The salinity problems have grave implications for the future food security and economic development of the country. With an annual population growth rate of 3%, securing food and improving livelihood of the rising population will be the biggest challenge. Even today, food shortages are widespread and since 1970 country is in the grip of consistent famines. It is reported that among children aged up to 5 years, around 25% are underweight and 40% are stunted due to malnutrition (UNCEF, 2016).

In the Rift valley and other irrigated areas of Ethiopia, salinity development is mainly caused by the presence of soluble salts in the irrigation water, hot and dry weather conditions and excessive use of poor quality

groundwater for irrigation. Development of large irrigation schemes in middle and lower Awash Valley without effective drainage systems along with poor water management practices have resulted in the gradual rise of saline groundwater. Due to high evapotranspiration, water evaporates from the soil surface leaving the salt behind causing secondary salinization in these areas (Frew et al., 2015). The farmers in Ethiopia are mainly using flood/basin methods to irrigate their poorly levelled fields. There is a tendency to over-irrigate because farmers usually do not have enough knowledge of actual crop water requirements. Therefore, if the current irrigation practices will continue, salinity problems will further exacerbate. Therefore there is an urgent need to take necessary measures to control spread of soil salinization.

In order to meet the future food security challenges, reclamation of existing saline soils and prevention of other areas from salinity development is of paramount importance. The low to medium salinity areas can be reclaimed through effective leaching and installation of appropriate drainage systems. However, these strategies are costly, time consuming and difficult to implement by farmers due to lack of financial resources and technical know-how (Qadir & Oster, 2004). The highly saline soils can be reclaimed by using chemical amendments and/or adoption of *biosaline* approach. The *biosaline* approach entails introduction of salt-tolerant food and forage crops, which can withstand higher salinity levels. These integrated food and forage systems can help in increasing flexibility of smallholder farmers to feed their families and livestock. However, this approach requires selection of diverse food and forage species which have the capacity to resist salts present in the soil (Qureshi, 2017).

One such crop is Quinoa (*Chenopodium quinoa* Willd), which has emerged as an ideal crop for drought prone and salinized agricultural areas due to its high nutritious value and wide adaptability and ability to grow in harsh climatic conditions characterized by high temperatures and poor soil and water quality (Ruiz et al., 2014; Bazile et al., 2016; Chukar-Allah et al., 2016). Quinoa is grown in Andes region for centuries, however, its production and consumption outside the Andes is relatively new (Jacobsen, 2017). Currently, quinoa is grown in more than 90 countries. Today, 80 percent of the quinoa production comes from Bolivia and Peru whereas the remaining 20 percent is produced in all other countries (Bazile et al., 2016). Despite this rapid exposition, quinoa farming is still in "experimental phase" in many countries. The yield differences are huge ranging from 0.6 to 3.9 t ha^{-1} depending on soil, water and climatic conditions (Scalin & Lewis, 2017). This clearly indicates the need for further research to develop varieties that can produce consistent yields under different agro-climatic conditions.

Quinoa has successfully been grown from non-saline to highly saline soils (15-20 dS m^{-1}) (Wilson et al., 2002; Bosque Sanchez, 2003; Adolf et al., 2012). At these salt levels other plant types either fail to grow or grow only poorly (Munns & Tester, 2008; Shabala et al., 2013)). Besides being gluten free, quinoa grain is rich in proteins and essential amino acids such as lysine, threonine, methionine, and much needed unsaturated fatty acids (*i.e.*, linoleic, oleic and linolenic), of minerals (Ca, Fe, Cu, Zn) and vitamins (A, B2, C and E) (Vego-Galvez et al., 2010). Quinoa is a good source of calcium and is suitable for lactose-intolerant consumers and those allergic to gluten (Repo-Carrasco et al., 2003; Vega-Gálvez et al., 2010). The leaves of the plant are frequently eaten as a leafy green vegetable just like as spinach. It can also be used as a highly nutritious feed for animals.

Quinoa is new in African countries, currently passing through pilot testing and field trial stages. Quinoa could withstand temperatures from -8 ^{o}C to 38 ^{o}C, at sea level or 4,000 meters above, which makes it viable for different agro-ecological conditions (Scalin & Lewis, 2017). The quinoa is heat sensitive and may encounter poor seed germination and crop establishment problems in very hot salt-affected areas (Chukar-Allah et al., 2016). In this study, response of five quinoa genotypes to different salinity levels (control, 5, 10, 15 and 20 dS m^{-1}) was evaluated with regard to agronomic parameters. It is envisaged that the outcome of this study will help in the selection of appropriate quinoa varieties for saline areas in different parts of Ethiopia.

2. Materials and Methods

2.1 Study Area

The experiments were conducted at the Werer Agricultural Research Center (WARC), Amibara, Ethiopia, which is located at 278 km to the east of Addis Ababa (9°12′8″ N latitude and 40°15′21″ E longitude). The study area is relatively flat with slope gradients of 1-2% (Figure 1). The mean annual rainfall is 570 mm with a minimum and maximum temperatures of 19 °C and 34 °C, respectively (Figure 2). Higher soil evaporation due to extreme temperatures causes the creation of saline soils and nutrient disparity in the soils causing poor plant growth. The Vertisols soil type of the area varies from silty clay to clay whereas the texture of the Fluvisols soils sandy loam to silty loam (Heluf, 1985; Wondimagegne & Mnalku, 2012).

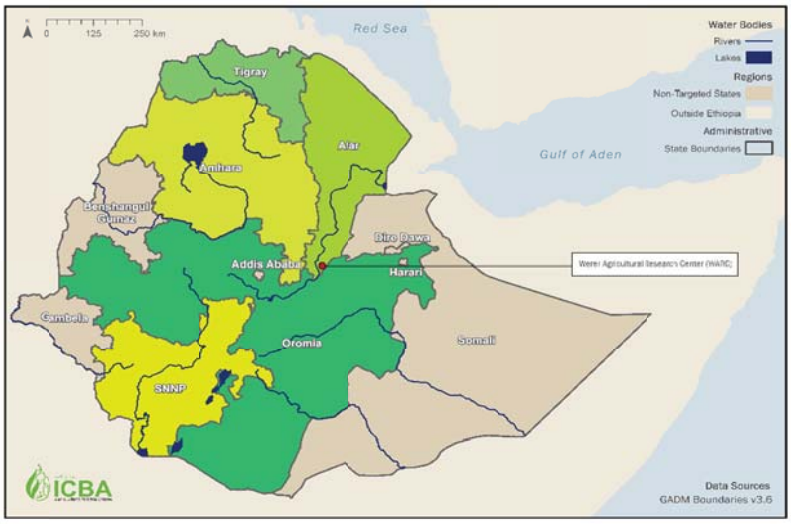


Figure 1. Location map of the study area

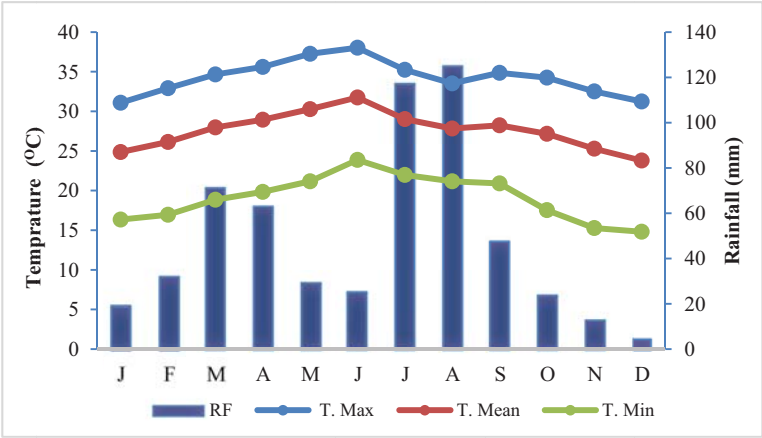


Figure 2. Mean monthly rainfall and minimum and maximum temperatures at the trial site

2.2 Trials Under Controlled Conditions

For pot trials under controlled conditions, treatments include two factors; food and forage genotypes and salt stress levels. The trials were conducted under 4 salt stress conditions to evaluate its suitability for different regions. Four salt stress treatments were prepared by mixing 7.28 14.57, 21.43 and 29.14 g of NaCl into 6.0 kg of soil packed per pot to produce salinity levels of 5, 10, 15 and 20 dS m^{-1}. Five quinoa genotypes (ICBA-Q1, ICBA-Q2, ICBA-Q3, ICBA-Q4, ICBA-Q5) were evaluated to test their performance under different soil salinity conditions. The treatments were organized in a completely randomized design with three replications.

Ten seeds of each genotype were sown in each pot. The seeds were surface sterilized using 70% ethanol (exposure for 10 seconds) followed by immersion for 10 minutes in sodium hypochlorite solution (NaClO; 5% active chloride). The treated seeds were washed thoroughly with distilled water and were placed on moist filter paper in petri dishes. Uniformity of seed size and quality was ensured before germination test. Irrigations were done with canal water (EC = 0.3 dS m^{-1}).

2.3 Trials Under Field Conditions

Field trials were conducted on the saline-sodic soil with an average ECe value of 19.50 dS m^{-1} and an exchangeable sodium percentage (ESP) value of 20. A plot size of 3 m × 4 m was used for field experiments. Soil samples were collected from 0-30 cm depth and analyzed for different soil parameters. The soil bulk density was determined according to the method described by Black (1965). The exchangeable bases (Ca, Mg, Na, and K) were determined from the extraction of neutral normal ammonium acetate extraction, Ca and Mg from EDTA titration method, while K and Na using flame photometer. The cation exchange capacity (CEC) of soil was

determined by the percolation tube procedure (Van Reewijk, 1992). The ESP was computed as the percentage of the exchangeable Na to the CEC of the soil (Table 1).

Table 1. Soil physio-chemical properties of the field trial site

Quinoa genotypes	Soil depth (cm)	pH	ECe (dS m^{-1})	Exchangeable bases ($cmol_{(+)}$ kg^{-1})			CEC ($cmol_{(+)}$ kg^{-1})	ESP (%)	BD (g cm^{-3})
				Ca+Mg	Na	K			
ICBA-Q1	0-30	7.9	19.32	36.94	8.00	1.04	41.16	19.44	1.38
ICBA-Q2	0-30	7.8	20.34	43.02	7.68	0.96	44.21	17.37	1.36
ICBA-Q3	0-30	8.0	20.01	38.83	9.67	1.01	39.83	21.28	1.37
ICBA-Q4	0-30	8.1	18.76	37.91	9.01	0.98	41.78	21.57	1.39
ICBA-Q5	0-30	7.9	18.98	38.02	8.98	0.87	43.57	20.61	1.36

Since soils of the study area are good in nutrients therefore no fertilizer was used for experiments. Irrigations were given according to crop evapotranspiration (ETc), which was calculated by multiplying reference evapotranspiration (ETo) with the crop coefficient (Kc). ETo was calculated using modified Penman-Monteith equation whereas the Kc values were taken from FAO-56 (Allen et al., 2006). In addition to total irrigation requirements according to ETc, an additional 10% of the total irrigation amount was applied for leaching of salts to manage root zone salinity.

2.4 Observations and Measurements

Mean germination time (MGT), germination percentage (GP), biomass and grain yield and shoot and root dry matter and other related data was measured. Seeds with full radicle were considered as germinated. Germination count was done on 5^{th}, 10^{th} and 15^{th} day after plantation. GP was calculated according to Ashraf and Foolad (2005) whereas MGT was determined using equation of Ellis and Roberts (1981). Chlorophyll content (SPAD units) of leaves was measured using Minolta Soil-Plant-Analysis Development (SPAD) meter. Plant height was measured with a standard ruler (*i.e.*, stem length from soil level to the top of the flower head).

$$GP = \frac{\text{Total germinated seeds}}{\text{Total number of seeds}} \quad (1)$$

$$MGT = \frac{\sum Dn}{\sum n} \quad (2)$$

Where, n = Number of germinated seeds on day D, and D = Number of days from the start of germination.

2.5 Statistical Analysis

Field and pot experiments were conducted for two years (2017-18) and the data was subjected to analysis of variance (ANOVA) technique (A. Gomez & H. Gomez, 1984) for factorial CRD using SAS 9.3 software (SAS Institute, Cary, NC). The significance of differences between the mean values at $p < 0.05$ was determined using Least Significance Difference (LCD) test. The comparison between all data obtained was made by using Duncan's Multiple Range Test (DMRT).

3. Results

3.1 Trials Under Controlled Conditions

3.1.1 Germination Percentage (GP), Mean Germination Time (MGT) and Germination Index (GI)

For all genotypes, increasing salinity affected seed germination. The GP was highest in ICBA-Q3, ICBA-Q4 and ICBA-Q5 in control and gradually decreased with the increasing salinity. The lowest GP was found in ICBA-Q1 and ICBA-Q2. The MGT also increased with growing salinity levels. The highest MGT was recorded in ICBA-Q1 at 20 dS m^{-1}, followed by ICBA-Q2 whereas the lowest was found in ICBA-Q3 in control. MGT for other three genotypes were comparable (Table 2). The GI followed the trends of GP for all genotypes. The maximum GI was observed in ICBA-Q5 followed by ICBA-Q3 and ICBA-Q4 at control. Lower GI values were observed at the highest salt concentration levels for all genotypes.

Table 2. Effects of salinity on GP, MGT and GI of five quinoa genotypes

Parameters	Genotypes	NaCl salt level (dS m^{-1})					LSD ($p \leq 0.05$)	CV (%)
		0	5	10	15	20		
Germination Percentage (%)	ICBA-Q1	36.67	23.33	10.00	16.67	10.00	6.00	17.93
	ICBA-Q2	16.67	16.67	13.33	20.00	16.67		
	ICBA-Q3	83.33	80.00	63.33	53.33	33.33		
	ICBA-Q4	83.33	83.33	56.67	60.00	40.00		
	ICBA-Q5	83.33	86.67	63.33	53.33	36.67		
Mean Germination Time (days)	ICBA-Q1	2.67	3.27	5.88	8.33	13.05	0.52	11.28
	ICBA-Q2	3.11	3.33	5.66	8.50	12.33		
	ICBA-Q3	2.61	3.83	5.27	5.77	10.27		
	ICBA-Q4	3.00	4.33	5.61	7.22	10.94		
	ICBA-Q5	3.16	4.33	5.67	8.07	10.27		
Germination Index (GI)	ICBA-Q1	1.01	0.45	0.23	0.24	0.10	0.22	26.84
	ICBA-Q2	0.31	0.21	0.19	0.23	0.24		
	ICBA-Q3	2.38	2.17	1.57	0.95	0.72		
	ICBA-Q4	2.02	2.32	2.01	1.10	0.76		
	ICBA-Q5	2.59	2.51	1.92	1.25	0.94		

3.1.2 Plant Height

For all quinoa genotypes, a decreasing trend in plant height was observed with the increasing level of salinity. The highest plant height was observed for ICBA-Q3 (92.7 cm) and ICBA-Q4 (92.3 cm) at 0 dS m^{-1}. However, at higher salinity level (20 dS m^{-1}), plant heights of ICBA-Q3 and ICBA-Q4 were reduced to 54.6cm and 51.0cm, respectively. Increase in salinity from 0 to 20 dS m^{-1} causes reduction in plant height of for ICBA-Q3 and ICBA-Q4 by 41% and 44%, respectively. Table 3 shows that plant height of all quinoa genotypes reduced significantly after 10 dS m^{-1}. These results agree with those of Jacobsen (2003) and Al-Dakheel et al. (2015) who found significant reduction in plant height with the increasing salinity levels in Phaseolus species and Lentils.

Table 3. Effects of salinity on plant height of five quinoa genotypes

Parameters	Genotypes	NaCl salt level (dS m^{-1})					LSD ($p \leq 0.05$)	CV (%)
		0	5	10	15	20		
Plant height (cm)	ICBA-Q1	68.67	67.00	67.67	61.67	42.47	4.01	8.05
	ICBA-Q2	68.33	67.67	67.33	63.67	45.67		
	ICBA-Q3	92. 67	84.00	74.67	63.00	54.66		
	ICBA-Q4	92. 33	85.00	69.47	59.33	51.00		
	ICBA-Q5	70.00	65.33	60.67	56.00	48.33		

3.1.3 Dry Biomass Yield

Dry biomass yield was attained by oven-drying fresh biomass at 65 ^{o}C to constant weight. In all quinoa genotypes, dry biomass yield conceded due to increased salt stress (Figure 3). The highest dry biomass yield was obtained in ICBA-Q3 at 0-5 dS m^{-1} whereas the lowest was obtained in ICBA-Q1 and ICBA-Q2. ICBA-Q4 performed better at elevated salinity levels (15-20 dS m^{-1}). The dry biomass yield decreased with the increasing soil salinity in the growth medium, although the response of all five genotypes to different salinity levels was heterogeneous. At 0 dS m^{-1}, dry biomass yield of ICBA-Q1, ICBA-Q2, ICBA-Q3, ICBA-Q4, and ICBA-Q5 was 15.5, 12.5, 29.6, 25.0 and 21.0 g/plant, respectively. However, the dry biomass yield at 20 dS m^{-1} was noted as 5.1, 8.6, 17.1, 18.1, and 13.5 g/plant, registering a reduction of 67%, 30%, 42%, 28%, 36% for ICBA-Q1, ICBA-Q2, ICBA-Q3, ICBA-Q4, and ICBA-Q5, respectively. For all salinity levels, minimum reduction was noted in ICBA-Q3, which shows that this genotype is most resistant to increasing salinity levels.

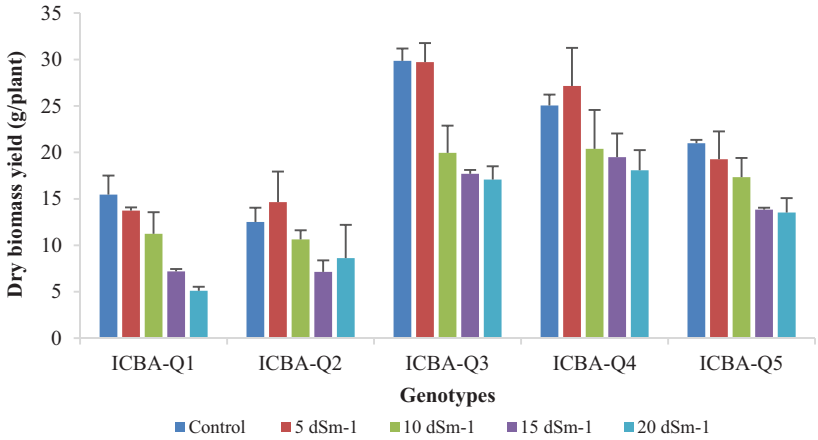
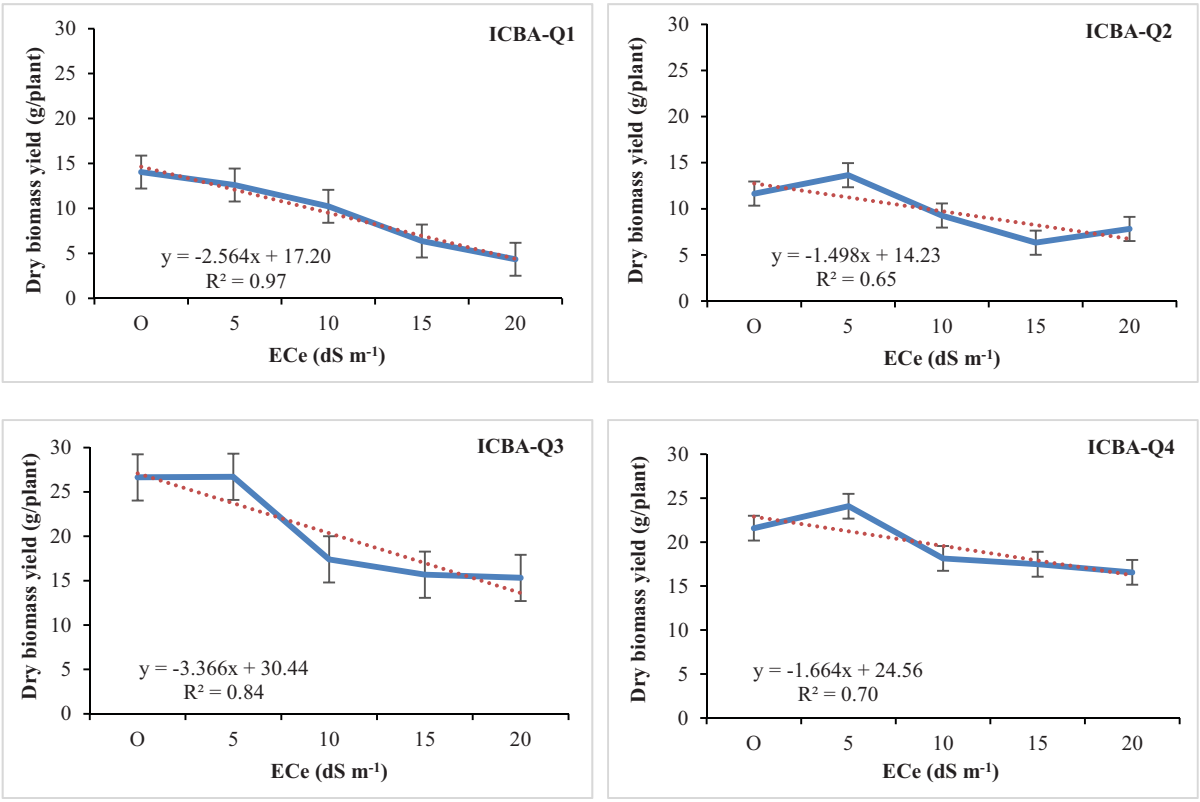


Figure 3. Dry biomass yield of five quinoa genotypes as affected by different salinity levels

The experimental data was also used to develop production functions for 5 quinoa genotypes under different soil salinity levels and the results are presented in Figure 4. The highest reduction in dry biomass yield per unit increase in salinity (1 dS m^{-1}) was observed in ICBA-Q3 (0.56 g/plant) followed by ICBA-Q1 (0.48 g/plant) and ICBA-Q2 (0.46 g/plant). The lowest reduction in dry biomass per unit increase in salinity was found in ICBA-Q4 (0.38 g/plant) and ICBA-Q5 (0.22 g/plant). These two genotypes showed more stable dry biomass yields under all salinity levels. The dry biomass yield for ICBA-Q3 sharply declined after 5 dS m^{-1} salinity level whereas this was not the case for ICBA-Q4 and ICBA-Q5. ICBA-Q5 showed more consistent dry biomass yield at all salinity levels. This shows that under moderate salinity levels (0-5 dS m^{-1}), ICBA-Q3 can be a good choice because of high yielding potential. However, for higher salinity levels (10-20 dS m^{-1}), ICBA-Q4 and ICBA-Q5 are more suitable due to their higher salt tolerance capacity.



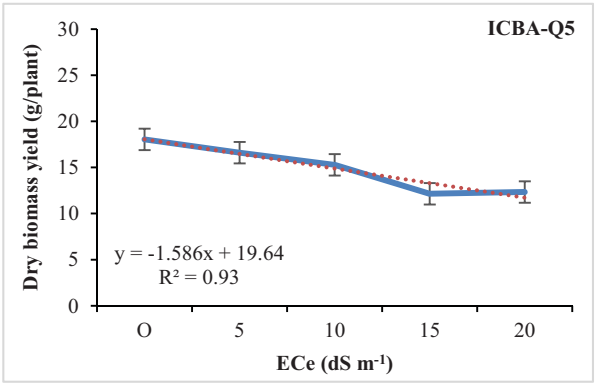


Figure 4. Dry biomass yield of five quinoa genotypes as effected by different salinity levels

3.1.4 Grain Yield

The grain yield was negatively affected by increasing salinity levels however, the impact was more noticeable at higher salt concentrations (Figure 5). Since ICBA-Q1 and ICBA-Q2 were poor in germination, they also produced lower grain yield. The highest grain yield was obtained for ICBA-Q3 at all salinity levels followed by ICBA-Q4 and ICBA-Q5. The differences in grain yields under ICBA-Q3 and ICBA-Q4 were non-significant. Under control, the grain yield of ICBA-Q4 and ICBA-Q5 was 10% and 42.5% less than the grain yield of ICBA-Q3. However, the reductions in grain yields at the higher salinity levels were comparatively lower than the control i.e., grain yields of ICBA-Q4 and ICBA-Q5 were 4.8% and 38% less than ICBA-Q3. The grain yields of ICBA-Q3 and ICBA-Q4 were comparable at 10-15 dS m^{-1}, however, there was a significant reduction in grain yields at higher salinity level (20 dS m^{-1}).

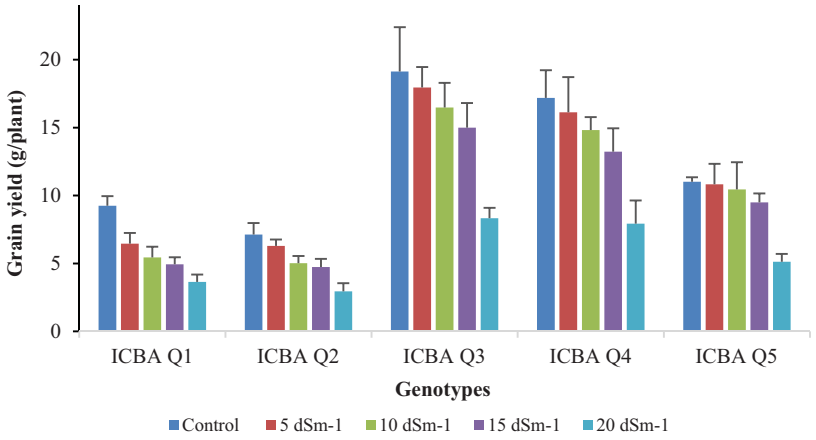


Figure 5. Grain yield of five quinoa genotypes as effected by different salinity levels

Figure 6 shows that the maximum reduction in grain yield per unit increase of salinity was recorded for ICBA-Q3 genotype followed by ICBA-Q4 genotype. Although ICBA-Q1 and ICBA-Q2 genotypes produced lower yields, the reduction in grain yield per unit increase in salinity for these genotypes was lower, *e.g.*, 0.28 g/plant for ICBA-Q1 and 0.21 g/plant for ICBA-Q2. The yield reduction per unit increase in salinity in ICBA-Q5 was 0.35 g/plant. Considering the overall dry biomass yield, grain yield and tolerance against higher salinity levels, ICBA-Q3 and ICBA-Q4 can be considered as the best genotype for all salinity levels under Ethiopian conditions.

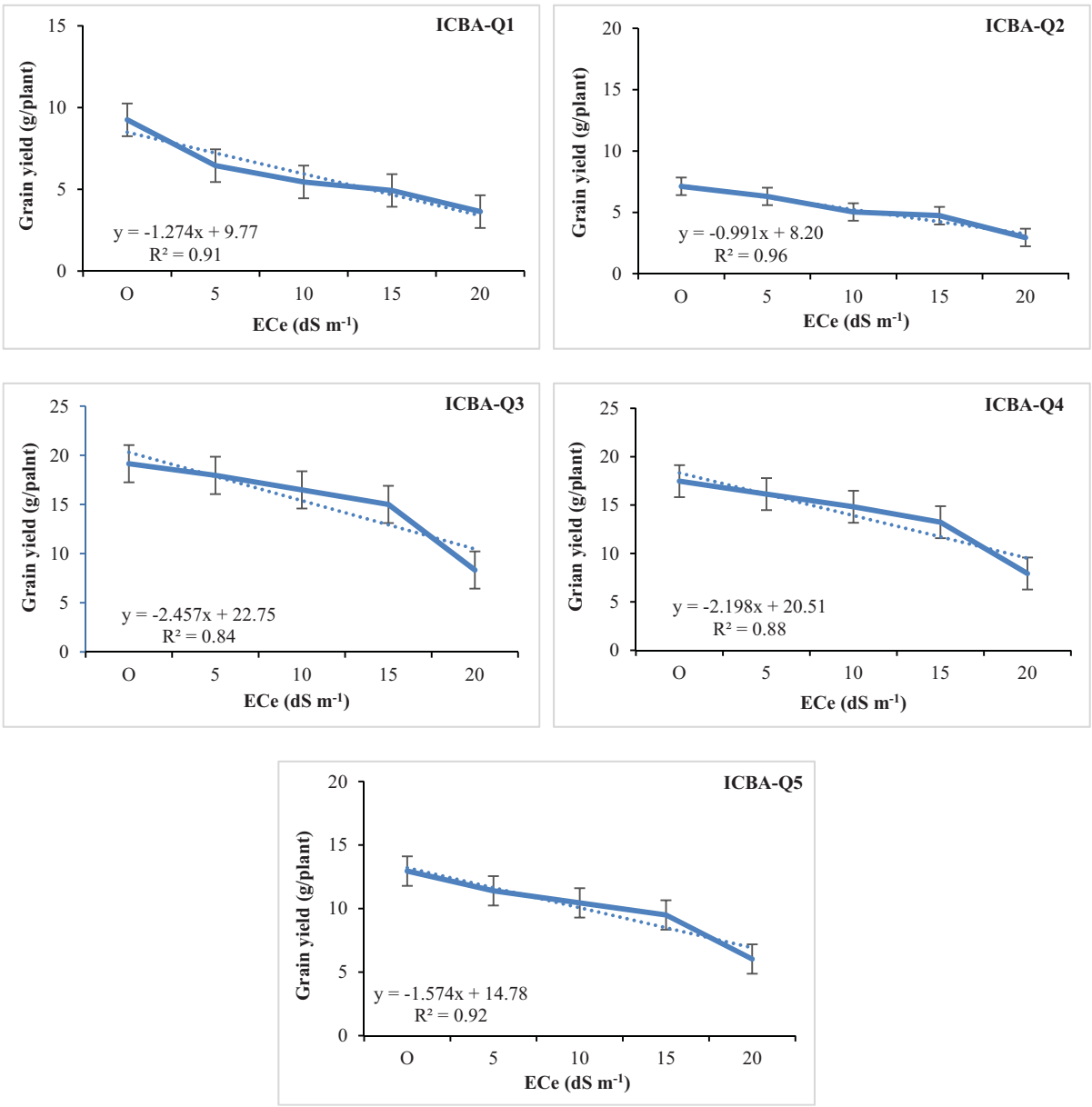


Figure 6. Grain yield of five Quinoa genotypes as affected by different salinity levels

3.1.5 Chlorophyll Content

Due to the distinct variation among all five quinoa genotypes, their responses to different salinity regimes for chlorophyll content were also different. Figure 7 illustrates that the chlorophyll content of all five genotypes tolerated salinity stress up to 10 dS m^{-1} but decreased significantly at higher salinity levels. The highest chlorophyll content was recorded at salinity levels of 0-5 dS m^{-1}. The results indicate a decreasing trend of chlorophyll content with increasing salinity stress except in ICBA-Q2, ICBA-Q4, and ICBA-Q5 where chlorophyll content was higher at 5 dS m^{-1} compared to control. At lower salinity levels (0-5 dS m^{-1}), the performance of ICBA-Q4 and ICBA-Q5 was superior than other three genotypes. Net decrease in the photosynthesis rates of quinoa by high salinity has also been reported (Eisa et al., 2017). They have also reported salt-induced growth reduction due to low photosynthetic supply because of impaired photosynthetic capacity.

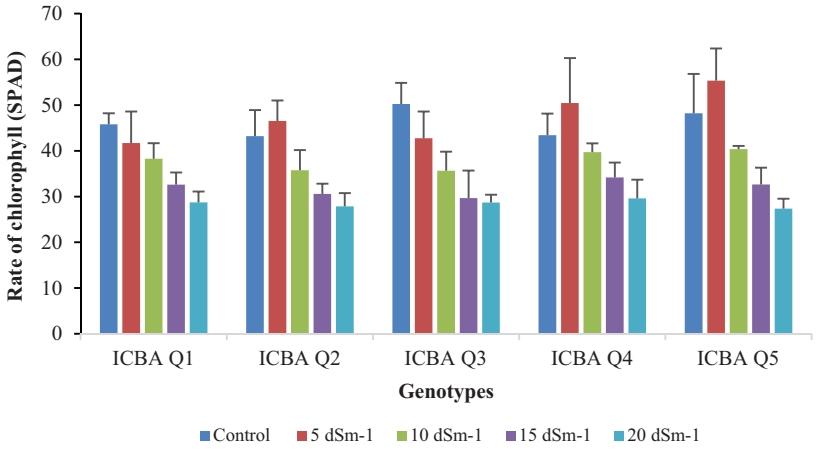


Figure 7. Chlorophyll rate of 5 quinoa genotypes as effected by different salinity levels

3.2 Trials Under Field Conditions

For the validation of pot trials results, selected five quinoa genotypes were also tested under field conditions with soil salinity (ECe) values approaching 20 dS m^{-1} (Table 4). The tested quinoa genotypes were found to be significantly different in biophysical parameters such as grain yield, days of 50% physiological maturity, days of 50% emergency, number of panicles per plant, plant height, number of days to pasty grain and milky stage and dry biomass yield. The results indicate that ICBA-Q3 performed superior with regard to grain and dry biomass yield followed by ICBA-Q4. The performance of ICBA-Q1 and ICBA-Q2 was the poorest under field conditions whereas ICBA-Q4 and ICBA-Q5 showed medium results. The field evaluation shows that ICBA-Q3 is the most suitable genotype for hot, dry and highly saline areas both in terms of dry biomass and the grain yield. As evident from the pot trial analysis, other genotypes such as ICBA-Q4 and ICBA-Q5 can yield satisfactory results in low to medium saline areas. Therefore, quinoa genotype for a certain area should be selected after due consideration of climatic and soil salinity conditions.

Table 4. Field evaluation of five salt-tolerant quinoa genotypes

Quinoa genotypes	DE (days)	DM (days)	PH (cm)	PPP (days)	DMS (days)	DPG (days)	DBY (ton/ha)	GY (kg/ha)
ICBA-Q1	12.33	93.00	138.60	8.00	80.00	90.67	1.239	464
ICBA-Q2	12.00	94.33	148.77	8.00	78.33	89.67	1.291	499
ICBA-Q3	9.00	86.33	144.00	11.67	72.33	84.00	7.211	2965
ICBA-Q4	8.67	89.00	152.13	10.00	75.67	88.00	5.885	1644
ICBA-Q5	8.67	86.00	156.13	9.00	71.33	78.66	4.023	1559
LSD ($P < 0.05$)	1.49	3.94	NS	1.41	NS	6.68	0.572	152
CV (%)	7.85	12.33	23.61	8.06	16.85	14.11	8.61	15.67

Note. DE = Days to 50% Emergence; DM = Days to 50% Maturity; PH = Plant Height; PPP = Panicles per Plant; DMS = Days to Milky Stage; DPG = Days to Pasty Grain; DBY = Dry Biomass Yield; GY = Grain Yield.

4. Discussion

The rising global demand for nutritious and healthy food has challenged scientists to look for alternate crops especially for the marginal areas where agricultural production is inefficient due to unfavorable climatic conditions, low soil fertility and lack of good quality irrigation water. In many countries of the Middle East and Africa region, scientists are experimenting quinoa production because it is rich in nutrients, tolerant to salinity and uses much less water than other crops. Against this backdrop, this study was focused on assessing the feasibility of 5 different quinoa genotypes for dry and saline soil conditions of Ethiopia under controlled and field conditions. The seed germination was adversely affected by the rising salinity. The salinity impedes seed germination either without loss of viability at higher salinities and/or by inducing stress to seeds (Breusegen et al., 2006). Gómez-Pando et al. (2010) did a study on 15 most salt-tolerant Peruvian accessions of quinoa and found that some genotypes of quinoa showed decline in germination and plant height under high saline conditions, while others did not or even register an increase.

The results indicate reduction in dry biomass yield with increasing salinity, which might be due to lack of water availability and hydrolysis of reserved foods and their translocation to the growing shoots. Other factors responsible for lower dry biomass yield may include panicle length, chlorophyll concentrations, number of productive tillers, number of primary branches per panicle, and fertility percentage (Ali et al., 2004). Studies have also reported reduction in plant growth and dry-matter accumulation under saline conditions in several grain legumes including *P. vulgaris* that can be ascribed by decrease in cell elongation (Turan et al., 2007; Gómez-Pando et al., 2010).

Gómez-Pando et al. (2010) have also found a remarkable influence of quinoa genotypes on root dry mass per plant under saline conditions. This was probably due to the stunted growth of plants caused by high salt concentration in the nutrient medium. The higher salt stress causes reduction in the rate of leaf surface expansion, which results in considerable decrease in the dry weights of shoot, leaves, and roots (Kandil et al., 2012). This can be linked to the limited supply of metabolites to young growing tissues. Metabolic production usually occur within the leaves and can be affected significantly at high salt stress conditions either due to the low water uptake or toxic effect of NaCl concentration (Hassen et al., 2014).

The results of this study also show decrease in chlorophyll content with the increasing salinity levels in all tested quinoa genotypes. This can be attributed to the fact that at the higher salinity levels, plants increase generation of reactive oxygen species as by-product. This damages the cellular components and cause chlorophyll degradation and membrane lipid peroxidation, reducing membrane fluidity and selectivity (Verma & Mishra, 2005). Reduction in chlorophyll content due to metabolic limitations of photosynthesis in leaves at higher salinity levels (above 250 mM) has also been reported by Munns et al. (2006). In another study decreasing chlorophyll content in trees is associated with aggravated salt stress due to enzymatic chlorophyll degradation (Xu et al., 2011).

The reduction in the photosynthesis under saline conditions mainly occur due to a reduction in leaf area, chlorophyll content and stomatal conductance, and decrease in photosystem II efficiency. The reduction in chlorophyll levels in plants under saline conditions is considered as a typical symptom of oxidative stress and is usually linked with the lack of chlorophyll synthesis, together with the activation of its degradation by the enzyme chlorophyllase (Netondo et al., 2004). The decrease in chlorophyll results in reduced biomass with respect to increasing salinity stress.

The decreases in chlorophyll with increased salt stress were found in *Phaseolus vulgaris* L., and cereals (Santos, 2004). Cocozza et al. (2013) has also shown that quinoa plant is more resistant to water and salt stresses due to its effective stomatal responses that helps plant growth and protected crop yield (Sai Ram et al., 2002). Jaleel et al. (2008) has shown reductions of chlorophyll content in *Catharanthus roseus* whereas Nazarbeygi et al. (2011) has got similar results in Canova. Accumulation of salts causes an irreparable damage to the photosynthetic mechanism due to dehydration of cell membranes and closure of stomata which reduces their permeability to CO_2 and thus chlorophyll formation.

5. Conclusions

There are considerable differences on various plant growth parameters with the increasing salinity on five quinoa genotypes. Results clearly revealed that nearly all parameters measured decreased with increasing levels of salinity stress. Most limiting factor for decreased plant growth was found to be reduction in photosynthesis expressed in the production of chlorophyll. We suggest that in future, plant breeding should focus on developing new genotypes that can withstand salinity and have high antioxidant activity. This study has shown that the performance of ICBA-Q3 is superior followed by ICBA-Q4 and ICBA-Q5. However, further optimization of these genotypes is recommended to enhance their productivity under different agro-ecological conditions. This should include testing of these genotypes for water and heat stresses to assess their suitability for hot and dry climatic conditions prevailing in many regions of Ethiopia. In this study, fresh water is used for all experiments. However, in many areas, availability of fresh water is limited, and poor quality groundwater is used for irrigation. Therefore, it would be worthwhile to evaluate the performance of these quinoa genotypes using different quality irrigation waters.

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Impact of ICT Based Extension Services on Dairy Production and Household Welfare: The Case of iCow Service in Kenya

Mwita Erick Marwa[1], John Mburu[1], Rao Elizaphan James Oburu[2], Okeyo Mwai[2] & Susan Kahumbu[2]

[1] Department of Agricultural Economics, University of Nairobi, Nairobi, Kenya

[2] International Livestock Research Institute, Nairobi, Kenya

[3] Green Dream TECH Ltd, Nairobi, Kenya

Correspondence: Mwita Erick Marwa, Department of Agricultural Economics, College of Agriculture and Veterinary Sciences, University of Nairobi, P.O. Box 29053-00625, Nairobi, Kenya. Tel: 254-723-536-703. E-mail: mwitaerick@gmail.com

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Abstract

Smallholder dairy farmers have the challenges of accessing timely and reliable agricultural information, and this limits them from realizing maximum farm output. The use of information and communication technologies (ICT) as a farming extension tool by smallholder farmers has the potential to reverse the scenario and improve farmers' outputs and incomes leading to increased welfare. This study employed the Propensity score Matching approach to evaluate the impacts of ICT-based extension services, in this case, iCow services on milk production, milk income, and household income using cross-sectional data from a survey of dairy farmers in Uasin Gishu, Nyandarua and Bomet counties of Kenya. The use of ICT-based iCow services is shown to increase Annual milk production per cow, milk income, and household income by 13%, 29%, and 22%, respectively. Partnerships between network providers and research institutes should be encouraged as part of bridging the extension gap occasioned by reduced public expenditure on extension services.

Keywords: iCow services, ICT, agricultural information, dairy farmers, propensity score matching, extension, husbandry practices

1. Introduction

Rural services are at the heart of thriving agricultural and rural development (ARD) in developing countries. Effective delivery of services is seen as 'essential if small farms in high potential areas are to intensify production, contribute to economic growth, and reduce poverty' (Milu & Jayne, 2006). Agricultural extension is one of the services that play an essential role in the growth and transformation of the agrarian sector in Sub Saharan Africa (SSA), Kenya included (Joseph & Polytechn, 2017; Mukembo & Edwards, 2016). Indeed, benefits like high productivity, quality of produce, reduction of diseases and pests, and subsequent increase in income among smallholder farmers can be attributed to access to quality extension service (Fu & Akter, 2012). Specifically, in livestock, such benefits are gained through use of information like patterns in livestock prices, good livestock management practices, and marketing (Milu & Jayne, 2006).

In Kenya, agricultural extension services are delivered by multiple providers, which include; National government, county governments, agro-inputs manufacturers and suppliers and Non-government Organizations (NGOs). Extension services in Kenya have evolved over time, beginning with the Transfer of Technologies (ToT) approach, which emphasized the adoption of technologies with little regard, if any, for how knowledge and skills about using these technologies were acquired. The system failed to inspire a wider uptake of techniques, and this led to introduction of the Farming Systems Research (FSR) in the 1970s. FSR focused on on-farm testing and refining of technologies but also could not adequately address the multiple and often diverse needs of farmers (Mukembo & Edwards, 2016).

Later in the 1980s, Training and Visit (T&V) approach was introduced with the aim of transferring information and technology through extension workers and contact farmers to the general farming community. However, T&V could not address the varying needs of farmers due to high expenses and low coverage of extension workers. To improve the situation, a new extension approach based on Farmer Field School (FFS) was introduced in 2001. The FFS uses participatory methods in enhancing farmers' knowledge and skills on the use of agricultural technologies. While the technique somehow improved the productivity of farmers, the approach did not fully meet their diverse needs (Mukembo & Edwards, 2016).

Given the limitations of previous approaches, extension services continue to evolve with players exploring new approaches, including the application of information and communication technologies (ICTs) in delivering advisory services. Studies show that the use of ICT in the farming community increases their production and income leading to increased welfare (Nyaga, 2012; Singh, 2006; Das, 2014). According to Duncombe (2012), the adoption of ICTs in the agricultural sector has yielded substantial economic, environmental and social benefits at local, nationwide and global stages. The use of ICTs by extension agents in gathering, retrieving, adapting, and disseminating a broad range of information needed by rural farming communities have made positive contributions towards rural development (Ajani & Agwu, 2012). A report by Fu and Akter (2012) concluded that use of mobile phone technology among farming communities improves the quality and speed of the delivery of extension services. In line with these developments, several ICT-based programs have mushroomed in Kenya to address the challenge of low farm productivity and improve agricultural performance among smallholder farm households. These programs include; iCow, Kenya Agricultural Commodity Exchange (KACE), National Livestock Market Information System (NLMIS), Regional Agricultural Trade Intelligence Network (RATIN), National Farmers Information Service (NAFIS) and M- farm among others.

This study focuses on the iCow's ICT-platform and services, as a tool for dissemination of information among smallholder dairy farmers. The iCow services are offered by Green Dream Technology (GDT) in partnership with the Safaricom Foundation and International Livestock Research Institute (ILRI) with the aim of improving extension services among smallholder farmers. The iCow platform achieves this through a cost-effective, scalable mobile phone extension service, which provides farmers with basic, pure, timely knowledge and digital solutions that would improve their production. The iCow platform offers innovative products that include weekly messages on various livestock and agricultural topics, livestock calendars, farmer SMS library, and an expert directory. It is conceptualized that the digital information on vaccination, spraying, mastitis control, deworming, hygiene, and other dairy management practices improves animal health, reduces incidences of disease outbreak, and consequently reducing the intake of antibiotics. Nutrition also improves due to information on fodder management, proper feeding, and feed quality. Additionally, information on record-keeping helps farmers to enhance planning and forecasting. The expectation is that farmers participating in iCow would use the acquired technical knowledge and apply superior production technologies and husbandry practices to realize higher outputs and consequently improving their incomes.

Although previous studies have assessed the use of ICTs in agriculture, most of these studies focused on the application of ICT tools in market information systems (MIS) (Okello et al., 2013; Oyeyinka & Bello, 2013; Hassan et al., 2008). Additionally, more attention has been given on the use of ICTs by extension officers (Fu & Akter, 2012; Tata & Mcnamara, 2018), there remains dearth research on the use of ICT extension tools by smallholder farmers. This study was designed to evaluate the impacts of the iCow services on milk production and household income among smallholder dairy farmers in Kenya. The paper is organized as follows. Section 2 describes the methodology used in the study. Section 3 presents and discusses estimation results, while section 4 makes concluding remarks and draws policy implications.

2. Method

2.1 Theoretical Framework

The study uses the theory of expected utility as developed by Bernoulli (1738), which has been applied in several studies on farmer decision-making in many aspects (Stearns, 2000; Babcock & Hennessy, 1996; Gómez et al., 2004). Following Bernoulli (1738), participation in iCow ICT program can be viewed as a binary choice decision problem by farm households that try to maximize utility or net returns. The utility is determined by a set of variables Z, which influences the cost of adjusting to a new extension approach involving ICT (such as the cost of acquiring a mobile phone and the time spent on reviewing messages relayed by the platform). Variables in Z also determine the relative returns that a farmer can earn from adopting iCow approach to extension. Thus, Z can include household characteristics such as educational status and farming experience,

both of which influence ability to synthesize relayed information and optimize farm decisions based on the provided information.

The probability that farmers participate in iCow platform is therefore determined by a comparison of the expected utility of participation in iCow extension program, U_{ip}, against the expected utility of participating in conventional extension program, U_{iN}. In making this comparison, farmers evaluate the benefits of adjustment mentioned above. Farmers, therefore, join in iCow program only if $U_{ip} > U_{iN}$, implying that the potential benefits outweigh the constraints, and this difference in utility can be represented by a latent variable, $R^* \cdot R_i^* = U_{ip} - U_{iN} > 0$. However, R_i^* is a latent variable; what is observed is actual participation in iCow program, R_i, with $R_i = 1$ if $U_{ip} > U_{iN}$ and $R_i = 0$ if $U_{ip} \leq U_{iN}$. Participation in iCow program can, therefore, be represented as follows:

$$R = Z\alpha - v \tag{1}$$

Where, α is a vector of parameters, and v is an error term with zero mean and variance σ^2. Since farmers are heterogeneous in their characteristics, not all of them will participate in the iCow program. For those who do, participation is expected to result in higher farm returns that may also affect household livelihood outcomes such as income.

2.2 Impact Evaluation

Program evaluation often follows approaches suggested by (Maddala, 1991)

$$y = \beta X_1 + \gamma R + \varepsilon \tag{2}$$

Where, y can be considered as one of the livelihood outcomes such as household income and milk yield.

In this study, milk production was computed accounting for the breed type and lactation length of the lactating cows, while milk production per cow was calculated by dividing total milk production in a household by the number of lactating cows. Household income was calculated by summing up, revenue from milk, farm income, and off-farm income. X_1 is a vector of the farm, household and contextual characteristics that could influence livelihood outcomes, and; R is a dummy indicating whether a household uses iCow services. We hypothesize that use of iCow services could influence livestock husbandry practices and uptake of improved technologies, leading to higher yields, adjusted dairy income and subsequently household income. Holding other factors constant, therefore, the coefficient (γ) captures partial effects of household uptake of iCow services. However, households may self-select into uptake of iCow services leading to bias estimates of treatment effects of iCow extension services. For instance, it is possible that some factors determining uptake of iCow services may also affect household income. If such factors are not included explicitly in Equation (2), as is the case when such variables are unobserved, then the indicator for uptake of iCow services in Equation (2) will be correlated with the error term (ε_1), leading to a biased estimation of γ.

One way to address this problem is to monitor users and non-users of iCow services over time and then apply difference-in-difference (DiD) analytical techniques to isolate the impact of iCow services on livelihood outcomes. However, such an approach cannot be applied to this study since monitoring of the use of iCow services was restricted to the treated households. An alternative approach that also addresses selection bias is the instrumental variables (IV) (Ichimura, 1997). However, the application of the IV approach is limited by the difficulty in finding a suitable IV that influences the probability of treatment without having a correlation to the error term (Wooldrige, 2011). One could also apply regression discontinuity (RD) method that fits regression line to estimate the average effects based on the outcomes of interest (Khandker et al., 2010). However, it is not always easy to establish selection criteria.

To address the potential selection bias, we, therefore, apply propensity score matching (PSM) approach that assumes that conditioning on observable variables eliminates sample selection bias (Heckman & Navarro-Lozano, 2004). Matching essentially creates an experimental condition in which uptake of iCow services is randomly assigned, allowing for identification of a causal link between the use of iCow services and livelihood outcomes. Instead of directly comparing outcome and impact variables between users and non-users of iCow services, PSM restricts comparison to households that are similar in terms of observable characteristics and therefore reduces the bias that would otherwise occur if the two groups were systematically different (Dehejia, 2002).

2.3 The Propensity Score Matching Method (PSM) Method

PSM involves constructing a comparison group based on an individual's probability of participating in a program conditional on observable characteristics (Ravallion, 2009; Khandker et al., 2010). This proceeds in

two-stage: first, we use the entire sample to estimate a probit/logit model that generates propensity scores $\boldsymbol{P}(\boldsymbol{z})$ estimates of the probability that a household with a vector of characteristics $\boldsymbol{z}$, will use iCow services. The vector $\boldsymbol{z}$ are assumed to be those observable variables that determine whether a household uses iCow services. In this estimation, households with similar observable characteristics are likely to have identical propensity scores $\boldsymbol{P}(\boldsymbol{z})$, even if some of them do not use iCow services.

Using similarity in propensity scores, we can, therefore, construct comparable groups of households with similar propensity scores $\boldsymbol{P}(\boldsymbol{z})$ but where one group uses iCow services while the other group of households does not use iCow services. In the second stage, we calculate the average outcomes for the two groups and then estimate the impacts of iCow services as the difference in average outcomes between these groups. This difference is known as the PSM estimator of average treatment effect on the treated households (ATT), which is expressed as follows:

$$\tau_{ATT}^{PSM} = E_{P(z|R=1)}\left[E\{Y_1|R=1, P(z)\} - E\{Y_0|R=0, P(z)\}\right] \quad (3)$$

Where, Y_1 and Y_0 are outcomes for users and non-users of iCow services respectively; $R = 1$ indicates that households use iCow services and $R = 0$ refer to a comparison group of households that do not use iCow services.

PSM rests on two assumptions; one is the conditional independence assumption (CIA), which states that unobserved characteristics do not influence participation (Heckman, 1998). The second assumption is the Common Support Assumption (CSA), which seeks to develop a common support overlap of propensity scores for users and non-users of iCow services. Fulfillment of CSA ensures implies that participants and non-participants in the iCow services have similar observable characteristics for proper matching of subjects (Richard & Monica, 2000; Khandker et al., 2010).

2.4 Sampling Procedure and Data

The study was implemented in Uasin Gishu, Nyandarua, and Bomet counties of Kenya, where iCow services have been in existence. The three counties were selected for the study because of the higher density of smallholder dairy farmers. The study used a two-stage stratified random sampling procedure to obtain respondents for the survey in the three counties. In the first stage, three dairy cooperatives, namely Sirikwa (Uasin Gishu), Olkalao (Nyandarua), and Siongiroi (Bomet) were purposively selected to form the sampling frame for users of iCow services. These are the counties that had been targeted by GDT for piloting and eventual rollout of the iCow services. Since GDT focused the entire membership of these cooperatives, it was not possible to find reasonable number of non-users of iCow services among members of the 3 dairy cooperatives. Moreover, any non-users may have been influenced in their livestock husbandry practices owing to their proximity to users. To reduce the challenge of spillovers, the study therefore also targeted three other dairy cooperatives within the same counties. These cooperatives had not participated in the iCow services and were identified approximately 15 kilometers from the dairy cooperatives that participated in the initial rollout of iCow services. These cooperatives were; Tarakwa, Miharati, and Ndanai in Uasin Gishu, Nyandarua, and Bomet, respectively and their members formed the sampling frame for non-users of ICow services.

In the second stage, respondents were randomly selected from the list of users of iCow service (members of the treated cooperatives) as well as non-user (members of control cooperatives where iCow services had not been piloted). The lists of members that formed the sampling frame in each case were obtained from the list of registered farmers as contained in the Kenya Dairy Farmers Federation (KDFF) registry. This resulted in a sample of 457 respondents, of which 209 farmers are regular users of iCow services, and 248 farmers were not enrolled in the platform.

Data were collected through personal interviews using a pre-tested questionnaire and administered using the Open Data Kit (ODK) platform. The household survey was conducted in June and July 2018, and information on farm-specific characteristics, farmers-specific characteristics, animal details, location characteristics, and household income were collected, the summary of which is contained in Table 1.

3. Results

3.1 Descriptive Analysis

Table 1 presents some of the descriptive statistics that reveal differences between users and non-users of iCow in outcome variables and other variables that have been used in the analysis. The t- values suggest that there are significant differences between the users of iCow and no-users with respect to milk production. Users realized higher average annual milk production per cow (2359.32 liters) as compared to non-users (1964.01 liters).

There are also significant differences in incomes earned by households with users of iCow services earning Ksh 50,625 and 132,031 more milk incomes and household income respectively than the non-users.

Table 1. Differences in means for users of iCow and non-users of iCow

Variable	Treated	Control	Differences
Outcome Variable			
Annual Milk production per cow (Litres)	2359.32	1964.01	395.31***
Annual Milk income (Ksh)	148277	97651	50625***
Annual Household income (Ksh)	411107	279075	132031***
Independent Variables			
Household head Education (Year)	09.85	8.35	1.49***
Household head Experience in Dairy farming (years)	12.78	13.00	-0.22
Household head Age (years)	43.77	44.87	1.09
Plot size under Dairy enterprise (Acres)	1.72	1.31	0.41***
Number of lactating cows	1.93	1.63	0.30
Number of breeds kept	1.15	0.97	0.17***
Distance from the farm to the road (Km)	2.46	1.63	2.75***
Membership period to Dairy cooperative(years)	7.54	6.54	1.00**
Household head Gender(1 = Male, 0 = Female)	0.76	0.70	0.05
Growing fodder(1 = Yes, 0 = No)	0.70	0.60	0.100**
Breed-type (1 = pure-exotic, 0 = Otherwise)	0.40	0.28	0.12**
Access to extension services (1 = Yes, 0 = No)	0.62	0 .54	0.07
Access to internet services(1 = Yes, 0 = No)	0.37	0.19	0.17***
Milking (1 = Yes, 0 = No)	0.96	0.93	0.02
Access to Credit services(1 = Yes, 0 = No)	0.31	0.25	0.06
Membership to other social groups(1 = Yes, 0 = No)	0.40	0.29	0.10**
Occupation (1 = Farmer, 0 = Otherwise)	0.71	0.73	-0.01
Marital status (1 = Married, 0 = Otherwise)	0.81	0.81	0.00
Household Decisionmaker (1 = Joint, 0 = Otherwise)	0.39	0.54	0.15***

Note. ***Significant at 1% level, ** Significant at 5% level and * Significant at 10% level.

On average, users of iCow had significantly more years of schooling; about ten years as compared to 8 years for the non-users. As for age, the average age of household head for users of iCow was 44 years while that of non-users was 45. The difference is, however, insignificant. At the time of this study, over 90% of the households had lactating cows, with each household keeping at least 2 lactating cows on average. Most of these cows were crossbreeds. With regards to membership to the dairy cooperative, most respondents interviewed belonged to dairy cooperatives, but iCow users had been members for significantly more extended periods than non-users of iCow services; average membership duration of 7 years for iCow users and 6 years for non-users. In terms of the gender of household head, most households were headed by males, even though there were significantly more male-headed households among users of iCow services (76%) than among non-users (70%).

On average, more iCow users had access to extension services (62%) compared to non-user (54%). This difference is, however, insignificant. These extension services are mainly provided by the extension agents belonging to the dairy cooperatives. With regards to accessing internet services, significantly more users of iCow users had access; about 37% compared to just 19% among non-users of iCow services. The low percentage across the board is probably due to the fact that most farmers live in remote areas where network connectivity is a challenge. Some of the farmers may also be in possession of phones that are unable to access internet services. These findings are similar to (Chilimo, 2009) who revealed that low network connectivity and low power supply are the main constraints that affect use of ICTs among farmers.

In relation to plot size under dairy enterprise, iCow users had significantly more acreage of their total land allocated to the dairy enterprise compared to the non-users. Additionally, more than 50% of the households interviewed grow their own fodder with nappier grass being a dominant fodder in the study regions.

3.2 Challenges Facing Both iCow Users and Non-users

Results from Figure 1 show various challenges both iCow users and non-users face in dairy production. Most farmers stated that feed shortages were the major challenge they faced, followed by livestock diseases and the high cost of feeds. Feed shortages can be explained by the fact most farmers in the study areas own small pieces of land that have been subdivided, making it a challenge to grow enough fodder. The reported high percentage of livestock diseases is because of the East Coast Fever (ECF) disease in the counties which has become a hindrance to optimal production and earnings among dairy farmers.

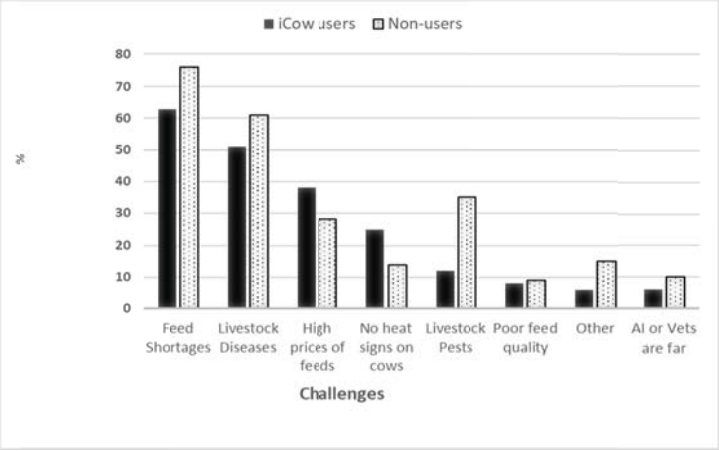


Figure 1. Various challenges dairy farmers face among the iCow users and non-users

Figure 2 presents results on the various iCow messages mainly put into practice once the farmers receive them. Most farmers reported that they had focused so much on practicing the tips on fodder management. This pattern can be explained in Figure 1, which pointed out that the significant challenge that both iCow users and non-users face is feed shortages. Therefore, most farmers focused on these messages by putting into practice the information that would give a solution.

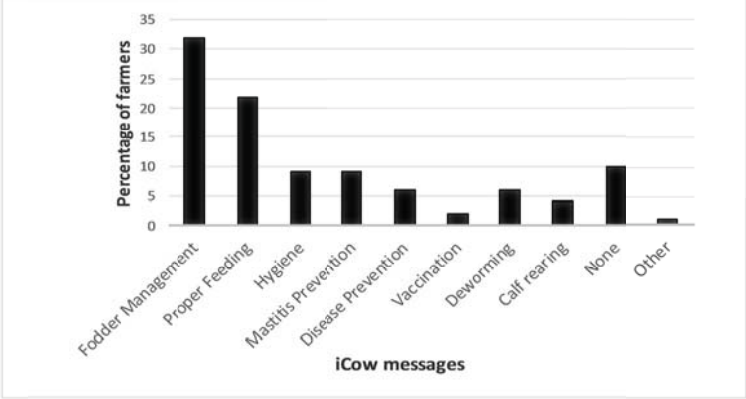


Figure 2. Various iCow words mainly put into practice by regular users of iCow

Results from Figure 3 shows that 57% of the farmers interviewed reported that hygiene had improved due to the information from iCow. Followed by 55% and 37% of the farmers stating that feeding had improved and had managed to control mastitis, respectively. These changes were highly noticed because, at many times, dairy farmers do suffer from lack of knowledge on nutrition, hygiene and how to control mastitis. With the information they receive from iCow, they were able to put it into practice resulting in observed changes.

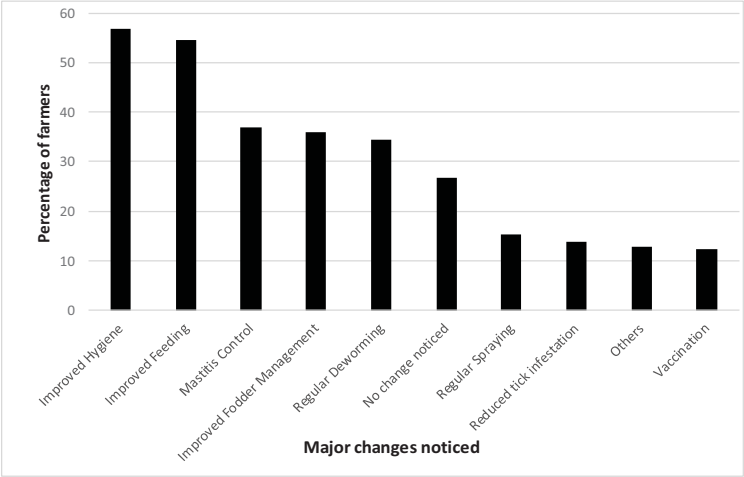


Figure 3. Changes noticed by iCow users after using the messages received from iCow

3.3 Impact of iCow Services

While the descriptive statics in Table 1 show differences in several variables between users and non- users of iCow services, one can hardly attribute the differences to the use of iCow services until the data is subjected to rigorous impact evaluation. In this sub-section, we discuss the results of the PSM estimation of the impact of iCow service on milk production, milk income, and household income. First, a logit model was employed to predict the probability of households participating in the iCow platform, results of which are presented in Table 2.

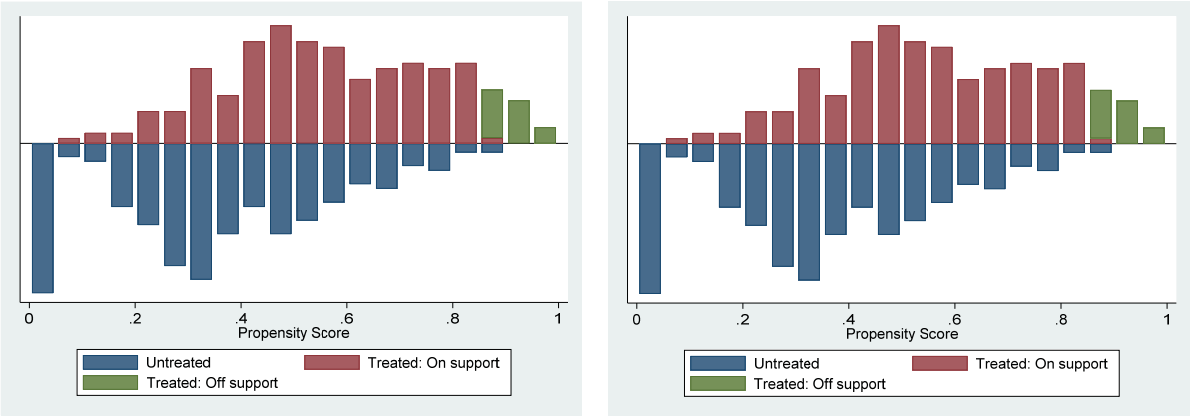
Table 2. A logit model of determinants of participation in the iCow program

Variable	Coefficient	dy/dx	S. E	P-value
Household head Gender(1 = Male, 0 = Female)	0.210777	0.0403317	0.256907	0.412
Household head Education (Year)	0.0762672***	0.0145936***	0.0261261	0.004
Household head Age (years)	-0.0103845	-0.001987	0.0080844	0.199
Experience in Dairy farming (years)	-0.0013415	-0.0002567	0.0130979	0.918
Growing fodder(1 = Yes, 0 = No)	0.1838024	0.0351702	0.2423104	0.448
Membership to Dairy cooperative(years)	2.983539***	0.5708932***	1.049018	0.004
Access to extension services(1 = Yes, 0 = No)	-0.0606871	-0.011612	0.2321239	0.794
Access to internet services (1 = Yes, 0 = No)	0.6520559***	0.1247694***	0.2548575	0.008
Milking (1 = Yes, 0 = No)	0.6696489	0.128135	0.5982336	0.263
Number of Lactating cows	0.3269762**	0.0625661**	0.1346353	0.012
Access to Credit services(1 = Yes, 0 = No)	0.1851316	0.0354245	0.2484814	0.456
Plot size under Dairy enterprise (Acres)	0.2873967***	0.0549927***	0.1149882	0.010
Membership to other social groups(1 = Yes, 0 = No)	0.4995711**	0.0955918**	0.2345287	0.030
Membership period to Dairy cooperative(years)	0.0436539**	0.0083531**	0.0215538	0.039
Occupation (1 = Farmer, 0 = Otherwise)	-0.3563509	-0.0681869	0.2763241	0.197
Marital status (1 = Married, 0 = Otherwise)	0.1434172	0.0274425	0.3003813	0.633
Household Decisionmaker (1 = Joint , 0 = Otherwise)	-0.4498008**	-0.08606**	0.2314903	0.048
Breed type (Local = 1, Otherwise = 0)	1.055652**	0.2019966**	0.4818823	0.025
Distance from the farm to the road (Km)	-0.0020488	-0.000392	0.0058846	0.728
Number of breeds	0.8289937*	0.158626*	0.4398011	0.056
Constant	-6.453247		1.325588	0.000
Log likelihood	-254.17437			
LR chi2(26)	120.63			
Prob > chi2	0.0000			
Pseudo R2	0.1918			
Observations	456			

Note. ***Significant at 1% level, ** Significant at 5% level and * Significant at 10% level.

Results from Table 2 indicate that several variables do influence the likelihood of households adopting the use of iCow services. In particular, level of education, membership to the dairy cooperative, access to internet services, number of lactating cows, land size under dairy enterprise, membership to other social groups, membership period to dairy cooperatives, breed type and number of breeds kept have a significant and positive effect on the adoption and use of iCow services. However, joint decision making at household level seems to affect the use of iCow services negatively.

Figure 4 shows the distribution of the estimated scores of PSM and the support region. The visual analysis showing the density of distribution for the treated and control groups as suggested by (Caliendo & Kopeinig, 2008) indicate that a majority of the treated and control individuals fall within the standard support region. In other words, most individuals had a positive probability of being users of iCow services. The support assumption (CSA) is, therefore satisfied, indicating that treated households had corresponding matches among the control households.



(a) Nearest Neighbour matching (b) Kernel-based matching

Figure 4. Distribution of the estimated propensity scores and the common support region

The results of the PSM model for quantifying the impact of iCow services on milk production and incomes are estimated with Nearest Neighbor Matching (NNM) and Kernel-based matching (KBM). In literature, various matching methods have been employed; however, in this study, we use the most common ones; Nearest neighbor matching (NNM) and Kernel-based matching (KBM), which are useful in checking the robustness of the results.Estimation results are presented in Table 3. We find that the use of iCow services has a significant and positive impact on milk production and household income. Based on the findings, we reject the null hypothesis that there is no significant difference between iCow users and non-users in terms of milk production and incomes and conclude that the iCow services had a significant influence on the stated outcome variables.

The average treatment effect on the treated (ATT) for the milk production per cow ranges between 298.15 liters (based on NNM) and 323.10 liters (based on KBM). The treatment effect is also significant at 5% for the NNM and at 1% for the KBM. The increment in milk production per cow can be attributed to iCow users being able to apply knowledge on husbandry practice that they acquire via use of iCow extension advise. These results are consistent with the findings by Das et al. (2016), which showed that use of ICT in accessing agricultural information increased production of rice in Bangladesh. Similarly, Hopestone (2014) and Ali et al. (2016) showed that use of ICT in agriculture has a positive impact on productivity.

We also find that the use of iCow services significantly increases milk income by between Ksh. 38,727 and Ksh. 38,309. It is likely that application of knowledge on livestock practices as advised via iCow services improves milk yield resulting in more surpluses for sale by farmers. The findings are similar to those of John and Barclay (2017) and Meydani (2017) who pointed out that the use of mobile phones among farmers in accessing agricultural information had positive impact on their income and productivity.

Additionally, impacts on annual household income are even high (Ksh. 62,381 to Ksh. 89,043) indicating a multiplier effect of iCow services. These results imply that revenue generated from dairy enterprise due to the use of iCow services are re-invested by households in further income-generating opportunities. A similar

observation was made by Halewood and Surya (2012), who showed that use of ICTs in accessing information led to increasing of farmers' income by up to 36% in countries such as Kenya, Ghana, Uganda, and Morocco. Also, Manyika et al. (2013) conclude that use of SMS by the Ethiopian Commodity exchange provided transparency on demand, supply, and prices and this increased farmers' share of revenue.

Following the results in Table 3, it can be argued that iCow positively influenced access to agricultural knowledge, leading to improved yields and increased surpluses that are sold for increased dairy income. This would subsequently impact household income depending on the role of dairy in a household income portfolio.

Table 3. Average treatment effects and results of sensitivity analysis

Outcome Variable	Matching algorithm	Treated	Control	ATT	SE	Bias (*gamma*)	Matched Observations		Total
							Treatment	Control	
Annual milk production per cow (Litres)	Neighbour matching	2337.89	2039.73	298.15 **	162.96	1.20-1.25	189	247	436
	Kernel matching	2337.89	2014.78	323.10 ***	133.45	1.35-1.40	189	247	436
Annual Milk income (Ksh)	Neighbour matching	140336	101608	38727***	11365	1.45-1.50	189	247	436
	Kernel matching	140336	102026	38309***	9990	1.40-1.45	189	247	436
Annual Household income (Ksh)	Neighbour matching	398907	336526	62381*	70984	-	189	247	436
	Kernel matching	398907	309863	89043**	44870	-	189	247	436

3.4 Testing for the Robustness of Results

3.4.1 Covariate Balancing

While the PSM procedure has the ability to control for selection bias, the estimates are only valid if two conditions are met: (i) balancing in covariates is achieved, and (ii) there is no systematic farmer heterogeneity due to unobservable (Caliendo & Kopeinig, 2008; Dehejia, 2002). The PSM estimation procedure aims to balance the distribution of variables relevant to the matching process in order to construct comparable groups. Balancing tests are therefore necessary after matching to establish if the matching process has indeed reduced the bias by eliminating differences in covariates. Only if this is achieved can the matched comparison group be considered as a plausible counterfactual (Caliendo & Kopeinig, 2008). We evaluate the balancing condition and bias reduction following (Rosenbaum & Rubin, 1985).

Table 4 presents the results of the covariates balancing, which was used to confirm the validity of the matching algorithms used to match the users of iCow and non-users. The results indicate that the majority of the covariates had insignificant differences after matching in all the matching methods as shown by the p-values under matched sample. This implies no systematic differences in these covariates, which confirms that a good counterfactual was generated by the matching process. Table 5 summarizes indicators of covariate balancing before and after matching. The results reveal substantial reduction of bias for both matching methods (78-89%). The pseudo R^2 and p-values of the likelihood ratio tests before and after matching are also presented in Table 5. The joint significance of regressors is rejected after matching, while it is not dismissed before matching. This underlines that systematic differences that are due to observable factors are properly eliminated.

3.4.2 Testing for Sensitivity Analysis

We also test for sensitivity of our results to hidden bias using Rosenbaum bounds (Rosenbaum, 1999; Hujer et al., 2004). Assuming two individuals have the same observed covariates z (as implied by the matching procedure), the two matched observations would differ in their odds of using the iCow services only by the difference in unobserved covariates, measured by the parameter Γ. The procedure involves changing the level of Γ and deriving the bounds on the significance levels of the ATT under the assumption of endogenous self-selection into the use of iCow services. This allows for identification of the critical levels of Γ at which the estimated ATT would become insignificant. The results of these bounding test are presented in Table 3 with the essential levels of gamma where the significant impact of iCow may be questioned.

Results show that the impact estimates are relatively insensitive to hidden bias in the outcome variables. For example, for the impact of iCow on milk production, the sensitivity analysis shows that at the gamma level of 1.20 to 1.40, ATT due to the use of iCow would need to be viewed critically. These critical values of gamma imply that if individuals who have similar observable covariates will differ in their odds of using iCow services due to unobserved heterogeneity by 20-40%, then the significant effect of iCow services on milk production may be questionable. The lowest critical value of gamma is 1.20-1.25, whereas the largest is 1.45-1.50. These

critical levels are pretty high. We can therefore conclude that our results are robust to unobserved heterogeneity among respondents.

Table 4. Results of covariate balance tests for propensity scores using NNM and KBM algorithms

Variables	Mean (NNM)					Mean (KBM)				
	Treated	Control	%Bias	%Reduction	p>|t|	Treated	Control	%Bias	%Reduction	p>|t|
Household head Gender (1 = Male, 0 = Female)	0.76	0.75	0.6	94.5	0.95	0.7619	0.72752	7.8	28.7	0.445
Household head Education (Year)	9.70	9.94	-5.1	84.4	0.62	9.709	8.9726	15.8	51.1	0.129
Experience in Dairy farming (years)	12.79	13.49	-7.3	-190.3	0.46	12.79	12.773	0.2	93.1	0.986
Growing fodder (1 = Yes, 0 = No)	0.69	0.60	20.7	10.2	0.04	0.69841	0.63998	12.3	46.4	0.228
Membership to dairy cooperative(1 = Yes, 0 = No)	0.99	0.99	1	98.2	0.75	0.99471	0.95955	13.6	75.8	0.022
Access to extension services (1 = Yes, 0 = No)	0.62	0.61	1.6	89.5	0.87	0.62434	0.57832	9.3	39	0.362
Access to internet services (1 = Yes, 0 = No)	0.34	0.37	-7.8	80.8	0.48	0.34392	0.24537	22.3	44.9	0.036
Milking (1 = Yes, 0 = No)	0.96	0.95	2.5	80.6	0.79	0.96296	0.95762	2.5	80.4	0.791
Number of Lactating cows	1.89	1.81	9.5	73.7	0.40	1.8942	1.7041	22.7	37.1	0.30
Access to Credit services (1 = Yes, 0 = No)	0.31	0.31	0.6	95.9	0.95	0.31746	0.28676	6.8	52.6	0.517
Plot size under Dairy enterprise (Acres)	1.47	1.52	-3.1	89.1	0.69	1.4788	1.3325	10.1	64.6	0.139
Membership to other social groups (1 = Yes, 0 = No)	0.38	0.39	-1.7	92.2	0.87	0.38624	0.33383	11	48.8	0.29
Membership period to Dairy cooperative (years)	7.67	7.53	2.5	87.1	0.81	7.672	6.7422	17.6	9.1	0.106
Occupation (1 = Farmer, 0 = Otherwise)	0.70	0.69	1.2	64.9	0.91	0.7037	0.72462	-4.7	-38.6	0.654
Marital status (1 = Married, 0 = Otherwise)	0.81	0.80	3.4	-259.4	0.74	0.81481	0.79727	4.5	-376.8	0.667
Decisionmaker (1 = Joint , 0 = Otherwise)	0.40	0.39	2.1	93	0.834	0.40212	0.45142	-10	67.2	0.334
Keep Dairy Local Breed (1 = Yes, 0 = No)	0.09	0.10	-2.7	93.5	0.797	0.09524	0.05704	12.9	68.7	0.162
Keep Dairy pure exotic Breed (1 = Yes, 0 = No)	0.38	0.317	14.6	46	0.162	0.38624	0.32719	12.5	53.6	0.232

Table 5. Results for statistical significance of matching algorithms

Matching Algorithm	Median Bias			Pseudo R2		p-Value of LR	
	Before Matching	After Matching	% Bias Reduction	Unmatched	Matched	Unmatched	Matched
Nearest Neighbour Matching	22.3	2.6	89	0.185	0.019	0.0000	0.938
Kernel-based Matching	22.3	10.6	78	0.185	0.058	0.0000	0.033

4. Conclusion and Policy Implications

The study employed PSM to examine the effects of the use of iCow services on milk production, incomes using cross-sectional data from dairy farmers in three counties of Kenya. Overall, the findings indicate that use of iCow services among dairy farmers has a positive and significant effect on milk production and income. The figures reveal that application of iCow services leads increase in milk production per cow, milk income and household income by 13%, 29% and 22% respectively, which can also be considered as an opportunity cost of not using iCow service. This positive impact shows the potential role of ICT-based extension in rural poverty reduction through increased household incomes. Therefore, these findings highlight the need to scale up the iCow services, due to its proven capacity of enhancing smallholder farmers' access to simple, timely information and digital solution, subsequently improving their production, incomes.

These findings also imply that ICT tools that enhance access and delivery of farm information should be integrated into the programs that aim at improving farm productivity and incomes. The positive correlation of use of phones in getting timely information among farmers suggests that policies should focus on improving infrastructure in the rural areas for the ICT usage: this includes, expansion of electrification programs for access to power for charging the ICT devices. Besides, there is also a need for development of mobile network coverage in the rural areas where the network is poor to facilitate exchange of information in uninterrupted manner.

Finally, partnerships between network providers and research institutes should be encouraged as part of bridging the extension gap occasioned by reduced public expenditure on extension services. It is through this that that research institutes will get the chance to refine the content of the e- extension approaches to meet the needs of farmers.

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Economic Analysis of Smallholder Maize Producers: Empirical Evidence From Helmand, Afghanistan

Hamidullah Elham[1], Jiajun Zhou[1], Mouhamadou Foula Diallo[1], Shakeel Ahmad[1] & De Zhou[1,2]

[1] College of Economics and Management, Nanjing Agricultural University, Nanjing, China

[2] China Center for Food Security Studies, Nanjing Agricultural University, Nanjing, China

Correspondence: De Zhou, College of Economics and Management, Nanjing Agricultural University, No. 1, Weigang, Xuanwu District, Nanjing 210095, China. Tel: 86-182-6007-5456. E-mail: zhoude@njau.edu.cn

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Abstract

Since war started at the end of 2001, the economy was severely devasted in Afghanistan, especially for the agriculture sector. Maize is the third most important cereal crop in Afghanistan, but the productivity of maize has a declining trend which may be caused by low efficiency of maize farmers nowadays. This study examines the production efficiency of maize producers and its important factors with the cross-sectional data form a multi-stage sampling survey of 250 maize producers in Helmand province in 2019. With the adoption of stochastic production frontier (SPF) model and production cost function, the paper gets the estimations of the average technical efficiency (0.737), allocative efficiency (0.65) and economic efficiency (0.568). The inputs, including land, labor, seed, fertilizer and pesticide/weedicides, have significant impacts on maize production and most of the farms exhibit an increasing return to scales. In addition, Tobit regression was applied to identify the influential factors of the production efficiencies for maize producers and the results indicate that education, family size, farm size, farming experience, contact to extension services and access to credit have significantly influence on the efficiency level. Finally, the study suggests that government should take some initiatives, such as extending the agricultural extension service, ensuring supply of high quality seeds and sufficient fertilizer with affordable prices and economical provision of mobile internet facility in remote areas, which will enhance the productivity and efficiency of the farmers and ultimately boost up their economic welfare and livelihood.

Keywords: maize production, technical efficiency, stochastic frontier analysis, Tobit model, Afghanistan

1. Introduction

Agriculture is the main contributor to the economy of Afghanistan as it accounted for 23% of the national GDP and employed 44% of labor force in 2017 (CIA, 2019). It also provides household income, food security and employment for more than 80% of the population in rural areas. However, agriculture sector is still associated with poor performance and not enough to feed the population. Since 2001, conflict with USA was emerged and political forces tried to establish a modernized economy. And there were drastic policy changes which would affect the agriculture sector tremendously, especially for the grain crops (Ahmadzai, 2017). But the value-added agriculture still declines since 2002.

In Afghanistan, maize is the third largest cereal crop and has a vital role in the developing economy of Afghanistan where the expanding population is still partially undernourished. When traditional wheat and rice become scarce, most of the people use maize as an alternative source of food. Therefore, maize is also important for the food security in Afghanistan as more than 54% population are living below the poverty line (CIA, 2019).

Maize productivity fluctuated and is decreasing in past years. In 2017, Afghan maize production was 0.174 million tons, 44% decrease of 0.312 million tons production in 2016 (FAOSTAT, 2017). The productivity of maize in Afghanistan is substantially lower than that in its neighboring countries (Table 1). The low productivity of maize is mainly caused by the lack of knowledge for efficient use of inputs and poor management skills. Given the importance of maize, the increase of maize productivity is very helpful for improving the food security in rural areas of Afghanistan. Thus, it is necessary for farmers to use the available resources in most efficient ways and to achieve a higher productivity in maize production and a better food security (Obaidi et al., 2012; Ngabitsinze, 2014).

Table 1. Production and productivity of maize in Afghanistan and its neighboring countries in 2017

Country/Region	Area harvested (million ha)	Yield (t/ha)	Production (million tons)
World + (Total)	197	5.75	1134
Asia + (Total)	67	5.37	361
Afghanistan	0.13	1.29	0.17
India	9.2	3.11	28.7
Iran	0.17	7.02	1.2
Pakistan	1.2	4.63	5.7

Source: FAOSTAT, 2017.

Agriculture sector is dominated by the small farmers in Afghanistan (Ngabitsinze, 2014). Most of the small framers hold very low productivity due to high inefficiencies (technical, allocative and economic). Some studies have identified the problems, such as lack of capital, fluctuated prices, shortage of crop storage facility, ineffective agronomic methods, poor management practices, low quality seeds, high cost of inputs and sudden changes in temperature, that affect the efficiency and result in low productivity of farming (Ahmadzai, 2017; Mangal et al., 2017; Rajiv Sharma, 2018). Several studies also highlighted many issues, like mismanagement practices at farm, high fragmentation of land, poor availability of credit, high costs of inputs, low adoption of advanced agricultural technology, low education level for farmers, lack of extension services, poor roads and infrastructure, are main impediments for the improvement of agricultural production in Afghanistan (Bell, 2012; Jilani et al., 2013; Maletta & Favre, 2003; Tavva et al., 2017; Thomas & Ramzi, 2011).

Increasing agricultural production and improving self-sufficiency of staple food have profound significance for the poverty elimination in Afghanistan and analyzing the efficiency of agricultural production, especially maize production is very important to reveal the approaches to improve the performance of the maize farmer. As one major maize production region, Helmand province encounters many restrictions including limited inputs and poor financial resources etc. (Saleem & Raouf, 2011; Sarhadi et al., 2014). The major objective of the present study is to evaluate the economic, allocative and technical efficiencies of maize producers in Helmand province in Afghanistan and explore the determinants of maize producers' inefficiency. In addition, this knowledge can help policymakers to reshape the policies and strategies to enhance the efficiency of maize production and improve the food security in Afghanistan.

The remaining parts of this paper are organized as follows: section 2 describes material and methods, while section 3 presents the empirical results and finally section 4 introduces the discussions. In the end, policy implications are concluded.

2. Materials and Methods

2.1 Study Area

The major maize cultivation region is the south-west region with 42% of total national maize production. Within this region, Helmand province was the second largest maize producer (Ministry of Agriculture, 2012) and maize is the largest crop produced in summer season with 60200 hectares in this province (DIAL, 2016). This research was conducted in Helmand province where maize was produced for both domestic consumption and export. Helmand Province is one of the largest provinces in Afghanistan, covering 58584 square kilometers. About 0.85 million population settle in this province and 94% of which are living in rural areas with poor literacy rate of only 5% and agriculture provides the main means of livelihood. In the past years, Helmand made a great contribution in agricultural production because of its organized irrigation canal system which was developed under the "Helmand River Valley Project". According to Ali Ahmad and George (2016), about 0.15 million hectares (70% of land) irrigated by the canal system.

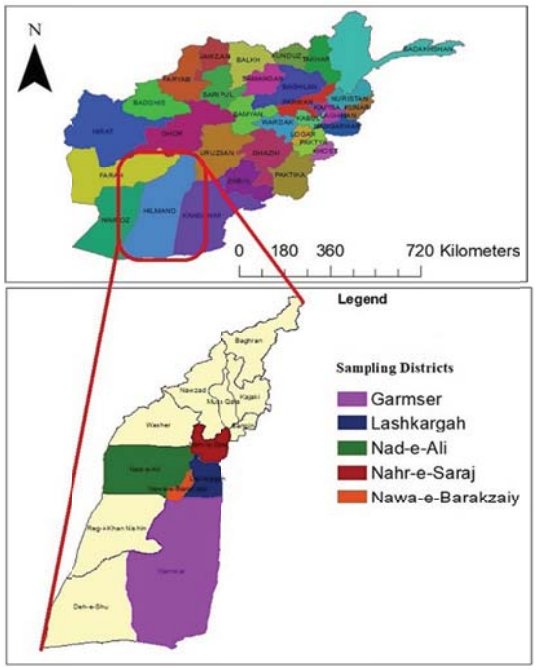

Figure 1. Map of study area

2.2 Sampling and Data Collection

The farm household survey was conducted in 2019 summer production season. 250 farmers were interviewed with multi-stage sampling technique. Four-stages were involved to select farmers in the study area: (1) 5 of 14 districts were selected in the southern part of the Helmand province as these (Nawa, Garamsir, NahreSarraj, Lashkar Gah, Nad Ali) districts are the main maize production areas (Table 2), (2) two units (towns) in each district were selected, (3) three villages were chosen from each unit, (4) 8-9 farmers who were producing maize were randomly selected in each village. The detailed information on socioeconomics and demographics of households, farm characteristics, inputs usage and outputs, institutional linkages and marketing were also collected.

Table 2. Maize production in Helmand Province in 2016

District	Maize Crop Area (ha)	Maize production (tons)
Nawa	7000	34300
Garamsir	7500	36750
Khanishin	2200	10780
Dishu	-	-
Lashkargah	4500	22050
Nad Ali	5000	24500
Marja	800	3920
Nahresiraj	13000	63700
Sangain	5000	24500
Musa Qala	1200	5880
Kajaki	2500	12250
Nowzad	6000	29400
Baghran	-	-
Washir	5500	26950
Total	60200	294980
Average yield per ha (tons)		4.9

Source: DAIL (Department of Agriculture, Irrigation and Livestock), 2016.

2.3 Empirical Model

Agricultural productivity can be measured as total output/input ratio. Productivity of maize crop can be improved with different means. For example, it can be enhanced by proper utilization of resources and improving the efficiency of maize producers. When it comes to the production efficiency, we followed the methodology of Stochastic Frontier Production function (SFP) to estimate maize production efficiency (Aigner et al., 1977). Cobb-Douglas Production Function for maize farmers was employed in this study.

$$\ln Y_i = \beta_0 + \sum_{n=1}^{5} \beta \cdot \ln X_i + (V_i - u_i) \quad (1)$$

where, Y_i represents the total output of i^{th} farmer; β is the vector of parameters to be estimated and X_i is the vector of inputs used for maize production, while V_i measure the random variation assumed to be an iid $N(0, \sigma_v^2)$ in Y_i due to the factors beyond the control of the farmer, and u_i is inefficiency error term which is a non-negative random variable associated with technical inefficiency of production and assumed to be an independent distribution of $N(u, \delta_u^2)$. Technical Efficiency (TE) of any farm could be defined as the ratio of observed output to the potential output defined by the frontier function. Following the Cobb-Douglas production function, TE can be measured with ratio of output values and estimated frontier values as follows.

$$TE_i = \frac{\exp(Y_i)}{\exp(\beta_0 + \sum_{n=1}^{5} \beta \cdot \ln X_i + V_i)} = \exp(-u_i) \quad (2)$$

By following the method of Efficiency Decomposition Technique (EDT) from Bravo-Ureta and Rieger (1991), on the basis of Equation 2 we can estimate the dual cost function and it will provide the basis to measure the economic efficiency (EE) and allocative efficiency (AE) of individual farm.

$$\ln C = \alpha_0 + \alpha_1 \ln C_1 + \alpha_2 \ln C_2 + \alpha_3 \ln C_3 + \alpha_4 \ln C_4 + \alpha_5 \ln C_5 + \varphi \ln Y_i + \mu_i \quad (3)$$

Where, C is the total cost of production for maize of farm *i*, C_1 is cost of land which is taken as the market price of rented land per hectare. C_2 is market price of labor per day, C_3 is the cost of fertilizer per kg, C_4 is the cost of seed per kg and C_5 is the total cost of other inputs such as pesticide, weedicides/herbicides per liter of market price. Whereas, the Y_i represents the output of maize in kg. We estimated the maximum likelihood parameters in stochastic frontier production function (SFP) and efficiency decomposition technique (EDT) with STATA 13.

Economic efficiency is the ability of farmers to use a minimum production cost of inputs while producing the maximum possible output, given the available technology. Bear in mind that economic efficiency is a product of technical efficiency and allocative efficiency (Abdulai , Nkegbe, & Donkor, 2017). Thus, the relationship is denoted as follows:

$$EE_i = TE_i \times AE_i \quad (4)$$

Where, EE_i is the economic efficiency of the ith farmer, TE_i is the technical efficiency of the ith farmer and AE_i is the allocative efficiency of the i^{th} farmer.

To estimate the impacts of demographic, socioeconomic, institutional linkages, farm characteristics and marketing variables on efficiencies, we regressed the efficiencies scores which are valued between zero and one on independent variables with Tobit model (Maddala, 1986; Dhungana et al., 2004; Ibrahim & Omotesho, 2013; Javed et al., 2008; Krasachat, 2004; Tobin, 1958).

$$E_i = E_i^* = Y^0 + \sum_{j=1}^{k} YZ_i + \varepsilon_i \quad (5)$$

Where, E_i represents the technical, allocative and economic efficiency respectively, E_i^* is the latent variable, γ is the vector of unknown parameters, Z_i = is vector of selected explanatory variables of i^{th} farmer and ε_i is the error term. The summary of all variables used in Tobit model is presented in Table 3.

Table 3. Descriptive summary of surveyed maize producers (n =250)

Variables	Unit	Description of Variables	Mean (S.D.)
Age	Years	Age of the farmer	43.92 (14.74)
Family Size	Numbers	number of persons in family	12.92 (4.12)
Education	Years	Formal Education in years	5.16 (4.72)
Farming Exp	Years	Farmer's Experience in maize production	19.69 (11.21)
Farm Size	Hectare	Total cultivation land	2.94 (1.42)
Liv Assets	Number of Heads	Total number of Animals on farm	4.48 (2.56)
CES	Number of times	Frequency of contact with extension officers	2.06 (0.07)
Access to Credit	Dummy	Access to get credit from banks or other sources	0.54 (0.03)
MFG	Dummy	Membership of Farmer Group	0.71 (0.03)
DtR	KM	Distance between farm and main road	1.45 (0.78)
DNM	KM	Distance from farm to the nearest market	4.33 (3.68)
Mobile/Internet	Dummy	Usage of Mobile or internet for information about production	0.64 (0.03)

3. Empirical Results

Estimations of SFP model and cost function are presented in Table 4. Results show that Land, seed, fertilizer, weedicides/pesticides and labor significantly affect the maize production. It also indicates that if labor increases just 1% maize output would increase 0.24%. The coefficients of remaining variables such as land, seed, fertilizer and weedicides are all positive which means more those inputs will lead the growth of maize production. The estimations of Likelihood ratio test, δ^2 and γ indicating that model was significant and the existence of inefficiency component. Returns to scale was 1.021 showing the increasing returns on the scale of maize production function.

Table 4. Maximum likelihood estimations of parametric SFP and cost function of maize production

Variables	ML Estimation of SPF Coef. (Std. Err.)	Variables	Estimation of Cost Function Coef. (Std. Err.)
Constant	2.98^{***} (0.291)	Constant	1.54^{**}
ln land	0.46^{***} (0.034)	$\ln C_{land}$	0.18^{***}
ln labor	0.24^{**} (0.028)	$\ln C_{labor}$	0.12^{**}
ln Seed	0.08^{***} (0.014)	$\ln C_{seed}$	0.19^{***}
ln Fertilizer	0.10^{***} (0.025)	$\ln C_{fertilizer}$	0.21^{***}
ln Weedicides	0.13^{***} (0.011)	$\ln C_{weedicides}$	0.10^{***}
Log-Likelihood	-238.14	$\ln C_{output}$	0.13^{***}
Returns on scale	1.021		
Sigma square $\delta^2 = \delta^2_u + \delta^2_v$	3.54^{***} (0.31)		
$\gamma = \delta^2_u/\delta^2$	0.64^{***} (0.05)		

Note. ***, **, and * denotes significance level at 1%, 5% and 10% respectively.

We also estimated the dual cost frontier function with which to measure the allocative (AE) and finally derived the economic efficiency (EE). All the variables in cost function include land, labor, seed, fertilizer and weedicides have positive and significant impacts on total cost of maize production. Particularly, 1% increase of cost of seed, fertilizer, land, output, labor and weedicides would increase the total production cost by 0.19%, 0.21%, 0.18%, 0.13%, 0.12% and 0.10% respectively.

Table 5. Summary statistics of estimated efficiencies

Type of Efficiency	Average	Maximum	Minimum	Std. Dev.
Technical Efficiency (TE)	0.737	0.971	0.216	0.163
Allocative Efficiency (AE)	0.652	0.932	0.392	0.964
Economic Efficiency (EE)	0.568	0.906	0.087	0.231

Table 5 presents the summary of all estimated efficiency—TE, AE and EE. The mean of TE for sampled farms is 0.737 with the range of [0.216, 0.971]. This implies that the average maize producer only attains 74% of potential output under the prevailing technology. Allocative efficiency is at an output level where the price equals the marginal cost (MC) of production. The average AE is 0.652 with a range of [0.392, 0.932]. It suggests that the maize farmers produced up to the average of 65% allocative efficiency point, and there is some space to optimize the inputs structure of maize production. Finally, with mean of 0.568, economic efficiency holds the interval of [0.087, 0.906], it is evident that there is a need to improve both technical and allocative efficiency in maize production in Helmand province. Particularly, the result suggests that a potential of 44% improvement of economic efficiency would be achieved by optimizing the inputs structure and improving production technical efficiency.

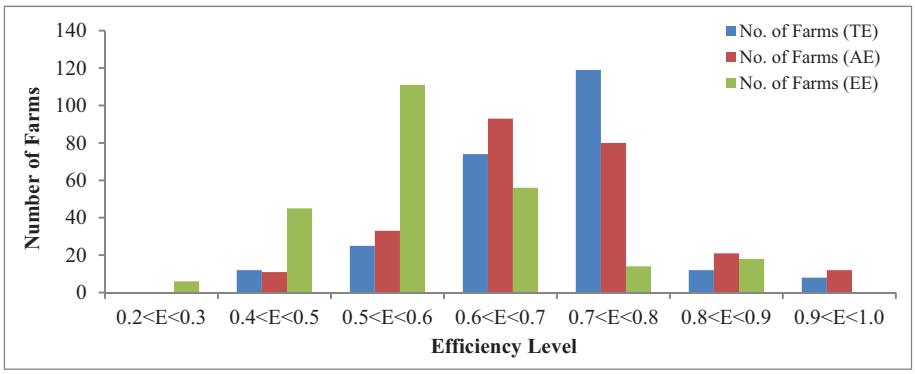

Figure 2. Distribution of technical, allocative and economic efficiency for farms in Helmand

The distribution of economic, allocative and technical efficiencies is presented in Figure 2. The technical efficiency of majority of farms (about 48%) locate to the category of $0.7 < E < 0.8$, while for more than 90 farmers, the allocative efficiencies are in the range of $0.6 < E < 0.7$. And 44% of the farms get economic efficiency of $0.5 < E < 0.6$. Moreover, less than 20 farmers achieve the higher level of AE, TE and EE. Thus, farmers have the potential to achieve the maximum yield of maize by improving their efficiencies.

The evaluations of the determinants of TE, AE and EE were also performed with the use of Tobit model. To achieve robust estimates, some verification tests were also conducted including multicollinearity and heteroscedasticity check with Variance Influence Factor (VIF) and Breusch-Pagan test respectively. The results indicate that VIF is of 2.85 which indicates there is non-existence of multicollinearity, while Breusch-Pagan chi-square values for EE, AE and TE are 1.84, 0.98 and 0.87 respectively, indicating that heteroscedasticity is not a problem in our models. Furthermore, Jargue-Bera test was applied to check for normality and the results (TE = 27.20, AE = 23.03 and EE = 20.83) verified that values of the efficiencies were normally distributed. The results of the Tobit models are presented in Table 6.

Table 6. Estimates of Tobit Regression Models

Variables	Technical Efficiency		Allocative Efficiency		Economic Efficiency	
	Coefficient	Std. Err.	Coefficient	Std. Err.	Coefficient	Std. Err.
Age	-0.003	0.002	-0.002**	0.001	-0.002	0.003
Family Size	0.002	0.004	-0.006**	0.003	-0.001	0.004
Education	0.004**	0.002	0.003***	0.001	0.002**	0.001
Farming Exp.	0.009***	0.003	0.004	0.003	0.005**	0.002
Farm Size	0.006***	0.002	-0.001	0.002	0.004***	0.001
Liv. Assets	0.002	0.004	0.002	0.004	0.001	0.002
CES	0.006	0.020	0.042**	0.018	0.024	0.017
Access to Credit	0.021***	0.002	-0.002	0.012	0.004	0.010
MFG	0.041***	0.015	0.029	0.019	0.037	0.024
DtR	-0.001	0.018	0.009***	0.003	-0.002	0.010
DNM	-0.002	0.010	0.004	0.022	-0.002*	0.001
Mobile/Internet	0.061***	0.019	0.047*	0.026	0.056*	0.030
Constant/intercept	0.032	0.021	0.030	0.051	0.067**	0.028

Note. ***, **, and * denotes significance level at 1%, 5% and 10% respectively.

According to the results, Age and household size negatively influence AE but are insignificant for TE and EE. This implies that older farmers and larger farmers were less efficient than their counterparts. The results also indicate that educated farmers hold higher technical, allocative and economic efficiencies and farming experience and farm size have positive and significant influences on TE and EE. This implies that farmers with a large land holding achieved higher levels of technical and economic efficiencies. Even though owning a large number of animals could provide manure, working power like oxen and household income, livestock asset (proxy of wealth status) is insignificant in the models. We also find that the frequency of contact with extension service agencies and distance between farm and main road increased farmers' allocative efficiency. The credit access and membership of farmer groups could improve technical efficiency. When it comes to the impacts of market, we find that those who are near the market get higher economic efficiency than the households that are far away from the market. Finally, mobile phone/internet usage empowers maize farmers to be more technically, allocatively and economically efficient.

4. Discussion

4.1 Stochastic Frontier Production Function, Cost Function and Efficiencies

The results indicate that land has positive relationships with maize production and cost which is consistent with the results from Ahmadzai (2017), and Debebe et al. (2015) who found that land had the largest effect on maize production in Ethiopia. We found that the most essential factor in maize production function is labor that is similar with the results from Sapkota et al. (2017), because most maize farmers in Afghanistan use labor-intensive methods in production. Regarding other inputs, several studies found that seed (Ng'ombe & Kalinda, 2015), fertilizer (Chirwa, 2007) and pesticides/weedicides (Debebe et al., 2015) also had significant and positive effects on maize output. It also performs the same way in the cost function (Abdulai et al., 2017; Debebe et al., 2015). Thus, taking the existing literature into consideration, the estimations in the present study are reasonable.

On average, the economic efficiency, allocative efficiency and technical efficiency for maize farmers are 57%, 65% and 73% respectively. As we mentioned before that Allocative efficiency (AE) is one indicator that the farmer could make a choice and utilize the inputs in maize production to reach a level where their factor prices equal marginal returns (Tijani, 2006). The results of the allocative efficiency indicate the inputs are either over-used or under-used in maize production in Helmand province. On the other hand, technical efficiency is the ability of a farmer to produce potential maximum output given a set of inputs and available production technology (Koopmans, 1951). The results reveal many maize farmers could produce at the level close to the potential production. Furthermore, improvement of AE and TE would greatly contribute the growth of EE. Thus, our results suggest that maize farmers in Afghanistan did not master the cost minimization in production which would lead the poor performance of maize production.

4.2 Determinants of Efficiencies

The results show that production efficiency tends to decrease as the age of farmers grows. Even though farmers acquire more experience in farming practice as time goes by and could efficiently utilize the resources for maize production (Amos, 2007; Chaovanapoonphol & Somyana, 2018; Msuya et al., 2008; Sibiko, 2012), farmers become less energetic as them get older and this affects their productivity which could be the reasonable case in our study. This result is in line with the finding from Taiwo et al. (2014) and Tavva et al. (2017) who found that efficiency in cassava and wheat production declined as the farmers got older. Amaza et al. (2006) and Chepng'etich et al. (2014) also reported that younger farmers are more energetic and keener to adopt advanced production technologies, thereby making them to achieve higher efficiency.

The household size is another important influential factor of the efficiency of maize production in Helmand province in Afghanistan. Particularly, larger families have enough labor forces to perform farming activities, especially for the labor-intensive maize production. This is consistent with the discourse that larger households have more domestic labor to deploy at the farm, especially in the time of labor shortage (Asefa, 2011; Aye & Mungatana, 2011; Debebe et al., 2015; Elibariki et al., 2008). However, we found that it negatively affects allocative efficiency, and this could be caused by the resource misallocation in productions such as overutilization of labor (Abdulai & Eberlin, 2001; Donkoh et al., 2013; Mwalupaso et al., 2019; Tavva et al., 2017).

Regarding education, most of the studies concluded that educated farmers are more efficient than their counterparts (Abdulai et al., 2017; Chaovanapoonphol & Somyana, 2018; Debebe et al., 2015; Mussaa et al., 2011; Nyagaka et al., 2010; Shehu et al., 2010; Tavva et al., 2017). Also, some researchers (Anang et al., 2016; Asante et al., 2014; Donkoh et al., 2013) reported contrary findings as they are more engaged in off-farm employments which may deter their farming activities. Our finding indicates that highly educated farmers could easily perceive instructions of maize production and are more apt to learn and adopt new skills and technologies in maize production and then get higher efficiencies.

Farming experience could increase the capacity of farmers in maize production. Hence, efficiency is positively influenced by the farming experience in present study. This is in line with the result of Abdulai et al. (2017), Gul et al. (2009), Mwalupaso et al. (2019), Okike et al. (2004), Olarinde (2011), and Sapkota et al. (2017) who found that experienced farmer can acquire better knowledge and skills to select the suitable farm equipment. When it comes to farm size, there are some studies identified that small farms hold higher production efficiency because of easy management and lower transaction cost (Adebanjo Otitoju & Arene, 2010; Amos, 2007; Elibariki et al., 2008; Idiong et al., 2009; Mwalupaso et al., 2019). Many other researchers found a positive relationship between efficiency and farm size as large farms are more efficient because modern agricultural techniques can easily be adopted and scale economies make them more productive (Chaovanapoonphol & Somyana, 2018; Chirwa, 2007; Endrias et al., 2010; Gul et al., 2009; Msuya et al., 2008). Our results also present positive impacts of farm size on efficiency.

Strikingly, institutional linkages like contact with extension service agencies perform a crucial role in the agricultural production and efficiency improvement for maize farmers in Afghanistan. More frequent contact with extensions services high likely provide farmers useful instructions and improve inputs allocation, cost reduction and augmented production (Abdulai et al., 2017; Aboki et al., 2013; Debebe et al., 2015; Peprah, 2010; Sapkota et al., 2017; Sibiko, 2012). However, the distance from the markets is one barrier for obtaining inputs and extension services which ultimately affects farmers' technical efficiency (Anang et al., 2016; Martey, 2019; Ng'ombe & Kalinda, 2015).

Credit access is pivotal for enhancing production efficiency and its impacts on TE are positive and significant, because financial credit enables farmers to extend and manage their farms and improves the agricultural production system (Chepng'etich et al., 2014; Nchare, 2007; Ng'ombe & Kalinda, 2015; Olarinde, 2011). Participation in farmers' organization/association/groups is another cardinal factor because farmers can enjoy the group benefits of scale economy, better extension services and access to inputs. The positive effect of membership of farmers group on efficiency level has also been reported in many studies that group membership reduced the cost of production and enhanced the productivity, profitability and production efficiencies (Aboki et al., 2013; Anang et al., 2016; Sudrajat et al., 2018). Finally, information technique adoption increases the potential production for adopting better practices and prevent farmers from making blind decisions which leads to the improvement in efficiency (Debebe et al., 2015; Sekabira & Qaim, 2017).

5. Conclusion and Policy Recommendations

Maize is the strategic crop and staple food in Afghanistan. However, its declining productivity in recent years poses a great threat to the poverty reduction and hunger eradication in the country. Therefore, this paper empirically examines the technical, allocative and economic efficiency of maize production with stochastic frontier analysis and cost function regression based on the data collected from Helmand province in Afghanistan. The results indicate that all the classical inputs significantly contribute to maize productivity, and fertilizer and seed have larger shares in the total cost of maize production. The average efficiency levels (EE = 0.56, AE = 0.65 and TE = 0.737) reveal that there is potential space to increase maize output with optimizing inputs structure and production technology adoption to improve the technical efficiency and allocative efficiency.

Furthermore, to scale up the efficiency levels, policies must be directed towards the positive determinants, such as education, farming experience, contact with extension services, farm size, membership of farmer group, access to credit and mobile phone/internet usage, as well as the those with negative influences, such as age and distance between local input/output market. Overall, the proper allocation and utilization of available resources (inputs) and advanced production technology should be prioritized to leverage the benefits maximization in maize production.

Specific measures for maize productivity improvement in developing country like Afghanistan should take the training and information for maize producers into consideration. Policymakers should also promote initiatives to make extension services for small farmers more effective. Furthermore, provincial administration should ensure the supply of quality seeds, and high prices of fertilizers must also be addressed through farmer inputs support program (FISP). Lastly, better internet and mobile networks are necessary for the availability of information on technologies and markets in rural areas which eventually could improve the productivity.

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Multivariate Approach in the Initial Development of Soybean as a Function of Co-inoculation and Micronutrients

Paloma Helena da Silva Libório[1], Ivana Marino Bárbaro-Torneli[2], Fabio Olivieri de Nobile[3], Fabiana Mota da Silva[1] & Sandra Helena Unêda-Trevisoli[1]

[1] Departamento de Produção Vegetal, Universidade Estadual Paulista, Jaboticabal, São Paulo, Brazil

[2] Agência Paulista de Tecnologia dos Agronegócios, Polo Regional Alta Mogiana, Colina, São Paulo, Brazil

[3] Centro Universitário da Fundação Educacional de Barretos, Barretos, São Paulo, Brazil

Correspondence: Paloma Helena da Silva Libório, Departamento de Produção Vegetal, Universidade Estadual Paulista, Via de Acesso Prof. Paulo Donato Castellane, s/n, 14884-900, Jaboticabal, São Paulo, Brazil. E-mail: paloma_liborio@hotmail.com

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Abstract

Co-inoculation in soybean is the mixed inoculation with bacterias of the genus *Bradyrhizobium* and *Azospirillum brasilense*. The applicability of this practice has become the subject of recent research that aims to overcome the main limitations of biological nitrogen fixation, obtained by traditional soybean inoculation with only *Bradyrhizobium*. This study investigated the interaction effects among cultivars, bacterium types, with and without micronutrients applied to the seeds on the initial developmental stages of soybean cultivars using multivariate analyses. The seedlings were cultivated in pots filled with soil in greenhouse conditions. The experiment was installed in the Alta Mogiana branch of the Paulista Agribusiness Technology Agency (APTA) in Colina, SP. The 32 treatments were arranged as 4 × 4 × 2 factorial with four soybean cultivars, four bacterium types, with and without micronutrients applied to the seeds. The evaluations were performed at 5, 8 and 32 days after sowing (DAS). The parameters analyzed in the pots showed that the cultivars behaved differently depending on the type of bacteria used. The co-inoculation promoted better nodulation and initial seedling growth in some cultivars. In general, cultivars without application of micronutrients were superior in terms of the parameters analyzed.

Keywords: *Azospirillum*, cluster analysis, cultivars, *Glycine max* (L.), mixed inoculation

1. Introduction

The soybean [*Glycine max* (L.) Merril] culture is among the most important cultures worldwide while being the main Brazilian commodity as well. The world production reached 369.32 million tons, with the United States and Brazil being the largest producers (USDA, 2018). In the 2018/19 harvest, Brazilian average yield was 3,182 kg ha^{-1} for a cultivated area of 35.8 million hectares (CONAB, 2019).

The soybean crop contributes significantly to the improvement of the productive systems since nitrogen is supplied to the soil via biological nitrogen fixation (BNF) besides the economic benefits generated by the grain production. Soybean is the best example in Brazil for the economic benefits provided by BNF since it generates an economy of approximately US$ 15 billion. In the countries where investment in research was not as strong, the biological process can only supply 50% of the demand, being complemented with nitrogen fertilization (Hungria et al., 2007).

Initially, the used turfus-type inoculants combined two of the four strains: *Bradyrhizobium elkanii* SEMIA 587 and SEMIA 5019 (29w), as well as *B. japonicum* SEMIA 5079 (CPAC-15) and SEMIA 5080 (CPAC-7) (Zilli et al., 2006; Santana et al., 2011). As the research advanced, liquid-type inoculants that are recommended for application in the sowing groove to avoid contact with other products have been developed and applied to the seeds (Santana et al., 2011).

Therefore, soybean cultivation is only economically feasible due to the inoculants containing *Bradyrhizobium* strains. The procedure should be done annually to maximize the benefits provided by the microorganisms, resulting in average yield increases of about 8% (Hungria & Nogueira, 2013).

Recent research on new technology for soybean culture has recommended using co-inoculation, which consists of adding more than one microorganism (*Azospirillum brasilense*) recognized as beneficial to the plants to maximize the gains obtained with BNF (Bárbaro et al., 2011; Hungria et al., 2013).

Also, to maximize the biological nitrogen fixation in soybean and increase yield, research to identify traits that help selecting more efficient genotypes regarding their symbiotic capacity should be considered by breeding programs. Therefore, it becomes necessary to understand better the interactions among different genotypes and bacteria involved in the process (Bárbaro et al., 2009). The multivariate analysis techniques are important tools for this type of study and have been widely used for the genetic analysis of the relationship between traits and existing genetic material (Iqbal et al., 2008). It is also noteworthy the need for research regarding the co-inoculation practice that has been recently confirmed as agronomical efficient in Brazil but the results in the literature still vary greatly (Gitti et al., 2012; Hungria et al., 2013; Zuffo et al., 2015; Zuffo et al., 2016).

This study evaluated the effects of the interaction among soybean cultivars, bacterium types with and without micronutrients on the early developmental stages of seedlings, using multivariate analyzes.

2. Materials and Methods

2.1 Description of the Experiment

Soil samples were collected from the experimental area cultivated with soybean in the Alta Mogiana branch of APTA, in Colina, SP. The soil samples were submitted to physico-chemical analysis, and counting of *Bradyrhizobium* bacteria and soil associative diazotrophic bacteria. The bacterial counts were performed in the Agricultural Microbiology Laboratory of UNESP, FCAV, in Jaboticabal, SP, following the recommendations of Dobereiner et al. (1995), and the results are detailed in Table 1.

Table 1. Results of the *Bradyrhizobium* and diazotrophic bacteria counts for the soil of the experimental area of APTA, Colina, SP, in the 2015/16 harvest

Sample	Soil moisture	Total bacteria	*Bradyrhizobium*	Diazotrophic Bacteria
	%	---------- CFU g^{-1} dry soil ----------		
APTA	11.63	8.23×10^6	2.14×10^7	1.1×10^6

Note. CFU: Colony Forming Unit.

The soil of the experimental area is a dystrophic Red Latosol. The results of the chemical and physical soil analysis are as follows: pH ($CaCl_2$) = 5.21; M.O. = 22.50 g dm^{-3}; CO = 13 g dm^{-3}; P = 18.54 mg dm^{-3}; K = 3.04 mmolc dm^{-3}; Ca = 18.67 mmolc dm^{-3}; Mg = 12.86 mmolc dm^{-3}; H + Al = 27.46 mmolc dm^{-3}; SB = 34.58 mmolc dm^{-3}; CTC = 62.04 mmolc dm^{-3}, and V = 55.73%, S = 3.57 mg dm^{-3}, Zn = 0.70 mg dm^{-3}, B = 0.18 mg dm^{-3}, Mn = 12.70 mg dm^{-3}, Cu = 0.45 mg dm^{-3}, Fe = 30.81 mg dm^{-3}; total sand = 804 g kg soil; clay = 150 g kg soil, and silt = 45 g kg soil, expressed as percentage: total sand = 80.40% (coarse sand = 55.50% + fine sand = 24.90%); clay = 15.00%, and silt = 4.50.

2.2 Material Used and Experimental Conduction

The soybean seedlings were planted in pots filled with the soil in the greenhouse in the Alta Mogiana branch of the Paulista Agribusiness Technology Agency (APTA). The 5-L plastic pots were filled with the properly corrected soil from the experimental area already cultivated with soybean. The soil was fertilized with N-P-K (4-20-20) at dosages calculated according to the chemical analysis of the soil.

The treatments were arranged as 4x4x2 factorial with four commercial cultivars of soybean: Brasmax Flecha IPRO, BMX Potencia RR, 5D634 RR and NS7338 IPRO, four types of bacteria [Control, *Bradyrhizobium* (traditional inoculation), *Azospirillum* and *Bradyrhizobium* + *Azospirillum* (Co-inoculation)], with and without cobalt and molybdenum micronutrients applied to the seeds, see Table 2.

Table 2. The 32 treatments investigated regarding the interactions among cultivars, types of bacteria with and without micronutrients

Treatment	Cultivar	Bacteria type	Micronutrients
1	Brasmax Flecha IPRO	Control	Without
2	Brasmax Flecha IPRO	Control	With
3	Brasmax Flecha IPRO	*Bradyrhizobium*	Without
4	Brasmax Flecha IPRO	*Bradyrhizobium*	With
5	Brasmax Flecha IPRO	*Azospirillum*	Without
6	Brasmax Flecha IPRO	*Azospirillum*	With
7	Brasmax Flecha IPRO	*Brady + Azos*	Without
8	Brasmax Flecha IPRO	*Brady + Azos*	With
9	BMX Potência RR	Control	Without
10	BMX Potência RR	Control	With
11	BMX Potência RR	*Bradyrhizobium*	Without
12	BMX Potência RR	*Bradyrhizobium*	With
13	BMX Potência RR	*Azospirillum*	Without
14	BMX Potência RR	*Azospirillum*	With
15	BMX Potência RR	*Brady + Azos*	Without
16	BMX Potência RR	*Brady + Azos*	With
17	5D634 RR	Control	Without
18	5D634 RR	Control	With
19	5D634 RR	*Bradyrhizobium*	Without
20	5D634 RR	*Bradyrhizobium*	With
21	5D634 RR	*Azospirillum*	Without
22	5D634 RR	*Azospirillum*	With
23	5D634 RR	*Brady + Azos*	Without
24	5D634 RR	*Brady + Azos*	With
25	NS 7338 IPRO	Control	Without
26	NS 7338 IPRO	Control	With
27	NS 7338 IPRO	*Bradyrhizobium*	Without
28	NS 7338 IPRO	*Bradyrhizobium*	With
29	NS 7338 IPRO	*Azospirillum*	Without
30	NS 7338 IPRO	*Azospirillum*	With
31	NS 7338 IPRO	*Brady + Azos*	Without
32	NS 7338 IPRO	*Brady + Azos*	With

The experiment was arranged as a randomized complete block design with the experimental plot being one pot with 8 seedlings, with four repetitions.

The seeds were treated with commercial liquid inoculants used in soybean culture at the following doses, 60 mL/50 kg seed of Biomax® Premium with *Bradyrhizobium* and 150 mL/20 kg seed of Biomax® Premium Maize with *Azospirillum brasilense* for grasses, as recommended by the manufacturer. Half the dose of both inoculants was used in the co-inoculation of seeds. A dose of 100 mL ha^{-1} of the product containing 1.5% Co and 15% Mo micronutrients was applied to the seeds before the inoculation.

A few days before sowing, all seeds were treated with 2 mL/kg seed of the Standak Top insecticide/fungicide, whereas the inoculants were applied last on the sowing day. In addition, some precautions were taken to ensure greater efficiency of inoculants, such as seed inoculation performed in the shade while the inoculant was uniformly distributed on all seeds.

2.3 Evaluations

At 5 and 8 days after sowing (DAS) following the recommendations of Brazil (2009), we evaluated the percent germination as well as the mass (MSR and MSPA) and length (CR and CPA) of the root and aerial shoot expressed as g $plant^{-1}$ and cm, respectively, at 8 DAS. The same parameters were also evaluated at 32 DAS, in addition to the number of nodes (NNOD) per $plant^{-1}$ and node dry mass (MNNOD) per mg $plant^{-1}$.

2.4 Statistical Analysis

The multivariate exploratory analyses were used to evaluate the means in the present study. Initially, the data were standardized following the equation $Z_{ij} = X_{ij} – X_j/S_j$, where: j = 1, 2, … p traits; i = 1, 2, … n, objects; X_j and S_j = mean and standard deviation of column j. Subsequently, two exploratory approaches were studied: principal component and non-hierarchical cluster analysis by the k-means method. The similarity between treatments was measured by the Euclidean distance and the mean binding/linkage between the clusters was performed by the Ward method, to determine the number of clusters previously. In the principal component analysis, the eigenvalues were extracted from the covariance matrix that generated the eigenvectors called principal components, which are determined from the characteristic equation of the matrix (Ferraudo, 2014).

For calculating the principal components, the variability was decomposed into four eigenvectors (principal components) constructed with the eigenvalues of the covariance matrix, which are linear combinations of the original variables seeking to maximize the relevant information (Hair et al., 2009).

For calculating the proportion of the total variance assigned to each principal component, we have the expression: $CP_h = ƛ_h/\text{trace (C)}\ 100$, where, C = covariance matrix of the standardized original data; ƛh = h-th characteristic root (eigenvalue) of Matrix C, Trace (C) = ƛ1 + ƛ2 ... + ƛh. Only eigenvalues ≥ 1 are considered since these components carry relevant amount of information from the original variables. On the other hand, eigenvalues < 1 have no relevant amount of information in the component. The correlation between traits/parameters and principal components was calculated using the equation: $r_{xj}\ (CP_h) = a_{ih}\sqrt{ƛ_h}/S_j$, where: S_j = standard deviation of the variable j; a_{ih} = coefficient of the variable j in the h-th principal component; ƛh = h-th characteristic root (eigenvalue) of the covariance matrix (Kaiser, 1958).

The non-hierarchical cluster analysis of the variables evaluated in the pots by the k-means method used a number of clusters previously determined for the calculation of the points representing the cluster centroids: $E = \Sigma^k_{k=1}\Sigma_{x1Eck}\ d(x_i, x_{ok})$, where, x_{ok} = cluster centroid Ck; d(xi, x_{ok}) = distance between points x_i and x_{ok}. The centroid can be either the mean or median of a group of points. The objective of k-means is to minimize the distance between each point and its centroid (Hair et al., 2009). All multivariate analyses were performed using Statistica software, version 10 (Stasoft, 2010).

3. Results and Discussion

Principal components analysis resulted in four principal components (PC), which explained 80.74% of the variance (Table 3).

Table 3. Eigenvector matrix and statistics of the twelve traits evaluated as a response to the interaction among cultivars, bacterium types with and without micronutrients, planted in pots and cultivated in the greenhouse

Eigenvalue number	Eigenvalue	Total Variance (%)	Accumulated eigenvalue	Cumulative variance (%)
1	4.277	35.640	4.277	35.640
2	2.284	19.036	6.561	54.677
3	1.893	15.776	8.454	70.453
4	1.234	10.285	9.689	80.738
5	0.704	5.867	10.393	86.605
6	0.580	4.833	10.973	91.438
7	0.335	2.789	11.307	94.227
8	0.226	1.887	11.534	96.114
9	0.193	1.610	11.727	97.724
10	0.155	1.295	11.882	99.020
11	0.093	0.772	11.975	99.792
12	0.025	0.208	12.000	100.000

The most important variables in discriminating PC1 treatments were CR32, MSPA32, MSR32, CPA8, CR8, MSR8 and G5, followed by PC2 with MSPA8, G5 and G8. The PC3 was explained by nodulation and PC4 by CPA 32 (Tables 3 and 4). Thus, all studied traits were correlated with the four principal components, becoming important to evaluate the initial development of soybean in this work. Toller et al. (2009) evaluated the parameters of BNF in 15 conventional soybean cultivars and reported higher node numbers and nodular dry matter for the cultivars IAC 23 and M-SOY5942. The cultivars BRS 133 and BRS 184 were highlighted for the

root dry mass whereas BRS 184, for the aerial shoot dry mass. The best correlation estimates were obtained for number of nodes x nodular dry mass and root dry mass x aerial shoot dry mass.

Table 4. Correlation coefficients between the traits and the four principal components that retained the greatest amount of information relevant to the studied treatments

Traits	CP1	CP2	CP3	CP4
CPA32	-0.417	0.008	-0.273	-0.772
CR32	-0.651	-0.114	-0.303	-0.451
NNOD32	-0.192	-0.210	-0.843	0.149
MSPA32	0.794	0.420	-0.179	-0.072
MSR32	0.833	0.370	-0.054	-0.032
MNOD32	-0.230	-0.243	-0.728	0.466
CPA8	-0.840	0.201	0.290	0.021
CR8	0.630	0.529	-0.096	-0.236
MSPA8	0.116	0.720	-0.569	-0.013
MSR8	-0.667	0.465	-0.081	-0.090
G5	-0.624	0.605	0.106	0.194
G8	-0.561	0.657	0.128	0.298

Note. G5 and G8 = germination percentage 5 and 8 DAS; CPA8 and CPA 32 = aerial shoot length; CR 8 and CR 32 = root length; MSPA8 and MSPA32 = aerial shoot dry mass; MSR8 and MSR32 = root dry mass; NNOD32 = number of nodes and MNOD32 = node dry mass.

Ferraudo (2014) defined as important the variables that have correlation values above 0.6 regardless of the signal, noting that correlations with equal signs indicate that the variables are positively correlated and those with different signs are negatively correlated. It is noteworthy that all the important traits in PC2 and PC3 are positively correlated, except for PC1 only, in which CR32, CPA8, MSR8 and G5 correlated positively with each other and inversely with the traits MSPA32, MSR32 and CR8.

The two-dimensional plane formed by the components PC1 (35.64%) and PC2 (19.04%) retained 54.68% of the original variance (Figure 1). The treatments are distributed on a coordinate factor plan considering the relationship between the variables.

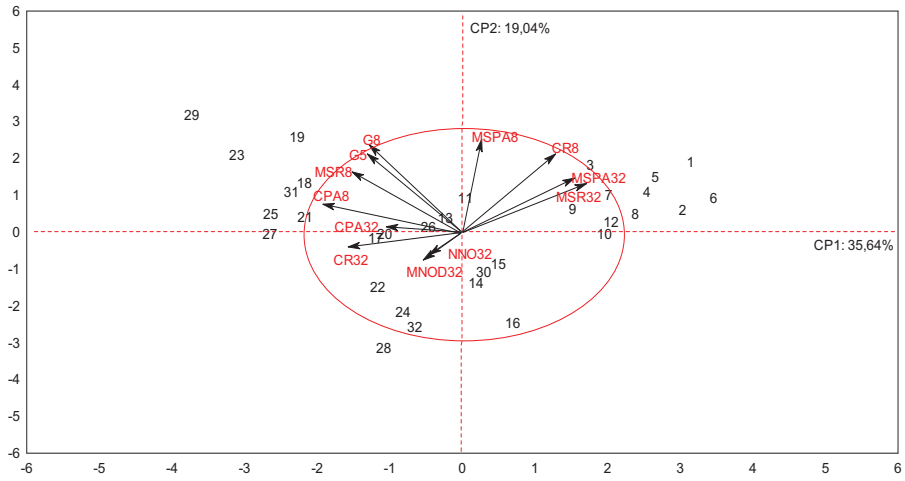

Figure 1. Biplot showing the dispersion of the 32 treatments using four soybean cultivars, four bacterium types, without and with micronutrients, for the principal components PC1 × PC2. G5 and G8 = germination percentage 5 and 8 DAS; CPA8 and CPA32 = aerial shoot length; CR8 and CR32 = root length; MSPA8 and MSPA32 = aerial shoot dry mass; MSR8 and MSR32 = root dry mass; NNOD32 = number of nodes and MNOD32 = node dry mass

The treatments located in the center of the plane, within the limits of the pre-defined scales, are not significantly different from one another so that the traits cannot be characterized as superior to one another in this amplitude range, making the group homogeneous. However, greater specificity for G5, MSR8 and CPA8 was observed for treatments 29, 23, 25, 31, 18 and 19 corresponding to NS7338 IPRO (*Azospirillum*, without micronutrients), 5D634 RR (*Brady* + *Azos*, without micronutrients), NS7338 IPRO (Control, without micronutrients), NS 7338 IPRO (*Brady* + *Azos*, without micronutrients), 5D634 RR (Control, with micronutrients), 5D634 RR (*Bradyrhizobium*, without micronutrients), respectively. The 25-NS7338 IPRO (Control, without micronutrients) and 27-NS7338 IPRO (*Bradyrhizobium,* without micronutrients) treatments were more specific for the CPA32 and CR32 traits (Figure 1).

The MSPA32 and MSR32 were higher for the treatments 1-Brasmax Flecha IPRO (Control, without micronutrients), 2-Brasmax Flecha IPRO (Control, with micronutrients), 3-Brasmax Flecha IPRO (*Bradyrhizobium*, without micronutrients), 4-Brasmax Flecha IPRO (*Bradyrhizobium*, with micronutrients), 5-Brasmax Flecha IPRO (*Azospirillum*, without micronutrients), 6-Brasmax Flecha IPRO (*Azospirillum*, with micronutrients) and 8-Brasmax Flecha IPRO (*Brady* + *Azos*, with micronutrients) (Figure 1).

Likewise, Bohrer and Hungria (1998) reported marked differences among cultivars regarding nodulation potential and BNF and concluded that the amount of shoot dry mass is a good parameter for selecting the most promising soybean symbioses.

The two-dimensional plan (PC1 × PC3) retained 51.42% of the variance, with treatments 29-NS7338 IPRO (*Azospirillum*, without micronutrients), 25-NS7338 IPRO (Control, without micronutrients), 18-5D634 RR (Control, with micronutrients), 21-5D634 RR (*Azospirillum*, without micronutrients), better discriminated for the CPA8 trait. The traits CR32, CPA32, NNOD32 and MNOD32 had the best results for the treatments 23-5D634 RR (*Brady* + *Azos*, without micronutrients), 27-NS7338 IPRO (*Bradyrhizobium*, without micronutrients), 31-NS7338 IPRO (*Brady* + *Azos*, without micronutrients) and 19-5D634 RR (*Bradyrhizobium*, without micronutrients) (Figure 2).

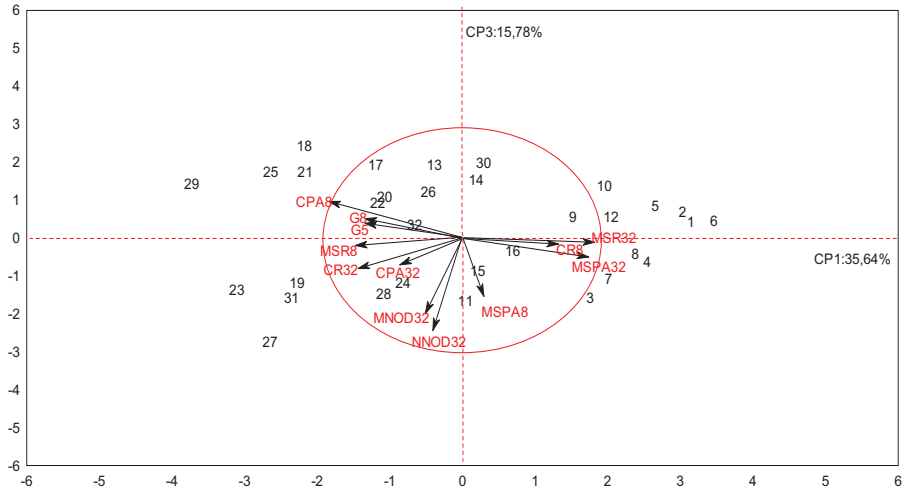

Figure 2. Biplot showing the dispersion of the 32 treatments with four soybean cultivars, four bacterium types, without and with micronutrients, for the principal components PC1 × PC3. G5 and G8 = germination percentage 5 and 8 DAS; CPA8 and CPA32 = aerial shoot length; CR8 and CR32 = root length; MSPA8 and MSPA32 = aerial shoot dry mass; MSR8 and MSR32 = root dry mass; NNOD32 = number of nodes and MNOD32 = node dry mass

The best results for MSR32 were observed for the treatments 10-BMX Potencia RR (Control, with micronutrients), 12-BMX Potencia RR (*Bradyrhizobium*, with micronutrients), 5-Brasmax Flecha IPRO (*Azospirillum*, without micronutrients), 2-Brasmax Flecha IPRO (Control, with micronutrients), 1-Brasmax Flecha IPRO (Control, without micronutrients), 6-Brasmax Flecha IPRO (*Azospirillum*, with micronutrients). The treatments 8-Brasmax Flecha IPRO (*Brady* + *Azos*, with micronutrients), 4-Brasmax Flecha IPRO (*Bradyrhizobium,* with micronutrients), 3-Brasmax Flecha IPRO (*Bradyrhizobium*, without micronutrients) and

7-Brasmax Flecha IPRO (*Brady + Azos*, without micronutrients) were discriminated by CR8 and MSPA32 (Figure 2).

Finally, the two-dimensional plane formed by the components PC1 (35.64%) and PC4 (10.29%) retained 45.93% of the original variance (Figure 3). Treatments 31-NS7338 IPRO (*Brady + Azos*, without micronutrients) and 18-5D634 RR (Control, with micronutrients) were more specific for G8 and G5, respectively. Whereas the treatments 29-NS7338 IPRO (*Azospirillum*, without micronutrients), 27-NS7338 IPRO (*Bradyrhizobium,* without micronutrients), 23-5D634 RR (*Brady + Azos*, without micronutrients), 25-NS7338 IPRO (Control, without micronutrients) and 19-5D634 RR (*Bradyrhizobium,* without micronutrients) were more specific for the traits CPA8, MSR8 and CR32.

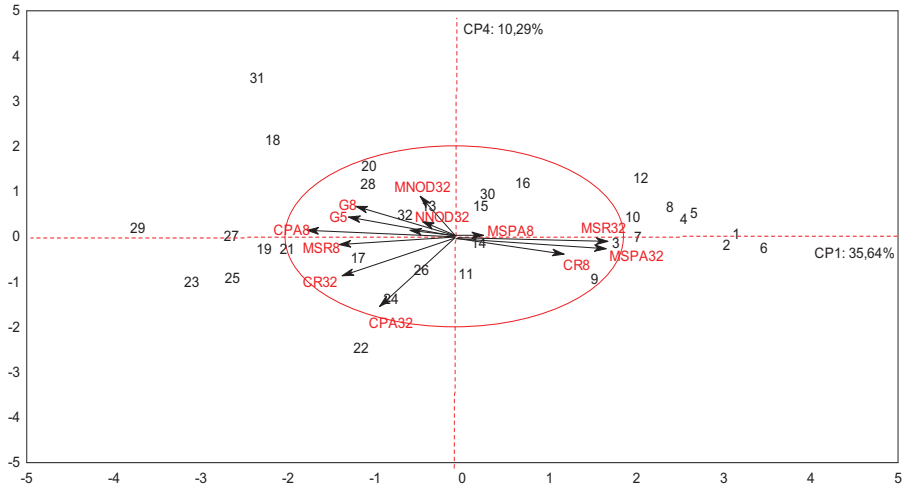

Figure 3. Biplot showing the dispersion of the 32 treatments using four soybean cultivars, four bacterium types, without and with micronutrients, in pots for the principal components PC1 × PC4. G5 and G8 = germination percentage 5 and 8 DAS; CPA8 and CPA32 = aerial shoot length; CR8 and CR32 = root length; MSPA8 and MSPA32 = aerial shoot dry mass; MSR8 and MSR32 = root dry mass; NNOD32 = number of nodes and MNOD32 = node dry mass

For CPA32, the treatment 22-5D634 RR (*Azospirillum*, with micronutrients) was better discriminated. On the other hand, the treatments 12-BMX Potencia RR (*Bradyrhizobium*, with micronutrients), 8-Brasmax Flecha IPRO (*Brady + Azos*, with micronutrients), 10-BMX Potencia RR (Control, with micronutrients), 4-Brasmax Flecha IPRO (*Bradyrhizobium*, with micronutrients), 7-Brasmax Flecha IPRO (*Brady + Azos*, without micronutrients), 5-Brasmax Flecha IPRO (*Azospirillum*, without micronutrients), 2-Brasmax Flecha IPRO (Control, with micronutrients), 1-Brasmax Flecha IPRO (Control, without micronutrients), and 6-Brasmax Flecha IPRO (*Azospirillum*, with micronutrients) performed better for the traits MSR32 and MSPA32.

The great variability observed among soybean-nodulating strains, regarding the efficiency of the symbiotic process (Araújo & Hungria, 1999), generates different interactions between bacteria and soybean genotypes (Bohrer & Hungria, 1998), corroborating the results shown in the 3 dispersion biplots of the different treatments (Figures 1, 2 and 3) in this work.

The 32 treatments and 12 traits evaluated previously by the Ward hierarchical clustering method formed nine clusters at the 18.18% cutoff limit in the dendogram, marked by abrupt level changes. This assumption was used in the non-hierarchical k-means method (Figure 4).

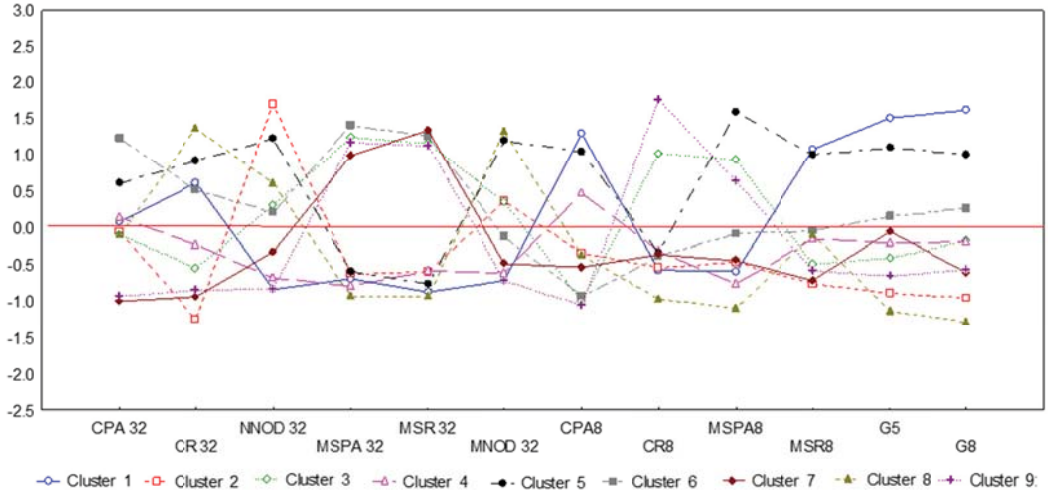

Figure 4. Distribution of the cluster centroids for the k- means clustering analysis obtained from the traits: G5 and G8 = germination percentage 5 and 8 DAS; CPA8 and CPA32 = aerial shoot length; CR8 and CR32 = root length; MSPA8 and MSPA32 = aerial shoot dry mass; MSR8 and MSR32 = root dry mass; NNOD32 = number of nodes and MNOD32 = node dry mass

Cluster 1 was formed by treatments 18-5D634 RR (Control, with micronutrients), 21-5D634 RR (*Azospirillum*, without micronutrients), 25-NS7338 IPRO (Control, without micronutrients), 29-NS7338 IPRO (*Azospirillum*, without micronutrients) and characterized by higher mean values of CPA32, CR32, CPA8, MSR8, G5 and G8 (Figure 4).

The treatment 15-BMX Potencia RR (*Brady + Azos,* without micronutrients) formed cluster 2, highlighting the nodulating parameters due to the high values of NNOD32 and MNOD32. Cluster 3 grouped the treatments 3-Brasmax Flecha IPRO (*Bradyrhizobium*, without micronutrients), 4-Brasmax Flecha IPRO (*Bradyrhizobium*, with micronutrients), 7-Brasmax Flecha IPRO (*Brady + Azos*, without micronutrients) and 8- Brasmax Flecha IPRO (*Brady + Azos*, with micronutrients), which was also characterized by CR8, MSPA8, MSPA32, MSR32, NNOD32 and MNOD32.

Cluster 4 grouped the treatments 13-BMX Potencia RR (*Azospirillum*, without micronutrients), 14-BMX Potencia RR (*Azospirillum* with micronutrients), 16-BMX Potencia RR (*Brady + Azos*, with micronutrients), 17-5D634RR (Control, without micronutrients), 20-5D634RR (*Bradyrhizobium*, with micronutrients), 22-5D634RR (*Azospirillum*, with micronutrients), 26-NS7338 IPRO (Control, with micronutrients) and 30-NS7338 IPRO (*Azospirillum*, with micronutrients) that displayed only CPA values slightly above average at 8 and 32 DAS.

Cluster 5, formed by the treatments 19-5D634RR (*Bradyrhizobium*, without micronutrients), 23-5D634RR (*Brady + Azos*, without micronutrients), 27- NS7338 IPRO (*Bradyrhizobium*, without micronutrients) and 31-NS7338 IPRO (*Brady + Azos*, without micronutrients), had the best results since the values were above the average for most of the evaluated traits CPA32, CR32, NNOD32, MNOD32, CPA8, MSPA8, MSR8, G5 and G8.

The application of cobalt and molybdenum by way of seeds can dramatically affect the survival of bacteria in standard inoculation (Campo et al., 2010; Santana et al., 2011), the same being verified in cluster 5 for *Azospirillum* and co-inoculation.

Cluster 6 formed by treatments 9-BMX Potencia RR (Control, without micronutrients) and 11-BMX Potencia RR (*Bradyrhizobium*, without micronutrients) had better results for the traits CPA32, CR32, MSPA32 and MSR32 and values slightly above the mean for NNOD32, G5 and G8. Cluster 7 formed by 10-BMX Potencia RR (Control, with micronutrients) and 12-BMX Potencia RR (*Bradyrhizobium*, with micronutrients) was characterized by high MSPA32 and MSR32.

Cluster 8 was composed of treatments 24-5D634RR (*Brady + Azos*, with micronutrients), 28-NS7338 IPRO (*Bradyrhizobium*, with micronutrients) and 32-NS7338 IPRO (*Brady + Azos*, with micronutrients) that had high values of CR32, NNOD32 and MNOD32. Finally, cluster 9 grouped 1-Brasmax Flecha IPRO (Control, without micronutrients), 2-Brasmax Flecha IPRO (Control, with micronutrients), 5-Brasmax Flecha IPRO (*Azospirillum*,

without micronutrients) and 6-Brasmax Flecha IPRO (*Azospirillum*, with micronutrients) with adequate values of MSPA32, MSR32, CR8 and MSPA8.

Therefore, the results show that the exploratory analyses of the principal components and k-means clustering were efficient and constitute a great tool to classify treatments that evaluate multiple traits, agreeing with several research works that used this technique for studying soybeans. There are reports in the literature stating that multivariate analysis allow identifying with good reliability the most important agronomic traits among many studied traits (Dallastra et al., 2014; Reina et al., 2014 Singh et al., 2020).

4. Conclusions

The multivariate analyses discriminate efficiently the different treatments tested.

The cultivars behaved different while the most promising interactions among cultivars, bacteria and micronutrients were observed for the 5D634RR and NS7338 IPRO cultivars in the standard inoculation (*Bradyrhizobium*, without micronutrients) and co-inoculation (*Brady + Azos*, without micronutrients) treatments.

In general, cultivars without application of micronutrients were superior in terms of the parameters analyzed.

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Adoption of Certified Seed and Its Effect on Technical Efficiency: Insights From Northern Kazakhstan

Muratbek Baglan[1,2], Xue Zhou[1,2], Gershom Endelani Mwalupaso[3] & Geng Xianhu[1,2]

[1] College of Economics and Management, Nanjing Agricultural University, Nanjing, PR China

[2] China Center for Food Security Studies, Nanjing Agricultural University Nanjing, Nanjing, PR China

[3] School of Natural Resources, Copperbelt University, Kitwe, Zambia

Correspondence: Geng Xianhu, College of Economics and Management, Nanjing Agricultural University, No. 1 Weigang, Nanjing, 210095, PR China. E-mail: gengxh@njau.edu.cn

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Abstract

Despite the economic and food security importance of the Kazakh wheat sector, current statistics suggest a yield gap between actual and potential yields. In view of this, farmers, stakeholders and the government are looking for agricultural technologies to increase the output. To this end, adoption of certified seeds is being promoted. The reasoning is that certified seed is produced from seed of known genetic origin and genetic purity, in a controlled and tested manner, processed and declared in accordance with the Law on Seeds and thus, could aid in producing maximum obtainable output. Unfortunately, little is known on whether this could affect wheat production and technical efficiency more than the conventional seed as such a subject has never benefitted from empirical analysis. To begin to fill this research gap, data from smallholder farms in Kazakhstan is used to evaluate the impact of adoption on technical efficiency by applying the stochastic production frontier. Results indicate that adoption of certified seed has productivity effects. Precisely, adopters are 20% more efficient than their counterparts. To a large extent, this is attributable to the quality of seeds used. Therefore, our study demonstrates the importance of certified seed adoption and accentuates the role governments can play in ensuring seed quality for enhanced technical efficiency.

Keywords: technical efficiency, stochastic frontier analysis, wheat, certified seed, Kazakhstan

1. Introduction

Agriculture is one of the prime movers of the national economy in most developing countries (Babu & Pinstrup-Andersen, 2000; Baydildina et al., 2000). For instance, in Kazakhstan, the wheat industry contributes substantial economic and social benefits. As a matter of fact, wheat accounts for 80% of total cropping land, making Kazakhstan the third largest wheat grain producer and the second largest net exporter among the Balkan sea countries (Pomfret, 2014). Particularly, its involvement in wheat production and export potential makes the country unique among central Asian countries because wheat is a strategic crop for food security and livelihood improvement. Since wheat is a dominant crop, any change in production is more likely to affect the producers' welfare and national economy (Satybaldin, 1998). In view of this, the government and development partners have been emphasizing the need for increased adoption of modern agricultural technologies to enhance productivity (Adesina & Zinnah, 1993; Admassie & Ayele, 2004; Boothby, Dufour, & Tang, 2010). In this regard, use of certified seeds is held as priority and prominent in their campaigns.

Different from other Asian countries, Kazakhstan seed system is strongly managed by the state under agricultural and research institute (FAO, 2013). The system has existed from 1992 and is quasi-monopolized with an inclusion of Scientific Research Institutes (SRI) which provides and inspects quality of certified seed. In addition, agricultural research institutes and universities are the sources for the pre-basic and basic seed. Kazakhstan seed

sector is administered and controlled by legislation and rules that were approved by representative parliaments. Seed certification and quality control services are provided by the public sector throughout the region. The inspection of seed producers' fields with some decentralized laboratories, control of the quality of seed marketed and inspection of processing plants remains the responsibility of the state (Abou-Jawdah, Sobh, & Saad, 2001; Auriol & Schilizzi, 2003). All administrative regions and district level decentralized laboratories report directly to the Ministry of Agriculture. Also, all development stakeholders in the region are sensitized on the importance of improved seed in contributing to boost agricultural productivity and production. The principal seed policy has been to ensure a continuous and wide use of high quality seeds. Therefore, in an attempt to incite adoption of yield-increasing technologies, the government of Kazakhstan has been promoting and actively participating in certified seed validation.

The discouragement of uncertified seeds is motivated by the fact that: (i) prolonged use leads to a decline in fertility (Jørgensen, Hauser, & Jørgensen, 2007); (ii) often infected with warehouse insects and diseases (Gaines, Preston, Byrne, Henry, & Westra, 2007); (iii) contain more weeds, broken seeds, and empty shells and (Gaines et al., 2007; Jørgensen et al., 2007); (iv) can only be protected with lower quality pesticides (Gaines et al., 2007). However, up till now, there is limited proof to validate whether use of certified seeds improves technical efficiency (the ability to produce maximum obtainable output from a given set of inputs under an existing technology). Thus, to a great extent, the lack of empirical evidence may have obstructed the formulation of policies capable of simultaneously addressing the current volatility in wheat productivity and rural development (Brown & Miller, 2008).

For the last two to three decades, a number of in-depth improved agricultural technology adoption studies have been conducted and agricultural transformation strategies were designed. Majority of the technological input studies focused on the promotion of improved crop varieties adoption to boost productivity and household income of rural poor farmers. Mathenge, Smale, and Olwande (2014), found that adoption of high yield variety of selected cereals (Maize, wheat and rice) impacts on household welfare and poverty reduction in Asia and Latin America. Langyintuo and Mungoma (2008) also add that to realize rural transformation and production enhancement, input technology change is a fundamental element. Thus, the assertion is that households who use ordinary seed have more challenge on food security and poverty than those who use improved seed. Likewise, Shiferaw, Kebede, Kassie, and Fisher (2015), support this logic and stated that, introducing new agricultural technology can benefit poor subsistence farmers and more educated farmers by providing basic information to enhance productivity. However, the major weakness of the aforementioned studies is the failure to state whether the improved seeds were certified and if so how the certification system was operated. Also, the methodological discrepancies of failing to account for biases from observed variables in estimating productivity effects.

Therefore, to bridge the research gap, this present study investigates whether farmers indeed adopt certified seed and the impact it has on technical efficiency. The results are very essential for policymakers because wheat is a staple food for most developing countries and as such measures that scale up the efficiency in production are pivotal for attaining zero hunger and poverty reduction. Particularly for Kazakhstan, since majority of the population live in rural area, increasing productivity to address food security is highly prioritized and considered as solution.

Consequently, the study contributes to the literature in two ways. First, we attempt to establish the adoption intensity of certified wheat seeds. This is very important because availability of agricultural innovations does not necessarily mean farmers are making use of it to leverage the benefits from espousal. Second, we employ a robust empirical strategy that addresses endogeneity, an element that leads to biased estimation which eventually makes policy formulation not cogent enough for the intended objectives. Given the need for agriculture to augment food production, ensuring that policy is informed using robust estimates is mandatory.

The remainder of the paper is structured as follows: Section 2 presents the materials and methods, Section 3 presents results and its concomitant discussion and Section 4 concludes and provides policy recommendations.

2. Materials and Methods

2.1 Study Site

The study was conducted in Kazakhstan's northern region (Akmola, Kostanay and North Kazakhstan) (Dara et al., 2018). The country has a population of 18,497,318 and is the ninth largest country in the world (2,717,300 sq. km). Agriculture is one of the leading branches of the economy, and the share of the agricultural sector in the Gross Domestic Product (GDP) is approximately 5-6%. More than 74% of the country's territory is suitable for agricultural production, but only 25% of the land is arable. The primary agricultural products are wheat, corn, rice, oats, cotton, potatoes, vegetables, sugar beets, and sunflowers.

Interestingly, Kazakhstan is one of the major producers and exporters of wheat in the world (Pavlova, Varcheva, Bokusheva, & Calanca, 2014).

The selected study area produces more than 80% of the wheat in the country (Figure 3). There are three prevalent groups of agricultural producers in the country, large agricultural enterprises, smaller individual farms mostly engaged in grain production, and tiny household economies focused on vegetables and livestock. Regarding wheat production, smallholder farmers dominate. The country has two planting seasons for wheat—spring and winter. The production is mostly rain fed, and the continental climatic condition has a range of annual precipitation from 150-400 mm. Additionally, on the grounds that the wheat grain has been deemed to be of poor quality, several certified wheat varieties have been released by Red Fall Experimental Station since 2000, including Naz, Sapaly, Yubilieynaya 60, Akterekskaya, Almaly, Alia, Egemen, Nureke, Ramin, Karasay, Maira, Konditerskaya, Rassad, Mereke 70, Farabi, and Alatau (Abugalieva & Peña-Bautista, 2010).

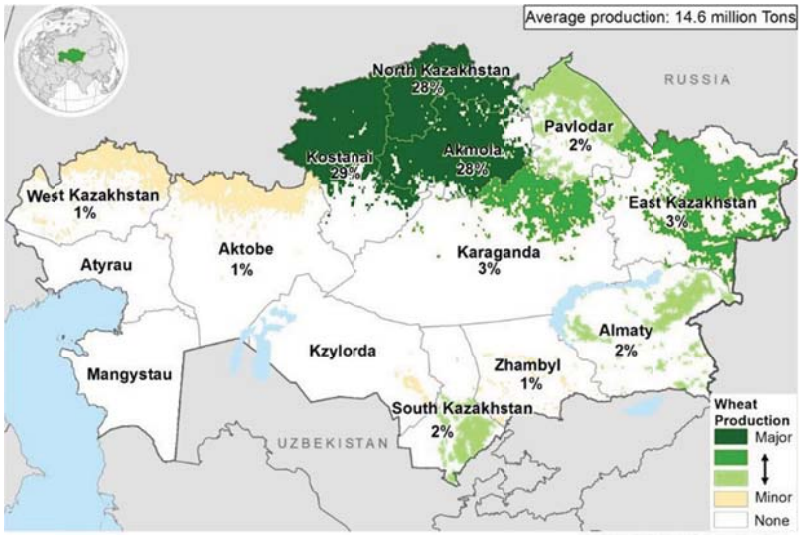

Figure 1. Wheat production in Kazakhstan by oblast

Source: State statistical agency of Kazakhstan.

2.2 Data and Variable Description

Cross-sectional data from a 2019 household survey (April-June) conducted in the northern part of Kazakhstan are used in this study. A multistage sampling procedure was employed to select the sample. First, three regions (Kostanai, Akmola and North Kazakhstan) with the same farming systems and agro-ecologies were purposefully nominated. This selection was based on the regional government, development of stakeholders' interventions, and strong certified seed campaign. The purposeful sampling technique allows the researcher to conveniently select the area of study on the basis of the potential respondents' knowledge of the subject under investigation and the availability of the practice (Saunders, 2011). Second, the quota-sampling technique was used to select 3 communities from each of the 3 regions. According to Saunders (2011), this sampling procedure permits researchers to limit the specific number that they select from a population. In the interest of having a proportional representation of members from different groups in a sample, quota sampling is the best approach (Owen, McNeill, & Callum, 1998). Finally, twenty-five wheat farmers (225 in total, *i.e.*, 3 regions × 3 communities × 25 wheat farmers) were randomly chosen using farmer lists obtained from the ministry of agriculture in the locality (Table 1).

Table 1. Sample from each community in each region

Region	Community	Sample	Total	Adopters	Non-adopters
Kostanay	Uzunkol	25	75	35	40
	Fedorovka	25			
	Zarechnoe	25			
North Kazakhstan	Petrovka	25	75	32	43
	Ruzayevka	25			
	Sovetskoye	25			
Akmola	Akmol	25	75	25	50
	Kapitonovka	25			
	Urumkay	25			
Total			225	92	133

Experienced and well-trained enumerators collected the data using a structured pretested questionnaire. This instrument was substantially rich in content because it included many variables related to the institutional center access, farming practices, farming inputs, actual production, and revenue from wheat sales. To comprehend the impact of certified seed adoption realistically and correctly, a question on the status of certified seed adoption was asked explicitly. Other demographic and socioeconomic data were also collected. The explanatory variables and factors in production disclose that there are significant differences in characteristics between adopters and non-adopters (Table 2).

Table 2. Variable description and summary statistics

Category	Description	Pooled (N = 225)	Users (N = 92)	Non-users (N = 133)
Explanatory variables				
Gender	Sex of household head (1 = male)	0.83 (0.06)	0.86 (0.03)	0.85 (0.02)
Experience	Farming experience of household head in years	21.71 (0.87)	18.07 (1.53)	22.67 (1.01)**
Schooling	Household head's number of years of formal education	11.24 (0.25)	11.62 (0.28)	8.35 (0.26)***
Cooperative	Households who are members of a cooperative (1 = member)	0.79 (0.06)	0.79 (0.01)	0.97 (0.07)***
Credit	Household's access to credit (1 = has access)	0.52 (0.04)	0.69 (0.07)	0.48 (0.04)**
Extension	Household's access to extension services (1 = access)	0.53 (0.03)	0.98 (0.02)	0.42 (0.04)***
Household size	Number of people in a household	6.14 (0.23)	6.30 (0.50)	6.09 (0.26)
Farm size	Total land owned per capita	3.62 (0.09)	3.15 (0.17)	3.74 (0.10)***
Market	Distance from the house to the market in kilometers	8.50 (0.26)	6.48 (0.53)	9.04 (0.28)***
Income	Annual income from wheat sales (USD)	210.33 (1.20)	200.54 (1.55)	170.20 (1.80)**
Classical inputs				
Seed	Quantity of wheat seed used in kgs	26.57 (1.41)	26.42 (3.37)	26.60 (1.55)
Land	Area cultivated for wheat production	3.75 (0.09)	3.45 (0.17)	3.84 (0.11)*
Fertilizer	Fertilizer quantity used (kgs)	580.45 (47.90)	612.20 (94.62)	478.05 (49.33)*
Labor	The hours of labor used in labor-days	134.74 (6.26)	134.86 (19.38)	134.71 (6.08)

Note. The figures in parentheses are the standard errors of the mean, while *, **, and *** indicate the statistical significance levels at 10%, 5%, and 1%, respectively.

2.3 Key Variable Measurement

Certified seed adoption is the primary explanatory variable and is captured as a dummy variable. We consider a household as an adopter if they used at least one type of certified wheat seed during the survey year. Accordingly, 1 is for adopters and 0 is otherwise. This approach is consistent with that of many researchers who have studied seed adoption (Admassie & Ayele, 2004; Shiferaw, Kassie, Jaleta, & Yirga, 2014; Teklewold, Kassie, & Shiferaw, 2013; Varma, 2018).

On the other hand, the technical efficiency level of each wheat farmer is a key output variable that is used to facilitate assessments of whether the adoption of certified seed is associated with improved efficiency. Thus, a stochastic frontier analysis is applied as explained in the empirical strategy section.

2.4 Econometric Framework and Estimation Strategy

A one-step stochastic frontier analysis (SFA) is applied to model the wheat farmers' productivity by employing the Cobb-Douglas production function on a matched sample (to address biases emanating from observed

characteristics using propensity score matching—'1-1 nearest neighbor matching without replacement'). The SFA is a parametric approach formulated by Aigner, Lovell, and Schmidt (1977) on which efficiency measurements rest; it is generally specified is as follows:

$$Y_i = f(x_i,\beta)\cdot\exp(v_i)\cdot\exp(-u_i) \quad (1)$$

where, Y_i is the output of the i-th farmer, x_i is a vector of the inputs, β is a vector of parameters to be estimated, and $v_i \sim N(0,\delta_v^2)$ and $u_i \sim N^+[f(\mu,\alpha),\delta_u^2]$ are the random error and the inefficiency term, respectively.

From Equation 1, Battese and Coelli (1995) indicate that the factors influencing technical efficiency can be estimated as follows:

$$u_i = f(\mu_i,\alpha) \quad (2)$$

Finally, TE is given by Equation 3,

$$TE_i = \frac{y_i}{y_i^*} = \frac{f(x_i,\beta)\exp(v_i - u_i)}{f(x_i,\beta)\exp(v_i)} = \exp(-u_i) \quad (3)$$

where, $y_i = f(x_i,\beta)\exp(v_i - u_i)$ is the observed production with inefficiency and $y_i^* = f(x_i,\beta)\exp(v_i)$ is the frontier output quantity with no inefficiency.

With the aid of several estimation tests and rational indicators relevant to the properties of the different production models (we shortlisted the translog and CD), the best model for the given data can be selected. In our case, the general procedure used to select the best model consists of conducting a likelihood ratio (LR) test after running both models as recommended by Belotti, Daidone, Ilardi, and Atella (2013), and Kumbhakar, Wang, and Horncastle (2015). This procedure ensures that a correct statistical decision is expressed based on the likelihood of how many additional times the data are under one model than its counterpart and the accuracy of the specified distributional assumption in the inefficiency estimation. The LR test general form is given by:

$$LR = -2\{\ln[L(H_A)] - \ln[L(H_0)]\} \quad (4)$$

where, $L(H_0)$ and $L(H_A)$ are the values of the likelihood function under the null and alternative hypotheses, respectively.

The LR test is employed as a statistical test to compare the goodness of fit of two models. Based on the likelihood ratio, a null model is compared to an alternative model. Despite the translog function being a general form in most productivity analyses, the parameters of CD are appropriate and suitable in our case, as confirmed by the statistical insignificance (0.741) of the LR test. The CD function and its respective inefficiency function are specified as follows:

$$\ln Y_i = \beta_0 + \beta_i \sum_{i=1}^{4} \ln X_i + v_i - u_i \quad (5)$$

$$u_i = \alpha_0 + \alpha_1 Certseed_i + \sum \alpha_i m_i + z_i \quad (6)$$

where, Y_i is the wheat output, X_i is a vector of the four classical inputs (land, fertilizer, seeds, and labor), β_0, α_0, α_1, α_i and β_i are parameters to be estimated, m_i is a vector of other determinants of technical inefficiency other than certified seed adoption, u_i is a non-negative inefficiency component that follows a truncated-normal distribution and v_i is a random error following a normal distribution for the production function while z_i is a random error for the inefficiency model.

3. Empirical Results and Discussion

3.1 Intensity of Certified Seed Adoption

Table 3 presents the adoption intensity of the adopters. The figures are conclusive in showing that certified seeds are being used by the wheat farmers (although not in high enough amounts) and that there are various varieties of certified wheat seed available. The data provided by the adopters during questionnaire pretesting revealed that they only use certified seed of certified genetic origin to attain full fruition, which is the goal of any producer (Turner, Ortmann, & Lyne, 2000). In support, Iqbal, Khan, Ahmad, and Ahmad (2001) contends that the prevalence of certified seed adoption for any crop will definitely lead to increased production, especially when the current environment (climate change, loss of soil fertility and increased pest attack incidences) does not favor full-fledged production under normal circumstances.

Table 3. Intensity of certified seed adoption

Certified wheat seed varieties	Number of Adopters	Percent
Naz	60	65.2
Sapaly	47	51.1
Yubilieynaya 60	80	86.9
Akterekskaya	78	84.8
Almaly	55	59.8
Alia	30	32.6
Egemen	84	91.3
Nureke	62	67.4
Ramin	58	63.0
Karasay	49	53.3
Maira	70	76.1
Konditerskaya	51	55.4
Rassad	69	75.0
Mereke 70	34	37.0
Farabi	44	47.8
Alatau	50	54.3

3.2 Technical Efficiency Effects

Table 4 presents the results of the SFA from which the impact of certified seed adoption on technical efficiency can be comprehended. We find that adoption of certified seed positively and significantly influences technical efficiency. This implies that given a fixed set of agricultural inputs, adopters produce wheat output closer to the maximum obtainable output than their counterparts. This result is plausible considering that a seed certification process guaranteeing seed quality has been developed in Kazakhstan (Abou-Jawdah et al., 2001; Auriol & Schilizzi, 2003). The process starts with establishing the seed crop varietal, crop nurture and testing by experts, and ends with harvesting, processing, quality control and labeling under the supervision of the Ministry of Agriculture. This is done in strict accordance and only producers formally listed in the seed register participate (Mangold & Bonner, 2006). Also, while seed processing is done at registered processing facilities, quality testing is carried out at accredited laboratories and package labeling is issued by the organization approved by the Ministry of Agriculture. It is anticipated that such an undertaking would assist farmers to realize a healthy and bountiful crop because the processed seed is considerably higher than the conventional uncertified seed on account of uniformity of seed size and quality (Goletti & Chabot, 2000; Meng, Longmire, & Moldashev, 2000). According to Iqbal et al. (2001), certified seeds are not only of good quality (*i.e.*, free from damage or immature seeds and diseases) but also high in germination and vigor as well as genetic purity (Bernard, Hellin, Nyikal, & Mburu, 2010). Considering that farmers are truly adopting certified seeds as revealed in Table 3, the results obtained in the SFA estimation is validated.

Similarly, Bernard et al. (2010) hypothesize that adopters of certified seed are likely to achieve higher yields (10-30%) than their counterparts because they are using higher quality seeds. The belief by adherents of certified seed adoption is that only seeds treated and processed at registered facilities meet the prerequisites of a productive crop, warranting a remarkable harvest. Therefore, certified seed adoption facilitates higher yields (Jang & Olson, 2010) consistent with the postulation by Iqbal et al. (2001).

Other significant determinants of technical efficiency include the household size, gender, education attainment and access to credit. The household size is very important for wheat farmers because it is a source of labor. We find that larger families have low technical inefficiency. As the household size increases, there is a more equitable labor distribution in the given task, leading to greater concentration, which facilitates improved technical efficiency. Our finding is similar to that of Amos (2007) but differs from the result of Mwalupaso, Wang, Rahman, Alavo, and Tian (2019), who found that the household size negatively influences the technical efficiency.

Gender is significant in improving the technical efficiency (Overfield & Fleming, 2001; Udry, Hoddinott, Alderman, & Haddad, 1995). One possible explanation is that most household heads are males and thus their numbers in the sample is higher. Another explanation is that more males than females are engaged in wheat production in Kazakhstan. In many studies evaluating the determinants of technical efficiency, gender has been found as a significant factor. While females play a significant role in agricultural activities, some tasks such as

plowing are not performed by women. For this reason, male-headed households are more efficient than female-headed households, consistent with the finding of Dadzie and Dasmani (2010) but contrasting with the result of Chirwa (2007).

Jaime and Salazar (2011) assert that education expedites learning, advances access to information, endorses forward-looking attitudes, and eases the adoption of new technologies. Other scholars (Binam, Gockowski, & Nkamleu, 2008; Külekçi, 2010; Rahman, 2010; Solís, Bravo-Ureta, & Quiroga, 2009) support this assertion, which could be the best explanation for the positive and significant impact of education on technical efficiency. Our finding is consistent with that of Hyuha, Bashaasha, Nkonya, and Kraybill (2007), who also indicated higher levels of education as a determinant of productivity.

Finally, for the significant determinants, access to credit allows farmers to acquire the required resources for success in farming. More specifically, it provides the finances required for the optimal growth of the crop and has the potential to lead to the maximum obtainable output. According to Pius Chinwuba and Odjuvwuederhie Emmanuel (2006), credit is an important source of financing that empowers farmers to procure agricultural inputs in a timely manner, and this credit increases the probability of obtaining the maximum output from the deployed inputs. In addition, since the availability of loans reduces capital constraints, it is anticipated that credit access could influence the technical efficiency. However, Feder, Lau, Lin, and Xiaopeng (1989) caution that, in most cases, this type of effect depends on the size of the available credit.

We also find that access to extension service is not significant for non-adopters but significant for adopters. This result suggests that the extension service or the approach used is not effective for achieving the desired objective. By default, households that frequently contact extension experts are expected to adopt new technology and have better access to information (Gelaw & Bezabih, 2004), which leads to farm productivity as concluded by Mwalupaso et al. (2019).

Contrary to our expectation, the experience of the farmer is insignificant. Since the expected acquisition of dexterity leads to auspicious outcomes (Ritson, 1997), an insignificant result suggests that experience alone is not enough in this case. There could be some conditions that need to be met for this factor to be significant, and adoption of certified seed could be one of them. Some factors (diseases) are outside of farmer control regardless of their experience, and so this could best explain the lack of cogent impact on technical efficiency. Our finding is similar to that of Oladeebo and Fajuyigbe (2007) but differs from that of Gul, Koc, Dagistan, Akpinar, and Parlakay (2009), who found that farming experience has positive and significant impacts on technical efficiency.

Table 4. SFA estimation for conventional and sample-selection SPF

Variables	Pooled	Adopters	Non-adopters
lnFertilizer	0.126 (0.179)	0.055 (0.058)	0.073 (0.136)
lnLand	0.608 (0.129)***	0.397 (0.122)***	0.792 (0.066)***
lnLabor	0.029 (0.038)	0.224 (0.055)***	0.008 (0.003)**
lnSeed	0.257 (0.062)***	0.693 (0.099)***	0.220 (0.044)***
Constant	6.703 (0.588)***	7.278 (0.501)***	6.630 (0.367)***
Technical Inefficiency Function			
CertSeed	-0.294 (0.099)***		
Household size	-0.040 (0.011)***	-0.504 (0.152)**	-1.430 (1.671)**
Gender	-0.052 (0.047)*	-0.926 (0.256)*	-2.789 (3.799)*
Experience	-0.008 (0.013)	-0.048 (0.074)	-0.270 (0.308)
Schooling	-0.023 (0.013)**	-0. 296 (0.153)*	-0. 988 (0.645)*
Credit	- 0.102 (0.051)*	-0.834 (0.414)**	-0.557 (0.275)*
Extension	0.055 (0.090)	1.113 (1.350)	1.824 (2.606)
Constant	0.372 (0.171)**	4.252 (1.137)***	-18.982 (24.985)
Model diagnostics			
Log likelihood	35.514	13.486	51.089
Mean TE	0.835	0.935	0.735
N	225	92	92

Note. Figures in parentheses are standard errors of the coefficient, while *, **, and *** indicate statistical significance levels at 10%, 5%, and 1%, respectively.

To understand the efficiency levels across groups to a satisfactory extent, Figure 2 presents the distribution. Compared to adopters, the majority of non-adopters have efficiency scores lower than 70 percent. As earlier stated, given that the government is involved in the registration and monitoring of registered seed certification outlets, an increased efficiency is expected.

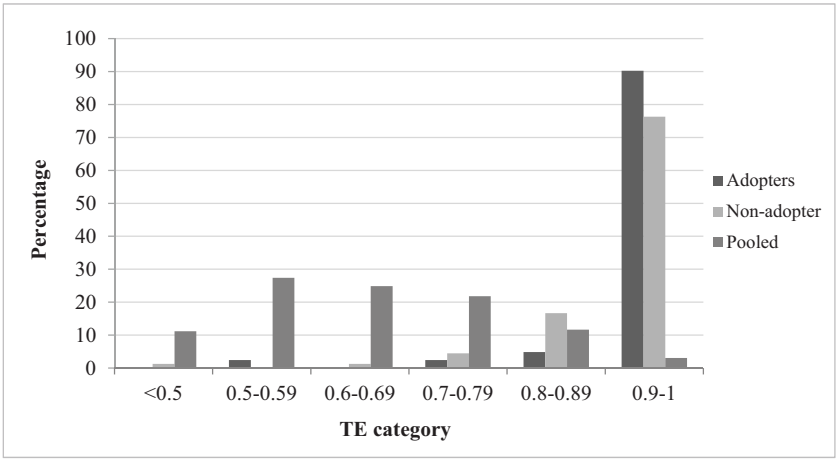

Figure 2. Technical efficiency distribution

4. Conclusion and Policy Recommendation

Seeds are the basic unit of plant propagation and as such they are the most pivotal agricultural input for increased agricultural productivity. In Kazakhstan, a leading producer of wheat, the government has been stimulating the adoption certified seeds in an attempt to augment farmers' technical efficiency. It is anticipated that such an undertaking would assist farmers to realize a healthy and bountiful crop because the processed seed is considerably higher than the conventional (uncertified) seed regarding uniformity of seed size and quality. However, there is limited evidence on the impact of certified seed espousal on technical efficiency. Therefore, the present study was undertaken to establish the adoption intensity and evaluate the technical efficiency effects.

The findings indicate that farmers are indeed adopting various available certified seed varieties and this has positively influenced their technical efficiency in wheat production. One distinct and cardinal element to this effect is the role of the state in actively participating in the seed certification process to ensure that the processing and distribution conforms to the seed laws. This suggests that there is authenticity in quality of the seeds considering that government is keen to achieve increased productivity in wheat production owing to the contribution it makes to the livelihoods of farmers and the national economy. Therefore, Kazakhstan provides a learning case for countries seeking to achieve improved technical efficiency on account of certified seeds.

Therefore, to make adoption of certified seed at a mind blogging scale, research, extension, input supply services, distribution and marketing are some of the major component recommended for an effective seed supply system. Also, quality, and timeliness of delivery, motivating and encouraging private sectors and community based seed multipliers could be considered as policy intervention.

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Water and Photosynthetic Rate Flows Under Drought Conditions in a Cork Oak (*Quercus suber* L.) Forest of Tunisia

Mokhtar Baraket[1], Sondes Fkiri[1], Kaoula Nefzi[1], Sihem Jebbari[1], Abdelhamid Khaldi[1] & Zouhair Nasr[1]

[1] Laboratory of Management and Valorization of Forest Resources (LR11INRGREF0), National Research Institute of Rural Engineering, Water and Forests, Carthage University, Ariana, Tunisia

Correspondence: Mokhtar Baraket, Laboratory of Management and Valorization of Forest Resources (LR11INRGREF0), National Research Institute of Rural Engineering, Water and Forests, Carthage University, Ariana 2080, Tunisia. E-mail: moktar.baraket@gmail.com; mokhtar.baraket@iresa.agrinet.tn

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Abstract

Relationships between drought, carbon and water fluxes have been rarely studied in south Mediterranean forests. The present research focused on the determination of seasonal and annual water and carbon fluxes of *Quercus suber* L. forests in northern Tunisia. The methodology was based on the calculation of the standard precipitation index, measurements of trees sap flow and net photosynthesis. Estimations of photosynthesis and transpiration during the 1965-2003 period were used on crop coefficients and water use efficiency terms.

Results indicate a wide evapotranspiration rates fluctuating from 354 mm y^{-1} to 784 mm y^{-1} with an average value of 553 mm y^{-1}. Extreme values of the standard precipitation index were -2.4 and +2.7. The carbon flux ranged from 0.255 to 0.586 kg y^{-1} m^{-2} with a mean value of 0.448 kg y^{-1} m^{-2} while average water efficiency reached 0.8 gr C kg^{-1} H_2O. Despite the fact, that there is a significant difference between the four studied sites and important annual variability of carbon fluxes, the correlations between water and carbon fluxes and drought index were very low. The results clearly indicate that deep transformations are occurring in the *Quercus suber* L. forests, as a result of carbon dioxide fertilization being cancelled by the drought effect.

Keywords: *Quercus suber* L., draught, net photosynthesis, evapotranspiration, mediterranean forests

1. Introduction

Global climatic models predict a change in rainfall pattern in Tunisia, characterized mainly by a decrease in summer rainfall coupled with greater inter-seasonal and inter-annual variability (IPCC, 2007; Hulme et al., 2001). These previsions for the near future reveal an accentuation of the drought, which means that increasingly longer and more intense dry periods to be expected (Giannkopoulos et al., 2005). The dry period of the year and the succession of two or more dry years would be greater when compared to the reference period (Nasr et al., 2008).

The study of the climate during the last century showed that drought remains a recurring and cyclical phenomenon in Tunisia (Benzarti, 1994). In fact, 50% of dry years were located in North of Tunisia where the climate is mostly humid. Severity of draught is dominated in and it is dispersed within the same region. Hajri (1996) showed that driest years occurred in the 1940s. However, in the 1960s it was more likely of the local type. The phenomenon of the isolated dry year is the most common in Northern Tunisia; it occurred 48% to 66% of the observed period. During the period 1985-1997, the succession of three consecutive dry years was recorded only in Beja province (1987-1990), but no succession of four consecutive dry years has been ever recorded. It is good to notice that the North-West Tunisia area is an important reserve of water and biodiversity. The *Quercus Suber* L. Forest is one of the most fragile ecosystems in this region. This forest has been an alarming deterioration, it now occupies an area of 90 000 hectares against 140,000 hectares 100 years ago (Boudy, 1952). However, it still offers several goods and services to society mainly the photosynthesis carbon capture insured by these forming trees which are threaten by the expected drought and the alteration of water and carbon flows.

The determination and modeling of water and carbon flows have shown the complexity of the exchanges between both forest and atmosphere (Le Dantec et al., 2000; Davi, 2000). These predictive models usually require a lot of data and observations (Dewar, 1992; Granier et al., 2000) of daily weather, soil and vegetation which are often unavailable. In this study, a simple approach based on accurate measurements of

evapotranspiration and photosynthesis for a full year and a historical simulation that assumes the consistency of water efficiency and crop coefficient were proposed. The main objectives are both the seasonal determination of flows and their simulation during the period 1965-2003.

2. Material and Methods

2.1 Study Site

The Tunisian cork oak (*Quercus suber* L.) forest is located on the northwest border of the country. It belongs to the humid and sub-humid bioclimatic stage (Figure 1). It is characterized by Mediterranean climate with four seasons where rainfall is mainly concentrated in autumn and winter and dry spring and summer. The maximum precipitations were 1550 mm and the isohyets indicated a strong NE-SW gradient. The landscape of the Kroumerie-Mogods region is typically that of a mountain forest with persevering hardwoods (43%), conifers (8%), maquis and scrubland (49%).

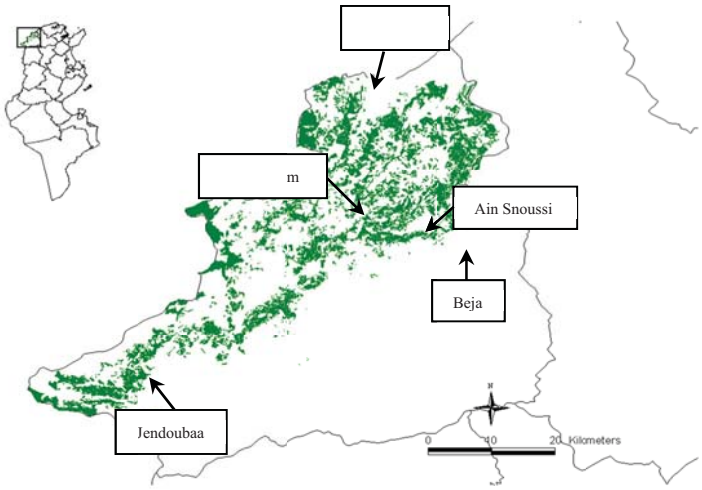

Figure 1. Geographical location of the Tunisian Suberie: experimental site and meteorological stations

The experimental site for the present study was located in Ain Snoussi forest (Lat N: 36°52′, Long E: 8°57′ and Alt: 640 m). This site belongs to the moisture cool winter bioclimatic stage. The average rainfall and temperature were, respectively, 1120 mm and 15.2 °C. The reference evapotranspiration estimated by the FAO-Penman formula is ETo = 1100 mm. The density of this forest varied greatly from 150 to 400 trees ha^{-1}. The soil is loam and rather deep with limited water reserves, Pf (0.3) = 15%; Pf (4.2) = 28%; Bulk density, ds = 1.35. A plot of 30mx30m oriented south-east was chosen. The perimeters of trees measured at 1.30 m from the ground vary from 70 to 130 cm. The average height of the trees was 10.3±1.2 m. The undergrowth is dominated by annuals and some shrubs. Vegetation cover was estimated at 78% during the wet season and 42% in the dry season. Eight trees, two per diameter class were chosen to measure sap flow and photosynthesis during the 2008 and 2009 season.

2.2 Measurements and Treatment of Climate Data

In Ain Snoussi, a HOBO weather station provides continuous measurements of air and soil temperatures (°C), solar radiation (μmol m^{-2} s^{-1}), wind speed and direction (ms $^{-1}$ and degree), relative humidity (%). An appropriate computer program allows calculation of sap flow as well as reference evapotranspiration (ETo, mm j^{-1}) using the FAO formula (Allen et al., 1996).

Historical temperature and precipitation data for representative forest stations (Table 1); Beja (BJA), Jendouba (JND), Ain Drahem (ADH) and Tabarka (TAB) were collected from the database of the National Institute of Meteorology of Tunis for the period 1965-2003. The temperature data (min and max) were used to calculate the reference evapotranspiration (Allen et al., 1996). The monthly precipitation was used to estimate the drought Index, SPI (Standard Precipitation Index) defined by McKee et al. (1993).

Table 1. Geographical Characteristics, temperature (°C) and mean precipitations (mm) of stations during the period 1961-1990

Stations	Lat N	Long E	Alt (m)	Tn (°C)	Tx (°C)	Rainfall (mm)
ADH	36°47'	8°43'	715	10.6	17.9	1488
BEJ	36°44'	9°11'	360	10.5	23.9	557
TAB	36°57'	8°45'	166	13.1	22.9	961
JND	36°29'	8°48'	143	11.1	25.2	460

2.3 Measurement of Sap Flow, Photosynthesis and Soil Moisture Content

Four trees were equipped with thermal sensors to continuously heating Granier. Tree diameters were between 20 and 40 cm. 2 cm deep is too shallow even after bark removal. Needles 5 and 10 cm long are available. The sensors were protected against radiation by an aluminum film. An acquisition unit type ΔT ($DL2^{-e}$) continuously (every 30 sec) measures signals that are averaged over 1 hour and stored in memory. The calibration equation established by Granier (1987) was used to calculate flow density;

$$SFd = 136.828K^{1.2997} \quad (1)$$

The index K of flux calculated by the formula;

$$K = \frac{dT_o - dT}{dT_o} \quad (2)$$

Where, SF_d: flow density (10^{-6} m/s); dT_o: temperature different (°C) when flow is zero, late night in wet period; dT: temperature difference for a positive flow density (°C).

An empirical relationship established in the study area connecting the tree diameter (DBh) to the sapwood section Sa (r = 0.65) by core sampling was used to calculate the daily flow;

$$Sa = 1.058DBh^{1.2889} \quad (3)$$

The average transpiration of the trees (Tr, mm j^{-1}) was calculated by weighting the DBhi of the tree i. The total daily flow was found by integrating the hourly flows and weighting by the diameters of the trees.

$$\frac{\sum_{i=1}^{i=4} SFDi \times DBHi}{\sum_{i=1}^{i=4} DBHi} \quad (4)$$

Soil water content was measured monthly by a TDR30 at depths of 10 and 30 cm at eight points, thus integrating the undergrowth cover. A simplified water balance was calculated on the 0-40 cm layer based on the soil field capacity value and precipitation recorded at the same site.

$$\Delta S = Es + P - D \pm R \quad (5)$$

Soil water content was measured monthly by a TDR30 at two depths (10 and 30 cm) at eight points, thus integrating the undergrowth cover. A simplified water balance was determined on the 0-40 cm layer based on the soil field capacity value and precipitation recorded at the same site.

The measurements of net photosynthesis were carried out on the eight trees chosen, for a full year by choosing to make these measurements in 5 typical days of each season of the year.. Net photosynthesis was measured by a Li-COR6400 device (Nebraska, USA) on the 4th leaf of young twigs, one from each orientation (North and South) (Nasr et al., 2012). The measurements included sun lit leaves (Pns), leaves in the shade (Pno) and dark respiration measurements (Rn). The total resulting was then calculated assuming equal leaf surfaces in the sun and leaves in the shade, such as:

$$Pn = \frac{Pns + Pno}{2} + Rn \quad (6)$$

2.4 Estimates of Seasonal Photosynthesis During the Climatic Period 1965-2003

Seasonal values of water efficiency, EUE = Pn/Tr and evapotranspiration coefficients, KT = Tr/ETo and KTo = (Tr + Es)/ETo were determined from the measurements made in the station of Ain Snoussi during the year 2008-2009. These values of EUE, KT and K To have been adapted after adjustment for the BJA, ADH, TAB and JND stations by a ratio of the vapor pressure deficits between that of Ain Snoussi and those of the other stations for the 2008-2009 periods. This assumes that the CO_2 and H_2O gas exchanges are essentially controlled by the stomatal conductance via the vapor air pressure deficit. These seasonal ratios ranged from 0.31 to 1.19. Thus, from the monthly temperature and precipitation data for the 1965-2008 periods, the terms SPI, ETo, ET and Pn have been calculated for each season and each station.

$$P \succ K_{To}.ET_o \Rightarrow ET = K_{To}.ET_o$$
$$P \leq K_{To}.ET_o \Rightarrow ET = P \quad (7)$$
$$P_n = EUE.K_T.ET_o$$

2.5 Statistic Analysis

SAS GLM procedure was performed for all collected data using the. The comparison of averages was performed by The Newman-Keuls test at the 5% risk threshold.

3. Results

3.1 Seasonal Photosynthesis and Evapotranspiration Values

Seasonal mean values of tree transpiration (Tr) and evapotranspiration of the undergrowth (Es) showed that maximum values were reached in spring being 1.4 mm/d and 1.3 mm/d, respectively (Figure 2). There was also a significant decrease in term of evapotranspiration and a slight decrease in tranpiration amounts during the summer season. It was recorded that annual water consumption of trees was 342 mm and evapotranspiration of undergrowth was 192 mm. For the growing season March-October, the tree transpiration (from trees sap flow) and the evapotranspiration (from soil water content variation) of the undergrowth were about 308 mm and 80 mm, respectively.

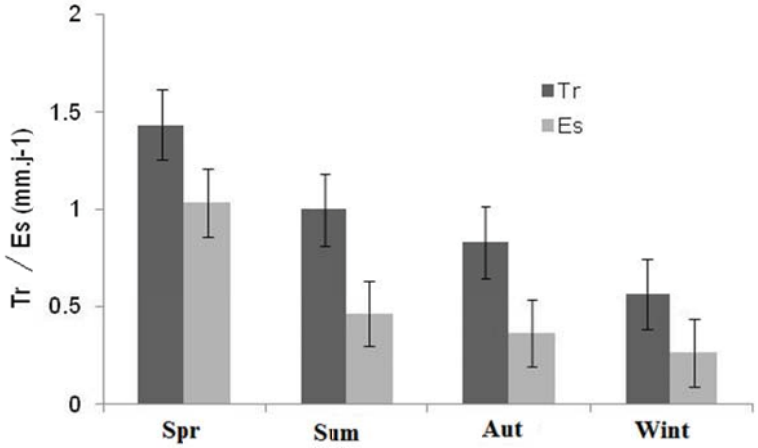

Figure 2. Seasonal evolution of tree transpiration and evapotranspiration of the undergrowth measured in a cork oak forest in northern Tunisia (2008-2009)

The highest values of the net photosynthesis were recorded in spring, with a mean value of 9 µmol m^{-2} s^{-1} for leaves in the sun in comparison with 4.3 µmol m^{-2} s^{-1} for those in the shade (Figure 3). During the dry season, there was a significant decline in net photosynthesis. Hereafter, net photosynthesis has increased significantly following probably the autumn rains to fall in winter as a consequence of lower temperature. Furthermore, nighttime breathing was maximal in summer, lowest in winter and average in autumn. The recorded values did not exceed (-2) µmol m^{-2} s^{-1}.

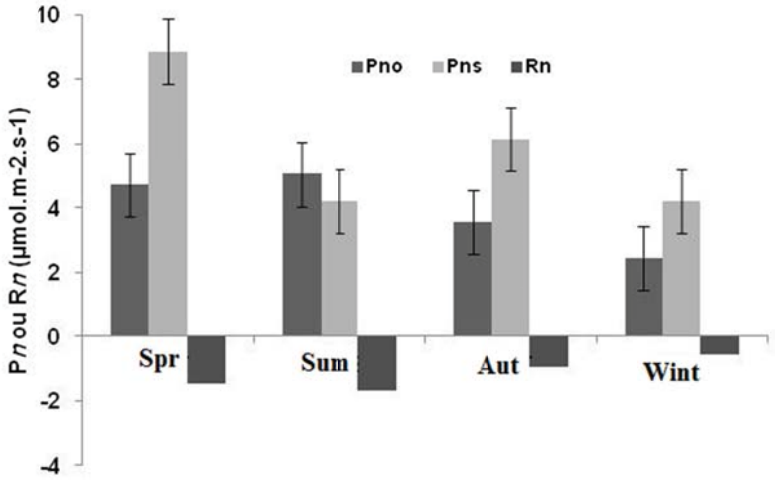

Figure 3. Seasonal values of Pno, Pns and Rn in a cork oak forest in northern Tunisia (2008-2009)

3.2 Historical Analysis of Drought and Seasonal Flows in 1965-2003

The estimation of drought by SPI showed very few wet years, 2 years at ADH. Normal years were dominant in 65% of cases (Table 2). However, we can note that a number of very dry years are more than very wet ones. Then, we showed an asymmetry between the numbers of dry and wet years which the percentages varied from 9 to 20%.

Table 2. Percentage of dry and wet years according to the SPI drought index for the 4 stations (TH: very wet year, H: wet year, N: normal year, S: dry year)

Stations	TH (Spi > 2)	H (1 < Spi < 2)	N (-1 < Spi < 1)	S (Spi < -1)	TS (Spi < -2)
TAB	0	20%	65%	12%	3%
ADH	2%	9%	67%	14%	5%
JDB	0	14%	67%	19%	0
BJA	0	18%	64%	18%	0

The analysis of simulated Fc fluxes showed to be greater in autumn and spring comparing to winter with the summer flows being the lowest (Figure 4). Summer fluxes are also the least variable from year flow to another. The most variable flows are those of the winter season where we note an increase in the carbon flux during mild winters such as 1987 or 2001. Furthermore, there was a slight upward trend in spring and autumn flows that started especially since the warming period of 1975.

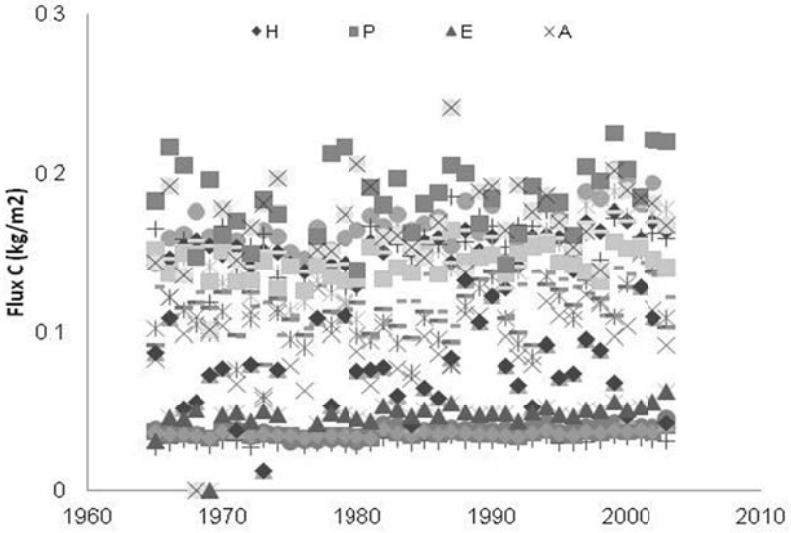

Figure 4. Simulated values of seasonal carbon fluxes (A: autumn, E: summer, H: winter, P: spring) for ADH, BEJ, TAB and JEN during the 1965-2003 simulation period. Ilm faut faire chaque station à part

3.3 Statistical Analysis of Tr, Fc and SPI Parameters in 1965-2003

For the different stations evaluated, Initial evapotranspiration (ET_0) varied from 354 mm $year^{-1}$ to 784 mm $year^{-1}$ with an average value of 553mm $year^{-1}$. The SPI values ranged from -2.4 to +2.7 and a variation of Fc from 0.255 to 0.586 kg an^{-1} m^{-2} was recorded with an average 0.448 kg an^{-1} m^{-2}. The water use efficiency reached 0.8 gr C kg^{-1} H_2O, which was slightly higher during a dry year (Table 3).

Table 3. Analysis of Fc, ET and SPI averages for DHA, BEJ, TEB and JEN stations during the period 1965-2003

Variable	Mean	SD	Sum	Min	Max
Fc	0.45	0.047	69	0.25	0.59
ET	1.5	0.35	233.4	0.95	2.15
SPI	-0.005	1	-0.8	-2.41	2.7

The GLM and SNK analysis indicated for Fc that the ADH station showed the highest mean comparing to the stations from BJA, TAB and JEN, but no significant differences between the other stations was recorded (Table 4). For Tr value, two groups can be distinguished, one with BEJ and ADH stations, the other with TAB and JEN stations (Table 5).Whereas, for the SPI index, no significant effect was recorded between the stations.

Table 4. Analysis of Fc, ET and SPI averages for DHA, BEJ, TEB and JEN stations during the period 1965-2003

Variable	DDL	Sum of Square Mean	Square Mean	Value F	Pr >F
Fc	3	0.04611361	0.0153712	7.92	<0.0001
ET	3	12.2891453	4.09638178	104.1	<0.0001
SPI	3	0.0359695	0.01198565	0.01	0.9983

Table 5. SNK analysis of the Fc, SPI and ET parameters for the ADH, TAB, BEJ and JEN stations during the period 1965 and 2003

Variable	SNK group	Mean	Station
Fc	A	0.47573	ADH
	BB	0.44859	JEN
	BB	0.44082	TAB
	B	0.42797	BEJ
ET	AA	1.80641	BEJ
	A	1.79514	ADH
	BB	1.26179	TAB
	B	1.21231	JEN
SPI	AA	0.01128	ADH
	AA	0.00256	TAB
	AA	-0.00474	BEJ
	A	-0.02949	JEN

Positive correlations between ET and Fc and low negative correlations with SPI were observed. It is evident that a significant station effect was present for the Fc and ET variables but no significant station effect at the 5% threshold for the SPI variable was found (Table 6).

Table 6. Correlations between the Fc, SPI and ET parameters for the ADH, TAB, BEJ and JEN stations during the period 1965 and 2003

	Fc	SPI	ET
Fc	1.000	-0.0876	0.456
SPI	-0.0876	1.000	-0.01023
ET	0.456	-0.01023	1.000

3.4 Evolutions of Fc and SPI in 1965-2003

From the results presented in Figure 5, it can be observed that there is certain cyclist of photosynthesis as well as for drought. On an annual scale, the synchronization between SPI and Fc is not established; in some cases it is even reversed. There is a clear downward trend in photosynthesis during major dry periods, such as that of 1987-1993. The lowest variation of Fc was recorded in JND, the highest one in ADH, while TAB and BJA were overall similar.

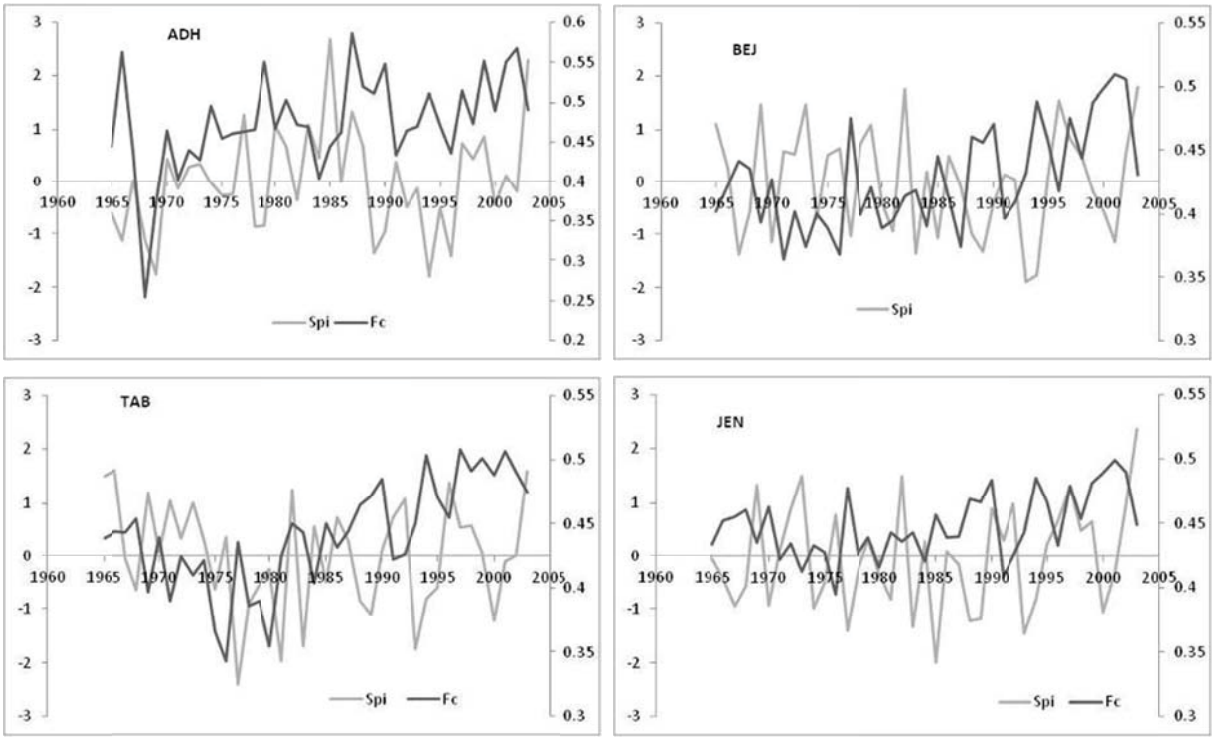

Figure 5. Variations in annual SPI and Fc values for BJA, ADH, TAB and JND stations during the period 1965-2003

4. Discussion

The physical errors in the estimation of sap flow by the Granier sensor have been widely discussed in the literature. They can be caused by the representation of the measurement according to the orientation and depth of the probes (Nasr et al., 2012). Our measurements were made at a depth of 2 cm assuming that most of the water flow passes through this slice. Thus, the South-East orientation chosen cannot represent the average of the flows. Poyatos et al. (2007) showed that 85% of the fluxes pass close to the cambium and a lower contribution of the heartwood. On *Quercus ilex*, Infante et al. (2007) used the Granier technique and showed a fairly large flux variation of 2 to 3.5 L dm^{-2} h^{-1} depending on the orientation.

Repetitive measurements of the net photosynthesis at the foliar scale on eight trees may not represent the average carbon fluxes of the forest. Indeed, the variability of measurements between trees, orientations and leaves was very important. The coefficient of variation has in some cases exceeded 50%.

Despite these sources of multiple errors, the average values of tree transpiration, soil evapotranspiration and net photosynthesis are quite comparable to the values observed in the Mediterranean forests. According to Tognetti et al. (1998), *Quercus ilex* sap fluxes were in the order of 50 L j^{-1} with a maximum hourly flux of 3 L hr^{-1}. Furthermore, Vincke et al. (2005) from sap flow measurements showed that declining trees responded to fluctuations in climate demand but their transpiration remained low and less than 1 mm/d. Under these conditions, the herbaceous layer can consume more water than the trees, up to 2.9 mm/day.

Using the flux method (Eddy covariance) on a 38% mixed forest (*Q. robur*/*Q. petraea*) and 31% Scots pine, Gerricle et al. (2005) estimated a primary productivity of 630 g C m^{-2} an^{-1}. In the same context, Periera et al. (2007) estimated by the eddy covariance in oak forest in Portugal that a primary productivity varied between 500 and 1000 g C m^{-2} an^{-1}. While, Wilkinson et al. (2012) obtained higher flows in the order of 1500 g m^{-2} $year^{-1}$ during the period 1999-2010 in a forest of Fraxinus and *Quercus robur*. The annual phytomass of Ain Snoussi Forest was evaluated at 5.98 T ha^{-1} $year^{-1}$ (Sebei et al., 2004), which equates to 0.29 kg of C m^{-2} $year^{-1}$.

Several authors have also highlighted the effects of drought on the carbon balance of forests. In fact, Breda et al. (2006) was able to show the consequences of an extreme event (drought in 2003) on typical Mediterranean stands. The low water availability of the soil in summer resulted in a very low carbon balance associated with low carbohydrate accumulations in the trunks.

For the simulations made, it was assumed that the ET/ETo and Pn/Tr ratios are constant from one year to another, as well as a constant value of the leaf area index (Lai) was assumed. This hypothesis remains valid for the leaf surface unit, but it did not allow the spatial integration of the simulated values. According to Davi et al. (2008), although the Lai varied slightly, it remained the main variable controlling both water and carbon flows and also the relationship between this parameter and the density for a Mediterranean forest of *Quercus ilex* and *Pinus halepensis*. Additionally, the control mechanisms for canopy transpiration are mainly stomatal regulation, hydraulic conductance and leaf area adjustment. The decrease in leaf area appears as the main mechanism for adjusting transpiration to new water conditions (Limosin, 2009) in *Quercus ilex* species.

During the period 1965-2003, the rate of atmospheric CO_2 had to increase approximately from 260 ppm to 380 ppm. This enrichment did not cause a net increase in calculated carbon fluxes. As if drought have counterbalanced the fertilizing effect of atmospheric carbon. However, in a controlled environment, it was previously highlighted that the effect of carbon enrichment on oak by an increase in the net photosynthesis capacity (Vivin & Gehl, 1997).

5. Conclusions

The simulations carried out showed evapotranspiration flux ranging from 0.95 to 2.15 kg m^{-2} d^{-1} wile photosynthesis flux ranged from 0.255 to 0.586 kg m^{-2} $year^{-1}$, SPI were -2.41 and 2.69 for a dry and wet year, respectively.

These simulations showed some inter annual variability of flows with a special site effect. However, synchronization with the climatic drought by the SPI index has not been established. In addition to this variability, it was not possible to observe clearly a trend upward or downward flows, but rather certain cyclicality was clearly noticed.

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Impact of Seed Size on the Seedling Vigour, Dry Matter Yield and Oil Content of Jatropha (*Jatropha curcas* L.)

Aderemi I. Makinde[1], Kehinde O. Oyekale[2] & David S. Daramola[2]

[1] Department of Agriculture, Federal College of Agriculture, Ibadan, Nigeria

[2] Department of Agriculture and Industrial Technology, Babcock University, Ilishan-Remo, Nigeria

Correspondence: Aderemi I. Makinde, Department of Agriculture, Federal College of Agriculture, Ibadan, Nigeria. Tel: 234-803-243-8757. E-mail: makindeaderemi30@gmail.com

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Abstract

Seed size is a trait of the plant that affects seed germination and seedling survival. This study aims to assess the growth response of *J. curcas* to different seed sizes. A pot experiment was conducted to evaluate the effects of *J. curcas* seed sizes on the seedling vigour and seed component. The seeds were fractioned into three sizes visually into: large, medium and small and by 1000 seeds weight (SW). Seedling vigour was assessed by: germination % (G%), seedling length cm (SL), seedling vigour index, SVI, seedling growth rate, SGR, and speed of germination, SOG as well as proportion of cake, shell and oil content (OC) expressed as% of the seed. Results obtained shows that G% and the SOG were not affected by seed sizes but by other factors within the seed. However, seedling vigour expressed as SL, SVI and SGR increased significantly ($P \leq 0.05$) with increase in seed sizes. Proportion of cake, shell and oil component of *J. curcas* seeds increased with increasing seed sizes while 60% of the seed is made up of the cake from where the oil is extracted. Dry matter yield, DMY significantly ($P \leq 0.05$) increased with increase in seed size from 6.41 g/plant in large seeds to 2.61 g/plant in small seeds. There is positive and strong significant correlation between, SW and DMY ($r = 0.91^{**}$), yield increase ($r = 0.82^{**}$), OC ($r = 0.85^{**}$), % cake ($r = 0.94^{**}$). Findings revealed that larger seed had higher potential of producing vigorous plants with eventual high crop yield and higher OC.

Keywords: seed size, seedling vigour, Jatropha, yield, oil content

1. Introduction

Jatropha curcas L. is a perennial shrub or tree belonging to the family Euphorbiaceae which in the last decades has received considerable attention from researchers and several stakeholders due to, among many uses, its potential as a feedstock, for renewable biofuel production and the ability to grow in marginal lands with less water and nutrients (Namasivayam et al., 2007; Wang et al., 2011). Nowadays, *J. curcas* grows in tropical and sub-tropical regions in a wide range of climatic conditions from semi-arid to humid (Achten et al., 2010).

J. curcas seeds contain about 25-35% of oil, which can be easily extracted and used both for biodiesel production, a renewable energy source alternative to conventional petrol diesel, and as cooking/lighting fuel, medicine, bio-pesticide, and for soap making. Additionally, the seed cake, an oil extraction by-product, can be used as organic fertilizer, combustible fuel, for biogas production (IFAD-FAO, 2010) and also feedstuff after detoxification (Wang et al., 2011). Besides the economic value attainable from *J. curcas* oil and its derivatives, its potential to adapt to low-nutrient soils and drier soils under arid and semi-arid conditions, minimize *J. curcas* competition against food crops. Furthermore, the plant offers an ecological advantage to mitigate soil degradation and to restore marginal land or abandoned farmlands (Reubens et al., 2011). Despite these potentials, *J. curcas* is still a semi-wild or wild undomesticated plant and has not received enough research attention to be able to understand its basic agronomic needs to improve its growing and management practices in many ecological areas.

Germination of seed indicates its power to reproduce a new plant. It is conditioned by a regular embryo development and the amount of available food reserves. These two components are necessary pre-requisites for the development of a normal embryo, a vigorous seedling and a well-developed plant (Adebisi et al., 2013). Therefore, the use of high-quality seeds is essential for a successful crop production and food security. Crop

yield and resource use efficiency depend on the successful plant establishment in the field, and seed vigor is what defines the ability to germinate and establish seedlings rapidly, uniformly and robustly, across diverse environmental conditions (Finch-Savage & Bassel, 2016). Seed size is an important physical indicator of seed quality that affects the emergence, plant growth and performance of the crop in the field (Adebisi et al., 2013). Indeed, the sowing of mixed seeds of a species may result in a non-uniform stand establishment, what may lead to heterogeneity in the plant vigor and size (Mishra et al., 2010). Distinct seed sizes have different levels of starch and other energy reserves which may be an important factor to improve the expression of germination and initial growth of seedlings (Shahi et al., 2015). Germination depends on the ability of the seed to use reserves more efficiently (Bewley et al., 2013), by mobilization of seed reserves for the germination traits (Sikder et al., 2009). However, these results vary widely between the crop species and the germination and growth environment. In general, large seeds have a higher seedling survival rate, higher growth and better field performance than small seeds, under non-stressful environments (Ambika et al., 2014).

To achieve success in crop production, the use of good quality seed is very essential which increase the yield by 15-20%. The extent of this increase is directly proportional to the quality of seed that is being sown. The seeds of a seed lot may differ by size, weight and density due to production environment and cultivation practices. Seed size is one of the components of seed quality which affects the performance of crop (Adebisi et al., 2011). Size is a widely accepted measure of seed quality and large seeds have high seeding survival growth and establishment (Jerlin & Vadivelu, 2004). A wide array of different effects of seed size has been reported for seed germination, emergence and related agronomical aspects in many crop species (Kaydan & Yagmur, 2008). However, these results varied widely between species. Generally, large seed has better field performance than small seed.

Bhatt et al. (1989) assessment of the effect of seed size on the nutrient composition and germination of potato seed showed that large seeds contained higher levels (% dry weight) of total proteins, ethanol soluble proteins and alkali soluble proteins than small seeds and they germinated faster and had the highest percentage of germination. Small seeds had the lowest levels of total lipids, phospholipids and water soluble proteins, the longest water saturation time and the lowest germination. In other words, seed size effects on canola emergence, yield or seed quality were not significant (Harker et al., 2015). Meanwhile, increasing seed size had a positive linear association with early canola biomass and 1000-seed weights, whereas, both days to flowering and days to the end of flowering had a negative linear association with seed size. Greater biomass from large seeds increases crop competition with weeds and also hastens flowering. Similarly, Adejare (2010) reported that large seed size of elite maize had higher seed quality and higher seed yield compared to other medium and small sizes.

If seed size influences seed quality and yield, it would be advantageous to sow seeds that give the best seed quality and highest yield. With the dwindling oil prices in the world and its corresponding effects on Nigeria income and economy, the development of research in a non-food crop like *J. curcas* may just be a way out of diversifying Nigeria economy as the cry for green, renewable energy continues because of its minimal contribution to global warming. Since the oil is contained in the seed of *J. curcas*, a better understanding of the seed in relation to oil production among other variables will provide agronomic information to the policy makers, growers and other stakeholders in alternative energy development, enhancing eco-friendly agricultural sustainability. Therefore, the objective of the study is to evaluate the growth response of *J. curcas* to different seeds sizes in a rainforest environment.

2. Materials and Methods

A pot experiment was conducted at the Seed laboratory of the department of Agriculture and Industrial Technology of Babcock University, Ogun State, Nigeria between January and March, 2019 to evaluate the effects of *J. curcas* seed sizes on the seedling vigour and the composition of the seeds.

2.1 Seed and Seed Source

Jatropha seeds used for the experiment were obtained from an already established farm in Oyo town, Nigeria. The seeds were harvested at the same physiological stage of maturity, that is, at yellowish-brown stage. This particular variety is commonly called “Linneaus” which begins to fruit from nine months after planting and can continue for between 20-25 years under good management.

2.2 Seed Size Allotment

The seeds were separated into three sizes visually and this was corroborated with weighing of 1000 seeds per size, using electronic balance (Model XY 1000C, Axion Medical Ltd, U.K) as well as width measurement of the samples using digital micrometer screw gauge. The mean seed weight varies from 717.9 g in large seeds to 652.9 g in medium seed and 505.1 g in small seeds. Similarly, average seed width of large seeds is 6.63 mm, medium

seeds is 5.87 mm and 5.04 mm in small seeds. Seed moisture content, MC varies from 3.52% in large seeds to 2.92% in small seeds.

2.3 Potting of the Seeds

Ten kg of sieved top soil was filled into 18 bottom-perforated plastic pots. The soil was saturated with water after which 20 seeds were planted in each pot at 3 cm depth. Subsequent watering was done at three days' interval using 120 ml per pot. Maximum relative humidity of the environment ranges from maximum of 85% to minimum of 67% while maximum temperature is 37 ^{o}C and minimum temperature is 25.5 ^{o}C.

2.4 Experimental Design

The experiment was a completely randomized design comprising of *J. curcas* seeds separated into three seed sizes and replicated six times.

2.5 Seed Viability and Seedling Vigour Assessment

Germination count began 4 days after planting after which germination count was done daily till 8 days after planting when germination peaked. The following parameters were taken to assess seedling vigour: germination % (G%), seedling length cm (SL), seedling vigour index, SVI, seedling growth rate, SGR, and speed of germination, SOG.

2.5.1 Germination %

Germination % was calculated based on the normal seedling evaluated on the 8th day and it was expressed in % (ISTA, 1999). Percent seed germination was then expressed as number of seed germinated over total no of seed planted (expressed in percentage).

2.5.2 Seedling Length

This was obtained with the aid of a ruler; by measuring the length (in cm) of the seedling from the base of the plant above the substrate to the apex of the last leaf on the 8th day.

2.5.3 Seedling Vigour Index

Seedling vigour index (SVI) was computed using the following formula suggested by Abdul-Baki and Anderson (1973), *i.e.*, SVI = Germination (%) × Seedling length (cm).

2.5.4 Speed of Germination

SOG was determined by dividing the number of first seedling emergence with day at emergence till the final count which are added together to give SOG according to AOSA (1983). *i.e.*, No of seeds at 1st count/day of 1st count + … No of seeds at final count/day of final count.

2.5.5 Seedling Growth Rate

SGR is the change in plant weight divided by the period of such change (www.wikihow.com) developed by Timothy Paine (2012).

2.6 Seed Component Determination

Proportion of cake and shell in the seed were also determined by their expression as a percentage of seed components while oil content of the seeds was determined in the laboratory using a Soxhlet extractor. Oil Content (OC) expressed in %: The oil content was determined gravimetrically after extraction using petroleum ether (n-hexane), in a Soxhlet instrument, technique 920.85 (AOAC, 1990), expressed in dry matter %. Air oven method recommended by ISTA (1985) was used to determine the moisture content, where the crushed seeds were oven-dried at 105 ^{o}C for 6 hours. Weight of a Thousand Seeds (WTS), expressed in grams/1000.

2.7 Dry Matter Yield

Dry matter yield was also determined by oven-drying the plant samples at 65 ^{o}C till constant weight was obtained and was expressed as g/plant.

2.8 Statistical Analysis

The data collected from the experiment were analyzed using SAS (Statistical Analysis Software) version 9.1 (SAS, 1999). Analysis of variance (ANOVA) was carried out on each variable and the Duncan Multiple Range Test (DMRT) was used for means separation ($P \leq 0.05$). Correlation analysis was also carried out on the parameters evaluated to ascertain the kind(s) of relationships that exist among the variables.

3. Results and Discussion

Growth response of *J. curcas* to different seed sizes was shown in Table 1. It was observed that the number of seedling emergence and the rate of such emergence from a seed lot was not determined by the size of the seeds but by other factors within and outside the seed. A glimpse of those factors were observed by (Shahi et al., 2015) where seeds of different sizes were found to have different levels of starch and other energy reserves which may be an important factor to facilitate germination and initial growth of seedlings. Hence, growers will have to look beyond seed size in selecting their seeds but also the genetic quality of such seeds. Also, ability of the seed to use reserves more efficiently (Bewley et al., 2013) could be a reason as well as heterogeneity in the plant vigor and size (Mishra et al., 2010). Expectedly, seedling vigour expressed as seedling length, seedling vigour index and seedling growth rate increased significantly ($P \leq 0.05$) with increase in seed sizes. Hence, seed size was found to be an important physical indicator of seed quality that affects the emergence, plant growth and performance of the crop in the field (Adebisi et al., 2013). This is because large seeds will be able to mobilize more seed reserves for the germination traits (Sikder et al., 2009).

Table 1. Effect of Seed size on the indices of Seedling vigour of *J. curcas*

Treatment (Seed size)	Germination % (G%)	Seedling length (cm)	Seedling Vigour Index (SVI)	Seedling Growth Rate (SGR)	Speed of Germination (SOG)
Large	39.17	25.00a	976.08a	1.59a	4.41
Medium	34.17	22.57b	790.67ab	0.80b	3.93
Small	27.50	22.43b	616.83b	0.59b	3.18
	Ns				Ns

Note. Means with same letter(s) in a column are not significantly different at 5% level of probability according to DMRT.

Effect of seed sizes on the dry matter yield of *J. curcas* and the attributes of seed was shown in Table 2. Results showed that cake, shell and oil component of *J. curcas* seeds increased with increasing seed sizes. A proportion (60%) of the seed is made up of cake from where the oil is extracted while the remaining 40% is the shell which can be compounded as manure and as input in biogas production (IFAD-FAO, 2010). Similarly, Bhatt et al. (1989) assessment of the effect of seed size on the nutrient composition and germination of potato seed showed that large seeds contained higher levels (% dry weight) of total proteins, ethanol soluble proteins and alkali soluble proteins than small seeds. Smaller seeds of harvested Canola offered lower seed attributes of lowest levels of total lipids, phospholipids and water soluble proteins (Harker et al., 2015). Growers can therefore focus on raising and selecting bigger seeds to ensure higher yield. Meanwhile, dry matter yield is yield component in plants showing their ability to produce and mobilize assimilates for the proper growth of the plant. Therefore, with increased dry matter yield, the plant will be able to grow vigorously, utilize resources for growth better and resist the pest interference better and thus enhance its productivity. With the observed increase in dry matter yield with increasing seed sizes, bigger seed will tend to produce better than plant raised from smaller seeds (Adejare, 2010; Harker et al., 2015).

Table 2. Effect of seed size on the seed components of *J. curcas* and dry matter yield (g/plant)

Treatment (Seed size)	% Cake	% Shell	% Oil content	Dry Matter Yield (DMY) (g/plant)
Large	60.42a	44.90a	59.93a	6.41a
Medium	58.97b	41.03b	57.87b	3.73b
Small	55.10c	39.58c	54.13c	2.61c

Means with same letter (s) in a column are not significantly different at 5% level of probability according to DMRT.

As shown in Table 3, positive and highly significant correlation was found between seed weight, SW and dry matter yield, DMY ($r = 0.91^{**}$), yield increase, YI ($r = 0.82^{**}$), oil content ($r = 0.85^{**}$), % cake ($r = 0.94^{**}$). These results shows that larger seed had higher potential of producing vigorous plants as described by (Zareian et al., 2013) where it is obvious that increase in biological yield by increasing seed size was related to higher seedling weight and weight of 100 plants were produced by larger seed sizes in wheat., increasing seed size had

a positive linear association with early canola biomass and 1000-seed weights, (Harker et al., 2015). In the same vein, positive association exists between seed size and oil content in soybean (Marega et al., 2001). However, there is positive and weak correlation between the seed weight and seedling length, SL (r = 0.51*) and seedling vigour index, SVI (r = 0.51*) showing that the rate of plant growth can be inferred from the plant seed weight. The was no correlation between the seed weight and germination percent as well as speed of germination which showed that other factors within the seed like availability of nutrient reserves, maturity of the seeds among other factors would affect rate of seedling growth other than the size of the seeds (Shahi et al., 2015). This is contrary to the general belief that, heavier seeds have a higher seedling survival rate, higher growth and better field performance than small seeds, under non-stressful environments (Ambika et al., 2014).

Table 3. Correlations among the eleven seed related variables in *J. curcas* (n = 18)

	SW	G%	SL	SVI	DMY	YI	SGR	SOG	% Oil	% Cake	% Shell
SW	1	.458	.499*	.514*	.909**	.862**	.858**	.369	.847**	.938**	-.938**
G%	.	1	.392	.983**	.361	.349	.347	.869**	.426	.459	-.459
SL	.	.	1	.552*	.580*	.577*	.578*	.416	.463	.411	-.411
SVI	.		.	1	.440	.428	.426	.872**	.472*	.491*	-.491*
DMY		.		.	1	.988**	.988**	.322	.789**	.819**	-.819**
YI	.		.	.	.	1	1.000**	.312	.726**	.767**	-.767**
SGR	.		.	.			1	.310	.721**	.761**	-.761**
SOG		.		.	.	.	.	1	.398	.409	-.409
% Oil	.		.					.	1	.879**	-.879**
% Cake		.			.				.	1	-1.000**
% Shell											1

Note. * Correlation is significant at the 0.05 level (2-tailed). SW = Seed Weight.

** Correlation is significant at the 0.01 level (2-tailed). YI = Yield Increase.

4. Conclusions

Seed size of *J. curcas* did not affect seedling emergence and rate of germination but significantly affect all the other seedling vigour indices, indicating early establishment and growth of crops raised from bigger seeds. Similarly, with increasing dry matter yield with increase in seed sizes, more vigorous growth against field interference and better utilization of resources is expected in larger seeds. Therefore, to ensure better survival of *J. curcas* on marginal land, bigger seeds should be selected to improve on its survival rate on the field. Since, seed size is directly proportional to oil content in *J. curcas,* therefore, in large scale oil production; bigger seeds should be used for better productivity and higher income while smaller seeds may be used for other industrial or domestic uses.

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Potential of Predator *Podisus nigrispinus* Dallas (Hemiptera: Pentatomidae) in the Control of *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae)

Juliana Simonato[1], Harley N. de Oliveira[2], José F. J. Grigolli[3], Mirian M. Kubota Grigolli[3] & Ivana F. da Silva[1]

[1] Programa de Pós-Graduação em Entomologia e Conservação da Biodiversidade, Universidade Federal da Grande Dourados, Dourados, MS, Brazil

[2] Embrapa Agropecuária Oeste, Dourados, MS, Brazil

[3] Fundação MS para Pesquisa e Difusão de Tecnologias Agropecuárias, Maracaju, MS, Brazil

Correspondence: Ivana F. da Silva, Programa de Pós-Graduação em Entomologia e Conservação da Biodiversidade, Universidade Federal da Grande Dourados, Dourados, MS, Brazil. E-mail: ivanaf.silva@hotmail.com

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Abstract

Managing *Helicoverpa armigera* is still a major challenge for Brazilian farmers, due to limited information available about chemicals and biological control of this pest in Brazil. This study focused on evaluating the biological aspects of *Podisus nigrispinus* fed with *H. armigera*, under laboratory conditions, as well as verifying the capacity of the *P. nigrispinus* in preying on caterpillars of *H. armigera* in the field, in soybean crops. The experiments were conducted in laboratory under controlled temperature conditions of 25±2 ºC, relative humidity (RH) 60±10% and 14-hour photophase, as well as in the field, at the experimental station of Fundação MS in Maracaju, MS. In the laboratory, second instar nymphs of *P. nigrispinus* were placed in plastic jars and fed with *H. armigera* larvae or *Tenebrio molitor* larvae throughout the nymphal phase. When they reach adulthood, 15 couples were formed, remaining with the same prey of the previous phase. For the field trial, soybean plants were caged, and inside these cages were released eight quarter-instar *H. armigera* caterpillars. Subsequently, in each cage were released an adult female or a fifth instar nymph, and after 24 hours, the number of prey caterpillars were assessed. Predator nymphal duration was shorter in treatment with *H. armigera* than with *T. molitor*. Nymphal viability was similar between treatments. Adult females and males fed with *H. armigera* presented greater body mass than those fed with *T. molitor*. The number of postures per female and the number of eggs per female were similar between treatments. The incubation period of eggs was longer for treatment with *H. armigera*, differing statistically from treatment with *T. molitor*. Eggs from treatment with *H. armigera* showed similar viability to treatment with *T. molitor*, not differing statistically. Females and males under treatment with *H. armigera* showed longer longevity compared to treatment with *T. molitor*. Adult females preyed on average 2.26 caterpillars within 24 hours and fifth instar nymphs preyed on 1.73 caterpillars/day. *P. nigrispinus* showed better development when fed with *H. armigera*, demonstrating that it can be used as an alternative host for breeding this predator. The predator showed satisfactory performance for predation rate/day. Release of fifth instar nymphs and adult females of *P. nigrispinus* may reduce the pest population in relation to the absence of the predator under field conditions, an important alternative to be used in *H. armigera* integrated management programs.

Keywords: predatory stink bug, asopinae, biological control, old-world caterpillar

1. Introduction

Brazil is one of the world's largest producers of soybeans, reaching record production of 114.843 million tons in the 2018/2019 crop and according to forecasts, soybean production will have a significant increase in the 2019/2020 crop, constituting a new production record (CONAB, 2019). However, this increase in production could be threatened due to phytosanitary problems that Brazilian producers have been facing, including the possibility of recurrence of *Helicoverpa armigera* caterpillars (Hübner, 1805) (Lepidoptera: Noctuidae) in the production fields (Dias et al., 2019).

Helicoverpa armigera has a worldwide distribution, occurring in countries in Asia, Africa, Europe and Oceania (Guo, 1997), and was first recorded in Brazil, officially, in the 2012/2013 harvest (Ávila et al., 2013; Czepak et al., 2013). It is a highly polyphagous species, and its caterpillars are found in more than 60 species of wild and cultivated plants, feeding on leaves and stems, however, they prefer shoots, inflorescences, fruits and pods, causing damage to both the vegetative phase and the reproductive phase of plants, impairing several crops (Oliveira et al., 2016). Managing *H. armigera* populations is still a major challenge for Brazilian farmers, given limited information available on chemicals and biological control of this pest in Brazil (Grigolli et al., 2016; Dias et al., 2019). However, due to awareness on the need to maintain environmental quality and human health safety, insect pest suppression methods have been a source of concern for society. In this context, it is necessary to seek an agricultural production system that contemplates environmental sustainability and promotes biodiversity in the agroecosystem (Oliveira & Ávila, 2010). Biological control using tactics to conserve or increase natural enemies in agroecosystems is a reality that needs to be investigated and explored under Brazilian conditions (Ávila et al., 2013), being an important tool for the control of *H. armigera* (Fathipour & Sedaratian, 2013). Among the predatory insects found naturally in agroecosystems, species of the *Podisus* genus present general feeding habits, being registered in several cultures feeding preferentially on lepidopteran caterpillars (Oliveira et al., 1999). The predatory stink bug *Podisus nigrispinus* (Dallas, 1851) (Hemiptera: Pentatomidae) is the subfamily species Asopinae most commonly found in Brazil and is found naturally in agricultural and forest ecosystems, preying on leafless caterpillars, as well as larvae and pupae of beetles and nymphs and adults of phytophagous stink bugs (Oliveira et al., 2008; Moura & Grazia, 2011). However, it is necessary to understand the interactions between ecological, behavioral, physiological and nutritional factors, as basis for the successful use of insects in pest control (Thompson, 1999).

The development and reproduction of *P. nigrispinus* may vary with the prey used (Lacerda et al., 2004; Lemos et al., 2005; Mahdian et al., 2006), which may have implications for mass production as well as the effectiveness of biological pest control in the field for different crops (Oliveira et al., 2004a). According to Oliveira et al. (2004a), predation potential studies should prioritize the mass breeding of these insects under laboratory conditions, and subsequently released into the field for biological control of key pests in various crops. In this context, it is vital to study the prey species most suitable for the production of *P. nigrispinus*, allowing mass multiplication; thus, its use in integrated pest management programs (Botteon et al., 2016). Despite the importance of *Podisus* sp. biological control in soybean cultivation and the existence of several studies on techniques for its mass rearing in the laboratory (De Clercq et al., 1988; Saavedra et al., 1992, 1995; Zanuncio et al., 1992; Torres et al., 2006), there is little information on the applied use of this biological control agent in soybean crop in Brazil (Bueno et al., 2014). Therefore, this study focused on evaluating biological aspects of the development and reproduction of *H. armigera* predator *P. nigrispinus* in laboratory, as well as to verify the predation capacity of *H. armigera* caterpillars in soybean, under field conditions.

2. Material and Methods

The experiment was conducted at the Entomology Laboratory of Fundação MS (22°08′S; 52°44′W, 425 m altitude), with the predator *P. nigrispinus* and prey *H. armigera* and *Tenebrio molitor* (Linnaeus, 1758) (Coleoptera: Tenebrionidae). Predator and prey rearing, as well as experiments, were maintained under controlled temperature of 25±2 ºC, relative humidity (RH) of 60±10% and 14-hour photophase. The field experiment was conducted in an experimental area of Fundação MS located in the municipality of Maracaju, MS. The *P. nigrispinus* individuals used in the experiment were kept in PVC pipes and were fed with *T. molitor* larvae as described by Zanuncio et al. (2001). *T. molitor* rearing was maintained in 29 × 23 × 11 cm (height × width × depth) plastic trays and fed with whole wheat flour (97%), beer yeast (3%) and chayote (*Sechium edule*), to provide the necessary water (Zamperline et al., 1992). The *H. armigera* caterpillars used in the experiment were obtained from the laboratory-maintained rearing, where adults were kept in 20 × 25 cm (diameter × height) PVC cylindrical cages, fed with 10% honey solution for posture increase. Eggs were removed daily and placed in plastic jars containing artificial white bean diet (modified from Greene et al., 1976).

2.1 Bioassay 1: Biological Aspects of P. nigrispinus Nymphs With H. armigera Larvae and T. molitor larvae as Prey

For the nymphal phase evaluation of *P. nigrispinus*, a completely randomized design was used, with two treatments (*H. armigera* larvae and *T. molitor* larvae) and 15 replications. Each repetition consisted of 1000 ml transparent plastic containers, with distilled water dampened cotton inside, dimensions of 15 × 10 cm (diameter × height) and capped with plastic lid. Five *P. nigrispinus* second instar nymphs were placed in each container, and they were fed with caterpillars of each treatment *ad libitum*, throughout the nymphal phase of the predator.

Material asepsis, water exchange and assessments were performed every 24 hours. The biological parameters assessed during the nymphal phase were: duration of each stage, nymphal phase duration and nymphal viability.

2.2 Bioassay 2: Biological Aspects of P. nigrispinus Adults With H. armigera and T. molitor Larvae as Prey

After the emergence of the adults, who were reared throughout the nymphal phase with *H. armigera* or T. *molitor*, 15 couples were formed per treatment (*H. armigera* larvae or *T. molitor* larvae), they were placed in 1000 ml transparent containers, with water dampened cotton inside, dimensions of 15 × 10 cm (diameter × height) and capped with plastic lid. One couple was housed per cage. Caterpillars and larvae used to feed couples were placed *ad libitum* in each treatment. The biological parameters evaluated were mass (mg) of newly emerged males and females, sex ratio, number of postures, total eggs per female, egg viability, incubation period and longevity of males and females. The experimental design adopted in this stage was completely randomized, with two treatments (*H. armigera* larvae and *T. molitor* larvae) and 15 replications.

2.3 Bioassay 3: Potential of P. nigrispinus Predation in H. armigera Caterpillars in Soybean Under Field Conditions

In an area of approximately 15 hectares sown with soybean (*Glycine max* (L.) Merrill), cultivar "BMX Potência RR" in a no-tillage system, cages made of pvc pipes and fittings, with a size of 1.00 × 1.00m (width × height) each cage, coated with *voile* fabric were randomly arranged, each covering approximately 12 soybean plants, free from pests and predators, with their bases covered with soil, to prevent their infestation by unwanted insects, as well as to prevent the escape of insects released inside. During the experiment, the soybean plants were between the R5 and R6 phenological stages (Fehr & Caviness, 1977). In the upper leaves of the plants, eight fourth-instar caterpillars of *H. armigera* were released in each cage 8 hours before the predators were released. Fifth-instar nymphs and adult females of *P. nigrispinus*, up to 24 h, were individualized and kept without food for 24 h. After this period a nymph or an adult predator was released from each cage. The evaluations were performed 24 hours after the predator release and the number of prey caterpillars recorded during this period. The experimental design was completely randomized, with 15 replications for both adults and predator fifth-instar nymphs, each cage constituting one repetition.

Gathered data were submitted to homogeneity and normality analysis, according to the conditions. Subsequently, the t-Test at 5% significance was conducted.

3. Results and Discussion

3.1 Biological Aspects of P. nigrispinus Nymphs With H. armigera Larvae and T. molitor Larvae as Prey

Regarding the nymphal phase, the duration of the second instar was longer in *H. armigera* treatment than in *T. molitor* treatment. Third and fourth instars showed no significant difference between treatments. Treatment with *T. molitor* presented a longer duration of the fifth instar than *H. armigera*. The duration of the nymphal period was shorter with *H. armigera* than with *T. molitor*. The nymphal viability showed no significant differences between treatments, with approximately 73.33% for *H. armigera* and 72.00% for *T. molitor* (Table 1).

Table 1. Period (days) of immature stages and nymphal viability (%) of *Podisus nigrispinus* fed *Helicoverpa armigera* and *Tenebrio molitor* in laboratory. T 25±2 ºC, RH 60±10% and 14-hour photophase

	Preys		t-test	CV (%)
	H. armigera	*T. molitor*		
2nd instar	4.26±0.16 a	3.66±0.17 b	6.24*	16.64
3rd instar	3.57±0.16 a	3.72±0.15 a	0.82ns	12.24
4th instar	3.81±0.23 a	4.28±0.14 a	2.52ns	19.85
5th instar	4.79±0.27 b	7.33±0.49 a	23.42**	23.67
Per. nymph (days)[1]	18.60±0.41 a	20.85±0.66 b	7.61*	11.34
Viab. nymph (%)[2]	73.33±4.22 a	72.00±5.45 a	0.03ns	26.99

Note. Means followed by the same lowercase letter on the line do not differ statistically from each other by the t-Test at 5% probability. ns Not significant, * significant at 5% probability, ** significant at 1% probability. [1] Per. nymph: Nymph period. [2] Viab. Nymph: Nymph viability.

Food quantity and quality affect parameters such as survival, mass gain, stage duration, egg number and longevity (Zanuncio et al., 2002; Peluzio et al., 2018). Comparing the nymph period found in this study (Table 1),

with the means obtained by Vacari et al. (2007) using caterpillars of *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae), by Espíndula et al. (2010) using caterpillars of *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) and by Santana et al. (2017), using caterpillars of *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), despite the variations in the different stages, when considering the nymphal period as a whole, the phase is similar, being 18.60 days for *H. armigera* (Table 1), 18,20; 19.40; and 19.61 days for *D. saccharalis*, *H. virescens* and *S. frugiperda,* respectively. The similarity in the duration of the nymphal phase indicates the adaptation of this predator to the different prey and also shows that the prey used does not influence the duration of the nymphal phase (Oliveira et al., 2004b). A shorter nymphal period is important, as it results in higher predator production, promoting predator population growth and, consequently, a better performance in field caterpillar control (Matos Neto et al., 2002; Juscelino-Filho et al., 2003), as well as the population increase in less time, in laboratory creations.

Nymphal viability did not differ statistically between treatments (Table 1). In other studies, the predator reached 89.00% of nymphal viability when fed with *D. saccharalis* caterpillars (Vacari et al., 2007), 88.60% with *Spodoptera cosmioides* (Walker, 1858) (Lepidoptera: Noctuidae) (Denez et al., 2014), 64.00% with *S. frugiperda* (Oliveira et al., 2004b) and 60.00% com *Thyrinteina arnobia* (Stoll, 1782) (Lepidoptera: Geometridae) (Oliveira et al., 2011). The results obtained in this work demonstrate the adaptation of the predator *P. nigrispinus* to the prey *H. armigera*. Insects, especially early instars, may require a period of time to adapt to the new diet, which may interfere with their development and survival (Santana et al., 2017). For the biological aspects evaluated, *H. armigera* proved to be suitable prey for the immature phase of *P. nigrispinus* with results similar to those obtained with the alternative host *T. molitor*, usually employed in mass breeding of this predator.

3.2 Biological Aspects of P. nigrispinus Adults With H. armigera Larvae and T. molitor Larvae as Prey

H. armigera fed adults had a sex ratio of 0.47 with no significant difference compared to *T. molitor* prey (0.54) (Table 2). *H. armigera* fed females and males had higher body mass than *T. molitor* fed females (Table 2). The result was similar to that reported by Oliveira et al. (2011) using *T. arnobia* caterpillars as food, being 65.39 mg for females and 45.61 mg for males; and when *S. frugiperda* caterpillars were used, the mass of females was 56.18 mg and 40.81 mg for males (Oliveira et al., 2004b), presenting lower results to those found in this study.

Table 2. Sex ratio, female and male mass (mg), number of postures and number of eggs of *Podisus nigrispinus* adults fed *Helicoverpa armigera* and *Tenebrio molitor* in laboratory. T 25±2 ºC, RH 60±10% and 14-hour photophase

	Preys		t-Test	CV (%)
	H. armigera	*T. molitor*		
Sex ratio	0.47±0.06 a	0.54±0.07 a	0.91^{ns}	39.98
♀[1] mass (mg)	62.80±1.77 b	52.83±2.15 a	16.64**	16.36
♂[2] mass (mg)	44.57±0.73 b	33.13±1.13 a	60.64**	14.64
Number of postures	14.60±2.27 a	15.73±1.27 a	0.21^{ns}	25.50
Number of eggs	378.06±61.04 a	311.67±35.18 a	0.97^{ns}	30.29

Note. Means followed by the same lowercase letter on the line do not differ statistically from each other by the t-test at 5% probability. ^{ns} Not significant, * significant at 5% probability, ** significant at 1% probability.

[1]♀: Females; [2]♂: Males.

The greater mass of females is due to the accumulation of biomass necessary for the beginning of reproduction, since this parameter has been related to the development of their ovaries, as well as egg formation (Oliveira et al., 1999, 2011). The predator's mass reflects its diet, that is, properly fed individuals gain more mass than those who eat less (O'Neil & Wiedenmann, 1990; Angelini & Boiça Junior, 2015; De Bortoli et al., 2016). Thus, it can be inferred that *H. armigera* confers adequate performance to the predator P. nigrispinus by providing greater body mass when compared to *T. molitor*. The number of postures per female in the *T. molitor* treatment did not differ from the *H. armigera* treatment (Table 2). Similar result was found when fed with *S. frugiperda* (Oliveira et al., 2004b) and with *T. molitor* larvae in another study (Oliveira et al., 2004b; Espindula et al., 2010). When the prey used was *H. virescens* the number of postures was 8.89 (Espindula et al., 2010) and when the predator was fed with *D. saccharalis* it was on average 5.63 postures per female (Vacari et al., 2007). Although there was no statistical difference regarding the number of postures and number of eggs between the treatments, *P.*

nigrispinus that fed on *T. molitor* presented numerically more postures, however, individuals from *H. armigera* treatment produced more eggs, demonstrating that the nutritional quality of this prey meets the predator's needs.

Fertility was similar between treatments (Table 2). The number of eggs per female of the *H. armigera* fed predator was similar to that found when fed with *S. frugiperda*, with a total of 447.62 eggs (Oliveira et al., 2004b); with *Alabama argillacea* (Hübner) (Lepidoptera: Noctuidae) it was 348.10 eggs (Oliveira et al., 2002); with *T. molitor* (392.76; 325.00) (Espindula et al., 2010; Oliveira et al., 2004b); 314.90 with *T. arnobia* (Oliveira et al., 2011) and larger than when fed with *D. saccharalis* (97.12) (Vacari et al., 2007). The higher number of eggs produced with the prey *S. frugiperda*, *H. armigera*, *A. argillacea* and *T. molitor* demonstrate that these species are nutritionally suitable for *P. nigrispinus*.

Regarding the egg incubation period, which was 6.04 days for *H. armigera* treatment, it was statistically different from *T. molitor* treatment, which was 6.62 days (Table 3). However, these values observed for both preys were lower than those reported by De Bortoli et al. (2016) when they used *S. frugiperda* (5.46 days) as prey *Musca domestica* (Linnaeus, 1758) (Diptera: Muscidae) (5.19 days) and *A. gemmatalis* (5.07 days).

Table 3. Incubation period (days), number of nymphs, nymphal viability (%), male and female longevity (days) of adult *Podisus nigrispinus* adults fed *Helicoverpa armigera* and *Tenebrio molitor* in laboratory. T 25±2 ºC, RH 60±10% and 14-hour photophase

	Preys		t-Test	CV (%)
	H. armigera	*T. molitor*		
Per. incubation (days) [1]	6.04±0.32 b	6.62±0.48 a	6.75**	6.74
Number of nymphs	356.60±54.64 a	291.00±34.19 a	1.10[ns]	52.91
Nymph viability (%)	96.13±1.32 a	92.46±2.06 a	1.83[ns]	7.86
Longev.[2] ♂[3] (days)	32.80±4.73 a	29.60±7.29 a	0.16[ns]	70.88
Longev.[2] ♀[4] (days)	27.93±3.86 a	26.33±4.60 a	0.09[ns]	53.52

Note. Means followed by the same lowercase letter on the line do not differ statistically from each other by the t-test at 5% probability. [ns] Not significant, * significant at 5% probability, ** significant at 1% probability.

[1]Per. incubation: Incubation period; [2]Longev.: Longevity; [3]♂: Males; [4]♀: Females.

The number of nymphs depends on egg quality and viability. Eggs from *H. armigera* treatment presented viability similar to those from *T. molitor* treatment, not statistically different (Table 3). Espindula et al. (2010) by feeding *P. nigrispinus* with *H. virescens*, obtained viability of 65.09%; with *D. saccharalis* the viability was 76.50% (Vacari et al., 2007), and the result was numerically similar to that reported by Oliveira et al. (2004) with the prey *S. frugiperda* (85.19%). The higher egg viability suggests that *H. armigera* has the necessary nutritional requirements for a good predator development. The longevity of *P. nigrispinus* males and females fed *H. armigera* was similar to that observed in *T. molitor* treatment (Table 3). Overall, females were found to be approximately 13% lighter than males. This shorter longevity of females compared to males was also observed by Oliveira et al. (2011) when the prey used was *T. arnobia*, being 35.54 days for females and 43.08 days for males (Oliveira et al., 2011). In a study conducted by Espindula et al. (2010) the prey *H. virescens* found shorter longevity of females (40 days) compared to males (49.21 days). This fact can be explained by the high energy use of females for egg formation and oviposition (Oliveira et al., 2011). This is due to the allocation of energy during physiological processes, increasing the energy demand for egg production, leaving less energy for other processes such as longevity, for example (Sibly & Calow, 1986).

Prey that is nutritionally adequate provides reduced development time, maximum survival rate and maximum reproductive rate, resulting in an increase in population. The development of this predator and its mortality rate may vary depending on the conversion efficiency of the food, so some predators are better suited to certain prey types that may provide faster development and longer survival (Stamp et al., 1991), as was verified when the predator was raised with *H. armigera* caterpillars.

3.3 Predatory Potential of P. nigrispinus in H. armigera Caterpillars in Soybean Under Field Conditions

After the predation period had elapsed, the cages were removed, the predators located and the collection of the remaining live and preyed caterpillars started, totaling the eight initially released caterpillars per cage (Figure 1).

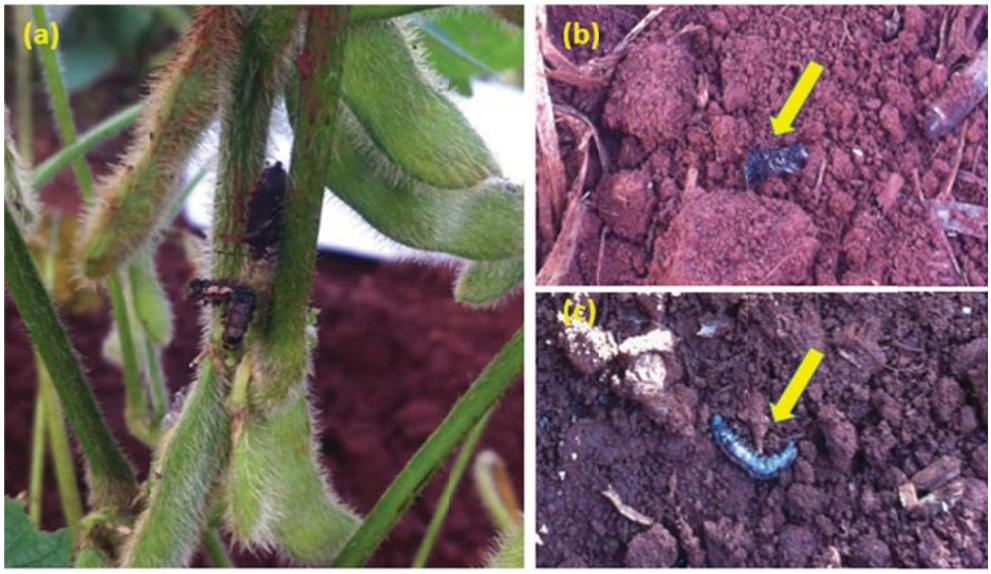

Figure 1. (a) Adult female of *Podisus nigrispinus* preying on *Helicoverpa armigera* caterpillar and (b, c) *H. armigera* caterpillars predated by *P. nigrispinus* in field on soybean crop. Photos: Juliana Simonato

Adult females preyed on average 2.26 caterpillars within 24 hours and fifth-instar nymphs preyed on 1.73 caterpillars/day, presenting statistical difference (Figure 2).

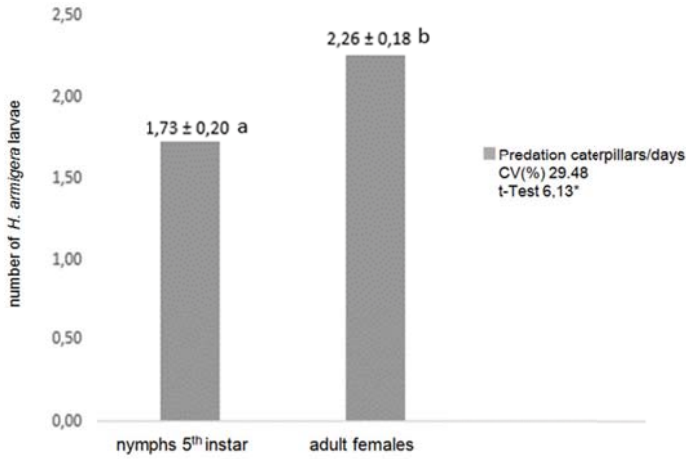

Figure 2. Averages (±SE) of the number of *Helicoverpa armigera* larvae predated by *Podisus nigrispinus* in 24 hours in soybean under field conditions. Means followed by the same letter do not differ statistically from each other by the t-Test at 5% probability. * significant at 5% probability

Study by Oliveira et al. (2008), where they evaluated predation by *P. nigrispinus* in third-instar larvae of *A. argillacea* in cotton plants under field conditions, under density of one or three caterpillars per plant, reported predation rates of 1.19 and 1.23 caterpillar/day for fifth-instar nymphs and adult females, respectively when exploring 20-day-old plants and daily availability of three caterpillars per plant. Predation rates were higher with three caterpillars per plant, regardless of leaf area, which may be due to the higher amount of prey available, which enabled the predator to locate the prey more easily (Oliveira et al., 2008). As found in this study, adult females preyed more caterpillars than fifth-instar nymphs, possibly because they are larger and need more energy for the reproductive phase. This fact was also observed by Oliveira et al. (2001) in a study on the functional response of this predator with *A. argilacea*, in which it evaluated the predation rate by adult females at the densities of one, two, four, eight and sixteen caterpillars/plant, in which a predation of 0.32 caterpillar was observed, as well as densities of one and two caterpillars per cotton plant in the flowering phase in the field,

increasing this rate according to the prey density, with up to 3.10 prey caterpillars/day, at the density of 16 caterpillars per plant.

Podisus predation rates on various prey species and under field conditions ranged from 0.32 to 2.46 attacks per female/day (Saavedra et al., 1997; De Clercq et al., 2000; Oliveira et al., 2001; Vivan et al., 2002), mainly affected by the area to be exploited by the predator, prey density and size, plant type and weather conditions (Oliveira et al., 2008). Some studies have shown that controlled (laboratory) conditions give the bug *P. nigrispinus* high predation rates due to the ease of finding its prey (Vivan et al., 2002; Oliveira et al., 2008), therefore, the prey search capacity of this predator in the field is lower due to the larger prey search area in this environment (O'Neil, 1988; Grants, 2015).

The results found in this study show that *H. armigera* is a nutritionally suitable prey, having the necessary requirements to promote a good development and reproduction of *P. nigrispinus* in a satisfactory way, and can be used as food in mass breeding of this predator. Release of fifth-instar nymphs and adult females of *P. nigrispinus* can reduce the pest population in relation to the absence of predator under field conditions, being an alternative to be considered in *H. armigera* integrated management programs in soybean crops.

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Technologies of Reproduction Terry Varieties of Clematis

Khanbabaeva Olga Evgenievna[1,2], Matsneva Anna Evgenievna[1,2], Kovaleva Irina Sergeevna[3], Tazina Svetlana Vitalevna & Tazin Ivan Ivanovich[1,2]

[1] Russian State University, RGAU-MSHA named after K. A. Timiryazev, Moscow City, Russia

Correspondence: Khanbabaeva Olga Evgenievna, Russian State University, RGAU-MSHA named after K. A. Timiryazev, Moscow City, Russia. E-mail: hanbabaeva@yandex.ru

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Abstract

The paper presents issues of optimizing the technology of growing popular terry varieties of clematis (*Clematis* L.) in Russia. Trimming groups, bookmark features of flower buds are described. The issues of reproduction and agricultural technology when laying uterine plantations are considered. A comparative analysis of traditional breeding technology with innovative technology (*in vitro*). The optimal nutrient medium for the stages of micro propagation, cultivation and rooting, enriched with vitamins and other substances. In experiments on green cuttings, 8 terry varieties were used. Estimated rooting of green cuttings by standard propagation technology. Spring cuttings are recommended for the varieties Bellof Woking (83%), Empress (81%), Blue Light (79%) and give a high yield of planting material from one mother plant. Two varieties with low rooting were selected—Purpurea Plena Elegans, Multi Blue. *In vitro* breeding technology has been developed for them. Accounting and observation of the development of two terry clematis varieties in the tissue culture: Purpurea Plena Elegans, Multi Blue. The aim of our study is to develop a technology for clonally propagation for these varieties, as well as the adaptation of micro plants of these varieties to non-sterile conditions. Therefore, *in vitro* propagation is recommended for these two varieties. An optimal substrate composition has been developed for the adaptation of clematis plants propagated *in vitro*, as well as for cuttings. The article provides recommendations for planting, pruning and caring for uterine plantings of clematis with double flowers in sheltered ground.

Keywords: clematis, varieties, terry, cuttings, pruning groups, uterine plants, flower buds, rooting, substrate for rooting, *in vitro* technology

1. Introduction

1.1 The Value of Terry Varieties of Clematis in Horticulture

Modern garden classifications of clematis are numerous. Some classifications divide varietal clematis into large-flowered (flower diameter 10 cm or more) and small-flowered (diameter less than 10 cm). According to the height of plants (length of vines), clematis is divided into 3 groups: the first—up to 1 m, the second—1.5-2 m and the third—2.5-3 m and higher. Varieties are also classified according to the trim group: from first to third (Ivanova & Khanbabaeva, 2013).

1.2 Pruning Varietal Clematis

To obtain a large number of green cuttings from varietal clematis in protected ground conditions, the use of strong pruning of uterine plants is recommended. Such pruning for 5-7 years of cultivation reduces the productivity of queen cells, but at the same time makes it possible to obtain a sufficient number of green cuttings. Under the condition of intensive use of uterine plants, it is recommended that they be completely replaced after 5-7 years of cultivation. During this time, unwanted viruses and diseases accumulate, so it is advisable to plant new mother liquids clematis with healthy planting material.

Terry varieties are very popular in gardening and landscaping in Russia. They have abundant and long flowering, large flowers. This group forms a few flowers on the shoots of last year in early summer, provided that the shoots wintered well. And then it gives a very plentiful flowering on the shoots of the current year in the second half of summer.

Since terry is partially associated with the transformation of part of the stamens into petals, the flowering of one flower lasts 5-7 days longer than in varieties with a simple flower.

1.3 The Appearance of Terry: Importance in Gardening

Terry flower in garden crops is an important decorative and economic feature. As a rule, in terry flowers, part of the stamens is reduced to petals, and sometimes completely. Most plants with double flowers bloom for a long time, more than 10-14 days. This is due to the lack of pollination. Fully double flowers, do not form seeds and have no aroma.

Terry flower is closely associated with polyploidy and mutagenesis. Polyploidy-hereditary changes associated with an increase in the number of whole chromosome sets. Polyploid plants are characterized by prolonged flowering, a larger flower, a corrugated edge of the petals, and high resistance to adverse environmental factors. Studies on polyploidy were carried out on a culture of iris bearded (Germanic) (Stern, 1956).

However, directly the double flower is associated with mutations that occurred during meiotic cell division. Mutations are sudden hereditary changes that represent the genetic basis of variability. Terry is a morphological somatic mutation. In ornamental plants, somatic mutations were found in color, shape and degree of terry of the flower.

Since they occur spontaneously and rarely in nature, in order to obtain terry, they use treatment or irradiation of plants with special substances-mutagens that cause the desired mutation (Khanbabaeva, 2011).

The combination of mutagenesis with hybridization is the most promising way in the selection of flower cultures. This combination accelerates the process of creating new varieties (Dryagina & Kudryavets, 1986).

1.4 Technology of Microclonal Propagation of Ornamental Plants

The culture of isolated tissues is used for propagation and for healing from viruses and other pathogens of planting material. This method is called clonally micro propagation of plants, allowing you to get hundreds of thousands of plants per year from one meristem. And also this method is used in plant breeding to produce fast-growing, resistant to pests and adverse environmental factors of plants (Kalashnikova & Rodin, 2004).

For clematis, this method is used only if, using standard propagation technology, a low reproduction rate is obtained.

Thus, the aim of our study is to select the optimal technology for the propagation of terry clematis varieties, characterized by low rooting and the yield of standard planting material with the traditional method of propagation. And also, the selection of substrate for the adaptation of micro plants of clematis varieties propagated *in vitro*.

2. Method

2.1 Objects of Study

The most common varieties belonging to the group of large-flowered terry varieties: Bellof Woking, Blue Light, Crystal Fountain, Empress, Evijohil, Mazury, Multi Blue, Purpurea Plena Elegans.

2.2 Technology of Green Cuttings

Terry varieties are propagated mainly by the vegetative method, using traditional technology-green cuttings or innovative-microclonal propagation.

Uterine plants of 7 varieties are located in protected ground, in a glazed, heated greenhouse. The age of the uterine plants is approximately 5-6 years. Grown under standard agricultural technology, in ridges, with a garter to the support.

Cuttings were carried out in two terms—spring and summer. In rooted cuttings, the length (cm) and the number of roots (pcs) were taken into account, and the percentage of rooting of each variety (%) was calculated.

For rooting, a substrate containing lowland peat, perlite (3:1) and mineral additives was used.

2.3 Technology of Reproduction in vitro

As the primary explants, shoot apexes taken from uterine plants in early March were used. The mother liquors are planted in ridges in the conditions of a heated winter greenhouse.

Before sterilization, the selected plant material was washed in running water with the addition of surfactants (surfactants) to remove contaminants for 9 minutes. Then the explants were treated with 70% ethanol for 2-3 seconds, then with 2.5% sodium hypochlorite solution for 7 minutes. Then the plant material was washed several times with sterile distilled water. Sterile explants were placed in test tubes on the Murasig-Skoog agar medium (MS) enriched with the following substances (mg/L): thiamine (B1), pyridoxine (B6), nicotinic acid (PP)-0.5; inositol-100, 6-BAP-1; sucrose-30,000, bacto-agar-7,000.

At the stage of micro propagation, the culture was planted in 250 ml glass vessels of 10 pcs. in each. The cultures were incubated in a light room at a light intensity of 1500-2000 lux, a temperature of 220 °C and a 16-hour photoperiod.

After the cultivation period, the height of the shoots, the number of additional shoots, and the number of nodes were taken into account. The multiplication factor was the sum of the single-node segments.

For the initiation stage, we used a nutrient medium based on mineral salts Murashige-Skoog (MS), enriched with the following substances (mg/L): vitamins B1, B6, PP of 0.5; iron 10000; inositol 100; glycine 1000; bacto-agar 7000; sucrose 30000 with the addition of VAR 0.1.

The percentage of contamination was 20%. At the stage of crop initiation, the duration of which was 5 weeks, conglomerates consisting of 1-2 shoots were formed on the explants. For further replication, conglomerates were divided into single microprobe and planted on fresh nutrient medium in 250 ml plastic containers.

At the stage of micro propagation proper, we used a nutrient medium based on the Murashige-Skoog mineral salts, enriched with the following substances (mg/L): vitamins B1, B6, PP of 0.5; iron 10000; inositol 100; glycine 1000, bacto-agar 7000; sucrose 30000 with the addition of the following concentrations of hormones (numbers-option numbers): 1. VAR 0.1 mg/L; 2. VAR 0.2 mg/L; 3. VAR 0.3 mg/L; 4. VAR 0.4 mg/L; 5. VAR 0.1 mg/L, 2-ip 1.0 mg/L; 6. VAR 0.1 mg/L, 2-ip 3.0 mg/L; 7. VAR 0.1 mg/L, 2-ip 5.0 mg/L; 8.2-ip 1.0 mg/L; 9.2-ip 3.0 mg/L; 10.2-ip 5.0 mg/L.

After the cultivation period, the height of the shoots, the number of additional shoots in one conglomerate, the number of nodes, percent rooting, and the length of the roots were taken into account. The multiplication factor was the sum of the single-node segments. The volume of each option was 20 explants.

Statistical data processing was performed using analysis of variance and the SPSS Statistics package.

3. Results and Discussion

The best propagation method for most terry clematis varieties is green cuttings (Figure 1).

Terry varieties can also be propagated by layering and dividing the bush. Laying out overwintered shoots in the spring around the bush, hilling them, you can get several rooted plants in 30-45 days. The division is best done in the spring, before the buds open. But the reproduction rate with such methods is not high.

When propagating such varieties of clematis by green cuttings, the following features should be taken into account. If reproduction occurs in a winter greenhouse, then the first cuttings should be carried out in late March-early April. To do this, in the fall it is necessary to apply a strong pruning of the uterine bushes, that is, remove all shoots to a height of 20-30 cm, which will allow you to adjust the tailoring intensity and the growth rate of the growing shoots. There will be no flowering on last year's shoots, as they are not preserved. But in the spring, shoots on the uterine bushes begin to grow rapidly, reaching a gain of up to 10 cm per day. Cuttings begin from the stage of budding. In spring cuttings, shoots, as a rule, have a large number of nodes (more than 8-10 pieces) and flower buds are laid closer to the top of the shoot, which significantly increases the yield of cuttings.

For rooting, cuttings are used only with vegetative buds in the axils of the leaves. Such cuttings are well rooted and quickly give growth. When rooting cuttings with generative buds in the axils of the leaves, the rooting percentage drops sharply, and there is no growth of shoots.

Terry varieties may be cut during the summer, but a number of difficulties arise in the summer. With an increase in air temperature (more than 25 °C), the number of nodes with each subsequent pruning is reduced in uterine plants. Flower buds are laid lower and lower, by the end of the season it is already 3-4 knots from the ground. At the same time, the yield of cuttings from the uterine plant is significantly reduced. Therefore, in June-July it is better not to cuttings, but to allow the plants to bloom and prune in early August, so that by the end of August,

when the temperature drops to 18-20 °C, shoots with sufficiently high flower buds can be obtained. However, it is necessary to ensure that the temperature during rooting of cuttings in autumn does not drop below 15 °C. If rooted cuttings remain in the greenhouses for the winter, then you need to provide them with a sufficient amount of light.

The study examined the rooting of the eight most common terry clematis varieties: Bellof Woking, Blue Light, Crystal Fountain, Empress, Evijohil (Josephine), Fair Rosamond, Multi Blue, Purpurea Plena Elegans.

Table 1. Rooting of green cuttings of the studied varieties of clematis, 2018-2019

No.	Sort	The rooting of spring cuttings (%)	The rooting of summer cuttings (%)
1.	Bellof Woking	83	49
2.	Blue Light	79	56
3.	Crystal Fountain	65	50
4.	Empress	81	45
5.	Evijohil	62	40
6.	Multi Blue	39	16
7.	Fair Rosamond	73	50
8.	Purpurea Plena Elegans	35	10

According to the data obtained, it can be concluded that most varieties give a large yield of cuttings during spring cuttings. Especially worthy of note are the varieties: Bellof Woking (83%), Empress (81%), Blue Light (79%). They gave the highest rates of rooting in the spring period of cuttings. Therefore, for the industrial propagation of these varieties, the traditional method is recommended-green cuttings (Table 1).

Two varieties (Purpurea Plena Elegans, Multi Blue) showed a low rooting percentage at two grafting periods; therefore, *in vitro* propagation technology is used for them (Korotkov & Korotkova, 2005).

Most varieties from the terry clematis group are imported from warm European regions, so plants need time to adapt to growing conditions to plant uterine plantings and landscaping. It is necessary to optimize the soil conditions, illumination, irrigation and top dressing, so that the plants take root better, take root and winter successfully.

Planting of young plants can be carried out almost throughout the growing season, but it is preferable to do everything in the early spring or autumn. For the arrangement of mother plants, it is better to use two-year-old seedlings in containers (with a closed root system), with well-developed 6-10 roots about 10-15 cm long.

For planting, choose a well-lit place, and the lower part of the plant may be in partial shade, which will favorably affect the growth of the vine. Shoots need strong support and protection from strong winds. Clematis does not tolerate stagnation of water in the root zone; this is one of the main causes of their death (McMillan Brown, 1987).

The plant is planted in a spacious (60 × 60 × 60 cm) pit with loose, nutritious soil, deepening the root neck by 1-2 knots (about 8 cm). From the buried buds, a tailoring center forms over time, and subsequently strong, abundantly flowering bushes develop that do not suffer from frost and overheating. However, weak seedlings cannot be deeply buried, as this will impede the development of young shoots. For planting, a light nutrient substrate is prepared in advance from peat, turf land, sand, and compost. Moreover, the acidity of the substrate should be in the range of pH 7.5-8 (slightly alkaline). Since most fertilizers acidify loamy and clay soil, when planting on these types of soils, you can add organic fertilizers or deoxidize the soil with lime.

Clematis, like all beautifully flowering vines, is responsive to the application of mineral fertilizers, especially trace elements. It is advisable to make basic fertilizers under the root in the first half of summer, combining this with foliar dressing with microelements (spraying on leaves). Extra root top dressings containing molybdenum, cobalt, silicon are especially effective. During the season, 3-4 such top dressings are carried out. Plants bloom profusely, leaves turn dark green (Sokolova, 2010).

For wintering, the vine should be removed from the supports and laid on the ground. In the early years of laying queen cells it is advisable to use a light shelter. You can cover it with peat, sawdust, light non-woven material.

Liana reaches decorativeness on the 3-5th year after planting, depending on the variety and growing conditions. In one place it can grow up to 30 years.

Thus, all the studied terry varieties are recommended for use in landscaping and landscape design (Ivanova & Khanbabaeva, 2013).

Subject to the above rules of agricultural technology, these varieties can be successfully propagated in nurseries of ornamental crops in winter glazed greenhouses, grow protected ground and obtain high-quality planting material (Khanbabaeva, 2013).

Table 2. Features of the development of clematis cultivars Purpurea Plena Elegans and Multi Blue in an in vitro culture, depending on the composition of the nutrient medium after 10 weeks of cultivation

Hormone concentration (mg/L) (Option number)	Shoot length (cm)		Reproduction coefficient		Root length (cm)		Spontaneous rhizogenesis (%)	
	PPE	MB	PPE	MB	PPE	MB	PPE	MB
1) BAP 0.1	4.2	2.2	4.7	4.5	2.7	0	70	0
2) BAP 0.2	3.5	1.9	**6.1**	**4.0**	3.8	0	60	0
3) BAP 0.3	3.8	1.2	**7.7**	**4.2**	**7.7**	0	30	0
4) BAP 0.4	4.6	1.9	**6.4**	**4.6**	6.4	2.1	20	10
5) BAP 0.1, 2-IP 1.0	3.5	1.6	5.3	4.4	4.0	4.1	10	20
6) BAP 0.1, 2-IP 3.0	3.2	1.7	5.2	3.0	0	0	0	0
7) BAP 0.1, 2-IP 5.0	2.3	2.1	3.8	3.6	0	0	0	0
8) 2-IP 1.0	4.9	2.1	6.1	2.8	0	0	0	0
9) 2-IP 3.0	3.3	1.3	5.4	3.1	1,1	0	0	0
10) 2-IP 5.0	2.0	1.9	2.5	3.3	0	0	0	0
HCP05	0.1	0.1	-	-	0.8	1.1	-	-

Note. PPE: grade Purpurea Plena Elegans; MB: grade Multi Blue; 3.3: differences between options are not significant.

If the difference between the options is more than the value of HCP05, then the differences on this basis are significant (Smiryaev & Kilchevsky, 2007). If the difference between the values is less than the value of HCP05, then the differences are not significant and, accordingly, the options do not differ. For example, according to the characteristic “shoot length, cm” in the Purpurea Plena Elegans cultivar, differences between options 6 and 9 are not significant and the options do not differ. Similarly, for options No. 2 and No. 5 on the basis of “Length of roots, cm” (Table 2).

For spontaneous rhizogenesis, it should be noted Option 1 (70%) and 2 (60%) for the Purpurea Plena Elegans variety. This variety has a high percentage of spontaneous rhizogenesis of 70% in a medium supplemented with a hormone in a BAP concentration of 0.1 mg/L. This feature of the variety for spontaneous rhizogenesis can be used and apply for it a one-stage technology of reproduction and rooting in one nutrient medium.

The Purpurea Plena Elegans variety had the highest breeding coefficient of 7.7 in Option 3 on a medium supplemented with a hormone of BAP concentration of 0.3 mg/L, while the height of the microprobe is 3.8 cm, the number of microprobe for 1 explants is 2-3. This medium can be recommended for use at the stage of micro propagation itself (Figure 1).

In the Multi Blue variety, the highest multiplication factor of 4.6 was observed in Option 4 on a medium supplemented with a hormone at a BAP concentration of 0.4 mg/L. At the same time, the height of microprobe is 1.9 cm, conglomerates with the number of microprobe 2-4 pieces are formed. In the Multi Blue variety, spontaneous rhizogenesis was 10-20% (Figure 2).

Options 2 and 4 should also be recommended for use, giving a high reproduction rate for both varieties.

According to the results of the experiments, it was decided to use a medium with the addition of a hormone in a concentration of BAP of 0.3 mg/L and BAP of 0.4 mg/L for the propagation of micro plants depending on the variety.

At the rooting stage, an optimal nutrient medium based on half concentrations of the Murashige-Skoog (MS) mineral salts is enriched with the following substances (mg/L): vitamins B1, B6, PP 0.5 mg/L; iron 10000; inositol 100; glycine 1000; bacto-agar 7000; sucrose 20,000 with the addition of the hormone IMC 0.2 mg/L. Cuttings with two nodes were planted for rooting.

The rooting stage lasts about 8-10 weeks. The rooting rate is 70% for the Multi Blue variety (Figure 2), and 80% for the Purpurea Plena Elegans variety (Figure 1).

Figure 1. Micro plants of Clematis cultivar Purpurea Plena Elegans

Figure 2. Micro plants varieties of Clematis Multi Blue

One of the important stages is the stage of adaptation of micro plants. In the process of reproduction, test-tube plants undergo anatomical and morphological and physiological changes: micro boots grown in tissue culture have low photosynthetic activity, poor water balance, roots, do not have root hairs, and function poorly (Butenko, 1975).

Adaptation to non-sterile conditions was carried out in a Veltorf brand substrate with the following recipe: horse peat, fraction 5-20 mm, PG mix fertilizer 1.2 kg/m^3, wetting agent 0.1 L/m^3, agroperlite 10%. The yield of plants during the adaptation of micro plants of two varieties was 90-95%. Three weeks later, young plants were released from the water-retaining film. Transplantation from cartridges into plastic containers was performed after six weeks (42 days). Plants transplanted from the cassettes fell into a relatively short dormant period, after transplanting, for about two weeks (14 days).

In vitro clematis plants of the first year differed from clematis plants obtained by green cuttings using thinner shoots (Figure 3). But after overwintering in a greenhouse, clematis plants obtained by the *in viro* method noted

an active growth of more powerful shoots (1-2 pieces) and seedlings were in no way inferior to plants obtained by the green cuttings method (Figure 4).

Figure 3. Clematis seedling of Purpurea Plena Elegans cultivar 1 month after transplantation from cassettes, the first year of cultivation

Figure 4. Clematis seedling Purpurea Plena Elegans cultivar, second year of cultivation

4. Conclusions

Thus, for most of the studied terry varieties of clematis, propagation is by way of cuttings. For a larger yield of planting material, spring cuttings in March are recommended. This requires the presence of uterine plants in a protected ground. Uterine plants should be kept at a high level of agricultural technology. To obtain more cuttings, a short pruning of uterine plants is recommended, as in varieties of the third group. It is recommended that the following varieties be propagated by the green cuttings method according to standard technology: Bellof Woking, Empress, Blue Light. They gave the highest rates of rooting in the spring period of cuttings. For two varieties Purpurea Plena Elegans and Multi Blue, which are characterized by low rooting using traditional technology, *in vitro* propagation is recommended. The maximum reproduction rate for these varieties was

obtained on MS nutrient medium with the addition of BAP hormones 0.2-0.4 mg/L (Options 2, 3, 4). To adapt micro plants to non-sterile conditions, horse mineralized peat with agroperlite is used. The growing time of planting material using the technology of cuttings is 4 months, with the technology of clonally propagation—5-6 months.

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Biochar Yield From Shell of Brazil Nut Fruit and Its Effects on Soil Acidity and Phosphorus Availability in Central Amazonian Yellow Oxisol

D. M. Oliveira[1], N. P. S. Falcão[1], J. B. D. Damaceno[2] & I. A. Guerrini[3]

[1] National Institute for Amazonian Research, Technology and Innovation Coordination, Manaus, Amazonas, Brazil

[2] Tropical Agronomy Graduate Program, Amazonas Federal University, Manaus, Amazonas, Brazil

[3] College of Agricultural Sciences, São Paulo State University, Botucatu, São Paulo, Brazil

Correspondence: D. M. Oliveira, National Institute for Amazonian Research, Technology and Innovation Coordination, Manaus, Amazonas, Brazil. E-mail: dmoliveira.am@gmail.com

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Abstract

Phosphorus is one of the most limiting elements in the amazon soil, requiring low cost alternatives that increase the agronomic efficiency of phosphate fertilizers for satisfactory crop production and biochar has been used as an option for increase soil fertility. The objective of this work was to evaluate the yield and properties of the biochar produced from the shell of Brazil nut fruit at 500 ^{o}C, as well as its behavior in the acidity and phosphorus availability from mineral source in Yellow Oxisol. The experimental design was completely randomized in a factorial arrangement (5 × 5) with five doses of biochar (0, 20, 40, 60 and 80 t ha^{-1}) and five doses of P_2O_5 (0, 100, 200, 300 and 400 kg ha^{-1}) in 20 kg pots. The trial was carried out at 365 days and the yield and properties of the produced biochar were evaluated, as well as the determination of acidity and total and available phosphorus. The biochar produced from feedstock was considered satisfactory, with 59%, which is a good alternative for producers. Aluminum contents were reduced confirming the potential of biochar as a corrective for acidity. Additionally, the amount of total and available phosphorus increased with increasing biochar doses. Thus, not only the feedstock but also the pyrolysis temperature showed hight potencial to improve the amount of phosphorus in the soil and decrease the soil acidity.

Keywords: soil fertility, pyrogenic carbon, perennial culture

1. Introduction

The Amazonian soils phosphorus fixation process is stronger than other Brazilian regions, due to the presence of large amounts of iron and aluminum oxides and hydroxides, kaolinite, which limit the crops production. Brazilian agriculture apply around of 15 million tons of P per year (Wang et al., 2012), of which only 5 to 20% is uptake by culture (Price, 2006). In addition, the estimated lifetime of the world's phosphorus reserves would be around 40 years (Streubel et al., 2012). These facts show the need to adopt strategies that increase available P and improve the agronomic efficiency of short term mineral fertilizers.

Peng et al. (2012), Streubel et al. (2012), and Yao et al. (2013b) demonstrated that biochar, besides being a source of P, could be capable of adsorbing phosphate. This suggests its potential in maintaining the applied P as fertilizer. Recently studies showed that the use of biochar can increase soil phosphorus availability (Atkinson et al., 2011; Edelstein & Tonjes, 2012; Farrell et al., 2014; Glaser et al., 2002; Gul et al., 2015; Sohi et al., 2010; Falcao et al., 2018; Glaser & Lehr, 2019), in addition, the use of biocarbonised organic waste (biochar) can have its effect confirmed as a soil conditioner and as a plant nutrient source based on Amazonian Dark Earth studies.

In the Amazon, due to soil origin and intense rainfall, inconveniences with phosphate fertilization become even more critical, but the maintenance of high levels of phosphorus, calcium, magnesium, zinc and organic carbon in the Amazonian Dark Earth is associated to a large and prolonged input of fresh and biocarbonised organic material (pyrogenic carbon) (Glaser et al., 2001; Lehmann et al., 2002, 2003b).

Thus, it is necessary to understand and rescue techniques that have been used by local Indians for centuries (Steiner et al., 2004), linking them with existing technologies such as mineral fertilizers. The practice of biocarbonization aims mainly at harnessing residues from agroindustrial activities with biochar production and its use in improving the physical, chemical and biological quality of degraded soils (Wang et al., 2012; Yuan et al., 2011a, 2011b). It also has a positive impact on reducing greenhouse gas (GHG) emissions by increasing carbon stocks in soils and improving agricultural production and recovery processes of degraded areas (Nóbrega, 2011). However, the levels of macro (P, K, Ca and Mg) and micronutrients (Mn and Zn) in the biochar composition are low, so the use of biochar in the soil requires additional nutrient sources to complement fertilization, increasing the agronomic efficiency of mineral and organic fertilizers, meeting the nutritional needs for growth and production of cultivated species (Falcao et al., 2018; Glaser & Lehr, 2019). Thus, the objective of this work was evaluate the potential of Brazil nut bark biochar to increase the amounts of total and available phosphorus and also the decrease the acidity index in the Amazonian Oxisol.

2. Material and Methods

The experiment was conducted in a greenhouse at the National Institute Amazon for Research-INPA, Manaus, (3°5′29″S; 59°59′37″W) Amazonas. The experimental design was completely randomized, in a factorial arrangement (5 × 5) with five levels of biochar (0, 20, 40, 60 and 80 t ha^{-1}), produced under a temperature of 500 ºC and five doses of P_2O_5 (0, 100, 200, 300 and 400 kg ha^{-1}), been as source triple superphosphate with 41% of P_2O_5, totalizing 25 treatments, with 4 replications and 100 experimental units. A complementary fertilization with 400 Kg ha^{-1} of N as urea, 532 kg ha^{-1} of K_2O as KCl and 80 kg ha^{-1} of micronutrients (FTE), all in a split form.

The soil used was a typical dystrophic Yellow Oxisol, clayey texture, collected from the 20-40 cm layer (Sombroek, 1966) with the following characteristics: pH CaCl (3.60), Ca^{2+} (6.00 $mmol_c$ dm^{-3}), Mg^{2+} (2.00 $mmol_c$ dm^{-3}), Al^{3+} (9.00 $mmol_c$ dm^{-3}), H+Al (31.00 $mmol_c$ dm^{-3}) K^+ (1.33 $mmol_c$ dm^{-3}), CTC (39.00), V% (22.00), P (3.00 mg dm^{-3}), S (47.00 $mmol_c$ dm^{-3}), Fe (251.40 mg dm^{-3}), Zn (1.13 mg dm^{-3}) and Mn (0.57 mg dm^{-3}) as determined by Resin method. The soil was passed through sieves with 2.00 mm diameter mesh and placed in 20 L plastic pots, filled with 20 kg of soil, and the treatments were all homogenized with biochar and mineral fertilizers.

The biochar was obtained from the biocarbonization of the dry biomass of the shell of Brazil nut fruit harvested in the 2014, in a retort located at the Forest Products Research Coordination of the National Institute for Amazon Research (CPPF-INPA). The biocarbonization was temperature of 500 ºC reached after 2h30 with residence time of 12 hours, the biochar was removed from retort after total cooling at room temperature, than was manually ground using a wood piece and passed through a 2 mm sieve.

The chemical characterization was done in order to obtain total contents of nutrients in the biochar, characteristics such as moisture and ash content were also determined, with biochar samples passed in a 1 mm sieve knife mill. To determine the humidity, a biochar mass was oven dried at 105 ºC for 24 hours and cooled in a desiccator at room temperature. The samples were weighed and calculated according to the equation 1:

$$U = (\text{dry } M)/(\text{wet}) \times 100 \quad (1)$$

The ashes contents was determined in a muffle (QUIMIS), with residence time of 3.5 hours, then weighed in analytical balance and the calculation of ashes performed as presented in the equation 2:

$$\%\ \text{ashes} = [(D - B)/(C - B)] \times 100 \quad (2)$$

Where, B = mass of calcined crucible (g); C = mass of crucible with initial sample on dry basis (g); D = mass of ash crucible (g).

The soil was collected twelfth month after the trial installation, been three single samples per pot, making up one compost sample, than put to dry environmental temperature, than a sieved in a 2.00 mm mesh sieve an than conducted to the soil laboratory for the following determinations: pH ($CaCl_2$) at ratio 1:2.5 (soil:solution) (Raij et al., 2001); exchangeable Al^{3+} was extracted by 1 mol L^{-1} KCl quantified in atomic absorption; Potential acidity (H+Al) was determined by the SMP buffer method (Shoemaker, McLean, & Pratt, 1961), and Ca^{2+}, Mg^{2+} and available phosphorus (P) were extracted by ion exchange resin and determined by colorimetry (Raij et al., 2001). Total phosphorus was extracted according to the method proposed by Hedley et al. (1982), with some modifications proposed by Codron et al. (1985) and ICP reading.

The results were analyzing by variance analyses (ANOVA) and subsequent regression analysis aiming to adjust the equations to the data obtained as a function of doses for each treatments. It was adopted as criterion in the choice of the model the interaction by the significant test F at 5% and 1%, with the aid of the R statistical program (R Core Team 2018).

3. Results and Discussion

The biocarbonization of the shell of Brazil nut fruit produced 59% for biochar, 33% for pyroligneous acid and 2% for tar. In general, most of the feedstock materials used for biochar production yield a maximum of 40%, depending on the temperature and the lignin content that is proportional to the yield. The high yield of biochar shows that this raw material has great potential for use not only by producers, but also by the traditional population living on extractivism, thus avoiding the disposal of this material in areas that may contribute to the proliferation of insect vectors of various tropical diseases.

Galinatto et al. (2011) assessing the economic potential of biochar in winter wheat crop, from production and use as soil conditioner to carbon stock estimates, concluded that depending on the market price of the product, biochar may be profitable and may yet, enter the carbon offset market.

The ash content of the shell of Brazil nut fruit biochar and the moisture content were 2.9% and 6.0%, respectively. According to Demeyer et al. (2001) and Barros (2006) a good biochar should be in a range of 3% and 4% ash and less than 8% humidity, demonstrating that the characteristics obtained here are with the average value within the proper range. Adsorption capacity due to low ash content can be positively influenced due to volatilization of organic material (Ramos et al., 2009; Cisnero and Gonzáles, 2010).

According to Cruz Júnior (2010), the basic ash composition of shell of Brazil nut fruit is of K_2O (30.8%), CaO (17.1%), SiO_2 (15.6%), Fe_2O_3 (13.6%), S, P, Mg, Al, Na, Mn, Ti, Cl, Ni and some metals in low contents. Table 1 shows the chemical attributes of biochar from Brazil nuts bark at a temperature of 500 ºC. We can observe by the chemical characteristics presented that this material should not be considered a source of fertilizer, but a conditioner of the soil, since when used alone, it influences much more in the soil structure, improving water retention, aeration, root system growth and soil biological activity. Lehmann et al. (2003b) and Van Zwieten, et al. (2010), attributed the higher plant growth to the positive changes in soil biogeochemistry resulting from biochar additions. However, Glaser and Lehr (2019), mentioned that biochar can be considered a source of phosphorus to low fertility soils.

Table 1. Chemical characteristics of the chestnut urchin biochar produced at 500 °C

Material	pH	N	P	K	Ca	Mg	S	B	Cu	Fe	Mn	Zn	Na
	$CaCl_2$	---------- g kg^{-1} ----------						---------- mg kg^{-1} ----------					
Biochar 500 ºC	9.1	7.0	0.6	23.0	6.0	2.4	1.4	41	28	575	264	25	159

In general, as higher the pyrolysis temperature, as higher the pH, ash content, carbon stability, biochar aromaticity, porosity and specific surface area (Wu et al., 2012; Zhao et al., 2013; Zhang et al., 2015b). Ringer et al. (2006) report that under slow pyrolysis conditions the yield is about 35% biochar, 30% bio-oil (pyroligneous acid and tar) and 35% gas and Wright et al. (2010) describe lower yields of biochar in rapid pyrolysis compared to slow pyrolysis, as it generates about 15% biochar, 70% bio-oil and 13% gas.

Based the reference the total N content found in organic compounds, the value found in the biochar used in this work was considered “low”, as well as the concentrations of P, Ca, K and Mg, however, these are strongly influenced by temperature, the temperature increase also provides the increase of trace elements that are present as Fe, Cu Mn, Zn and Cd, however the bioavailability for the plant is very low and these concentrations are decreased with increasing temperature by changing their chemical forms during the process, however, Fe and Mn concentrations were elevated.

The nutritional characteristics of biochar vary depending on the material used in pyrolysis, but in most cases there is an increase in pH, CTC, macro and micronutrient contents (Hossain et al., 2011; Jeffery et al., 2011; Agrafioti et al., 2013; Yuan et al., 2013; Masek et al., 2013).

Similarly, very different responses to growth of different crops have been found for sugarcane bagasse biochar and biosolids (Chen et al., 2010). Based on the results observed in the present study, they emphasize the importance of quantifying the yield of biochars made from different raw materials before large-scale application.

3.1 Effect of Treatments on Acidity, Available and Total P Contents

The results revealed that there was interaction between the doses of biochar and phosphate fertilization ($p < 0.05$), for pH values in $CaCl_2$, with an increase of 1.44 pH units, when comparing treatments without biochar and 80 t ha^{-1} both in the absence of P_2O_5 (Table 2). Van Zwiten et al. (2010) assessing the effect of biochar on acidic soils in Australia found that applying only biochar increased soil pH considerably, while treatment that received biochar and mineral fertilizer decreased soil pH.

Table 2. Effect of different doses of biochar and P_2O_5 in Yellow Oxisol on pH ($CaCl_2$) at 365 days after the installation of the experiment

P_2O_5 (kg ha^{-1})	Biochar t ha^{-1}				
	0	20	40	60	80
0	3.55 Ac	3.53 Ac	3.76 Abc	4.27 Ab	4.99 Aa
100	3.46 Ac	3.45 Ac	3.84 Abc	4.33 Aab	4.75 Aba
200	3.45 Ac	3.48 Ac	4.08 Ab	4.58 Ab	5.32 ABCa
300	3.40 Ab	3.51 Ab	3.73 Ab	4.71 Aa	4.72 BCa
400	3.42 Ac	3.47 Ac	4.11 Ab	4.74 Aa	4.34 Cab

Note. Averages followed by the same lowercase letters in horizontal do not differ statistically from each other. Averages followed by the same capital letters in vertical do not differ statistically from each other.

In this experiment, it was also noted that treatments without biochar showed a slight acidification with increasing doses of P_2O_5, decreasing by 0.13 pH units. Agreeing with Solaiman et al. (2012), who evaluated the effects of biochar applied to soils under different management systems, observed that in the treatment that received biochar with NPK fertilizer, the soil pH increased in relation to the control treatment, and those treatments that received only NPK the pH decreased in the corn, cowpea and peanuts crops.

The pH values increased considerably in the treatment that received 40 t ha^{-1} of biochar, this results may be explained by the high biochar adsorption capacity, since the temperature of 500 °C has a high surface area and a more condensed carbon structure. (Downie et al., 2009), which may have facilitated the retention of cations such as Al^{3+} and Fe^{2+}.

In general, the doses of biochar presented a linear effect on the pH values, however, considering the ideal pH range for soil fertility, it can be seen that the doses from 40 t ha^{-1} of biochar independent of the P_2O_5 doses provided more adequate values, not only for plant nutrient availability but also in economic terms, since, from the 100 kg ha^{-1} doses of P_2O_5, the variation of values was minimal.

Jeffery et al. (2011) in a review of the effects of biochar on soil, concluded that such practice provides a gain in crop yield, regardless of the type of feedstock and the amount of application, with an average increase of about 10% and attributed this gain. The contribution of biochar in increasing pH, water retention capacity and nutrient availability.

These pH values are a reflection of the process of neutralization of exchangeable aluminium. According to Raij (2011), aluminium the main element that is associated with the negative effect of soil acidity on plants. In Brazil, the use of limestone to increase pH values is common, however, Caires et al. (2008), reports that this may have limited action since it is not promoting effect of acidity reduction in the subsurpeficial layers, which are dependent on the carbonate leaching process. In on other hand, the present study the limestone was not applied and the process of neutralizing exchangeable acidity was attributed to biochar. These results corroborate with Van Zwiten et al. (2010) and Berek et al. (2011) that in a field experiment installed on Hawaii's acid soils using 3.0 levels of biochar (0, 2.5 and 5%) cultivating *Desmodium ovalifolium* reported a decrease in Al content from 1.4 to 0.6 $cmol_c$ kg. Similar results was found (Figure 1) in this study when increased the levels of biochar the exchangeable Al decreased in all treatments.

Although for the Al contents, there was no significant effect among the studied factors, it can be observed that the application of biochar, started the process of neutralization of Al at the first applied dose and was reduced as the doses increased biochar, (Figure 1)which allows us to infer about the power of biochar as an acidity corrector, this results may be occurred due to its ash content or physical characteristics, which makes it a great ally in the pH change processes. Malavolta et al. (2006) reports that high levels of Al impair the development of the root

system and reduce the availability of nutrients such as P and S, making biochar a promising material in reducing such inconveniences.

The application of 80 t ha^{-1} of biochar reduced the Al content in soil by over 90%, which may be linked to the amount of ash present in the biochar (2.9%) which represents a "good" indicator of agronomic use.

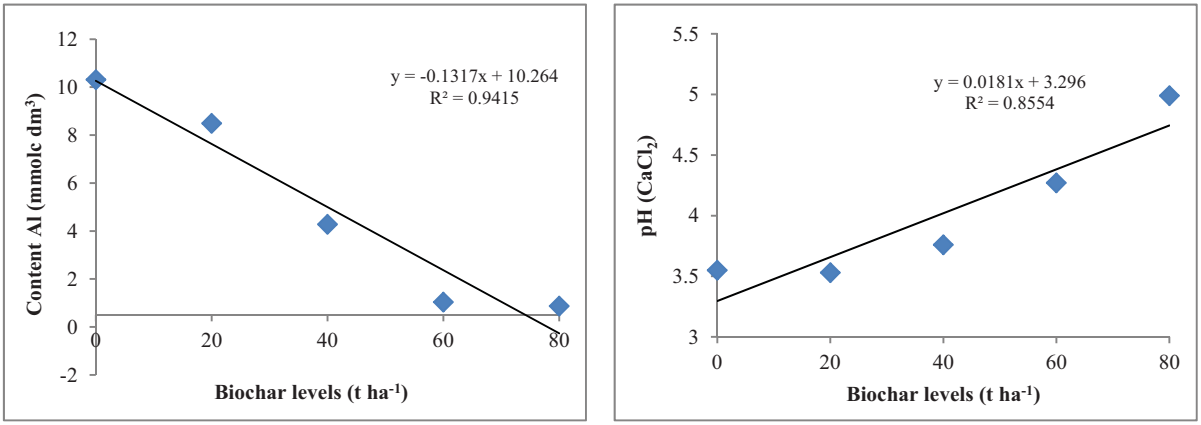

Figure 1. Acidity indices as a function of biochar levels after 365 days of application

Similar to Al^{3+} contents, H+Al contents were strongly influenced by biochar application. The application of 60 t ha^{-1} of biochar provided potential acidity reduction by approximately 50% (Figure 2).

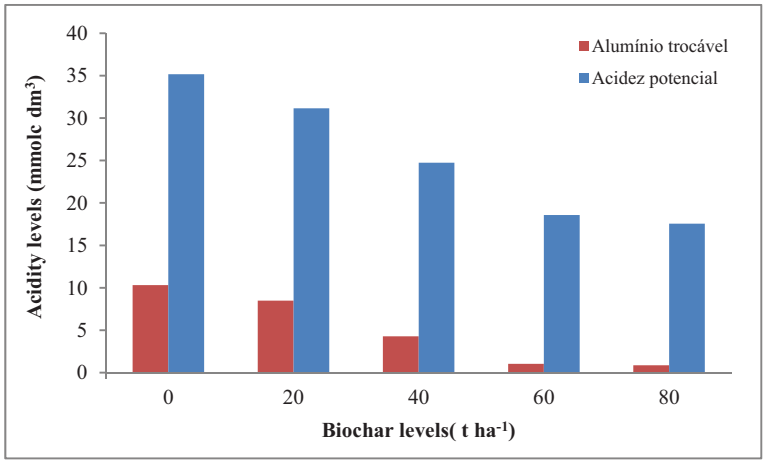

Figure 2. Exchangeable aluminum content and potential acidity 365 days after application of biochar levels

3.2 Available and Total Soil Phosphorus Content

The treatments resulted in significant differences in the phosphorus contents, being more pronounced between the doses of P_2O_5 applied. However, there was a considerable increase among treatments that received 400 kg ha^{-1} P_2O_5, where in the absence of biochar the P content was 21.70 mg kg and with only 20 t ha^{-1} biochar the P was 38.77, which was statistically equal to the other biochar levels.

Coal samples taken from different black earth sites and at different depths showed different P adsorption and desorption properties (Falcão et al., 2003). These results allowed us to infer that this coal has the potential to retain significant amounts of solubilized P from mineral fertilizers, thus avoiding its chemical fixation by iron and aluminum oxides and type 1:1 clay present in high concentrations in tropical soils.

Table 3. Effect of different doses of biochar and P_2O_5 in Yellow Oxisol on Phosphorus contents available 365 days after the installation of the experiment

P_2O_5 (kg ha^{-1})	Biochar (t ha^{-1})				
	0	20	40	60	80
0	2..44 C	2.17 D	2.48 D	2.56 C	2.68 B
100	8.57 BC	9.68 CD	7.91 CD	6.70 C	6.58 B
200	19.71 Abab	16.46 BCab	16.75 BCab	14.19 BCb	28.27 Aa
300	24.47 A	25.22 B	22.81 B	21.19 B	25.14 A
400	21.70 Ab	38.77 Aa	41.55 Aa	35.78 Aa	32.19 Aab

Note. Averages followed by the same lowercase letters in horizontal do not differ statistically from each other. Averages followed by the same capital letters in vertical do not differ statistically from each other.

With the application of P_2O_5 doses without biochar, a significant increase up to 300 kg ha^{-1} P_2O_5 dose was observed, but the application of 20 t of biochar the increase was linear until the maximum dose of P_2O_5. The levels of phosphorus decreased when 40 t of biochar was applied, which may be linked to the increase in pH, leading to the increase of available micronutrient contents in the soil solution and thus forming insoluble compounds such as Fe phosphate and Mn phosphate, the higher the amount of biochar, the greater the adsorption effect of P. However, Nelson et al. (2011) and Qayyum et al. (2015), reported that the application of 5,0 t of biochar temporarily reduced the available P content in two soil types, but in Oxisol did not significantly affect the availability of P with extra amounts of P was applied.

Since approximately 75% of P applied to Brazilian soils, as soluble phosphate fertilizer, is sorbed on the colloidal soil particles resulting in a low agronomic efficiency of this element (Raij, 2011), the presence of biochar may represent an “ally” in the soil cost reduction process in phosphate fertilizer.

Oliveira et al. (2019), working with bamboo biochar under different temperatures and different phosphate sources, reports that the P levels were higher in the treatment with biochar (500 ºC) and triple superphosphate, in a rotation of 4 successive cycles, also allowing the observation of a longer effect of this soluble source

It is also noted that P levels, regardless of P_2O_5 levels, were affected by the presence of biochar, which may be associated with P sorption capacity by biochar, and may play an important role in the sorption and desorption of P in the process soil, acting as a more efficient P adsorbent per gram of material than soil clay fractions. Rajapaksha et al. (2015) reported that the presence of biochar in the soil resulted in increased pH, CTC and P content.

These results may also be related to the characteristics of biochar, since pyrolysis performed at high temperature generally produces biochars with high surface area (> 400 m^2/g) (Downie et al., 2009; Keiluweit et al., 2010), highly aromatic and consequently very recalcitrant to decomposition (Signh et al., 2010), and is considered good adsorbent (Mizuta et al., 2004; Lima & Marshall, 2005), of cationic and anionic charges (Morales et al., 2013).

The reasons for the high efficiency of pyrogenic carbon in nutrient retention are: (a) biochar has a larger specific surface than coal resulting from burning wood at higher temperatures and (b) has a higher negative charge density per unit area consequently a higher charge density (Liang et al., 2014). This high charge density may, in principle, cause greater oxidation of the pyrogenic carbon itself or by non-pyrogenic carbon adsorption (Lehmann et al., 2005).

Although no significant difference was observed, in the absence of P, the treatments that received 80 t ha^{-1} of biochar showed a slight increase compared to the control. Comparing the control treatment with that received 40 t of biochar in the absence of P_2O_5 and the treatment that received 40 t of biochar with 100 kg of P_2O_5, we can see a highly significant increase in soil available P content, with a variation from 2.44 to 2.48 jumping to 7.91, going from a range “very low or low” to “medium”, considering ideal levels according to Raij (2011). In an economic aspect, it can be noted that the lower amount of P_2O_5 may be more efficient in the availability of P.

The total phosphorus contents increased as increased the levels of biochar independent of the amounts of P applied. A big part of this P liberated from different sources of fertilizer was, probably, linked to formations with Fe and Al. It is also observed that only the application of 80 t ha^{-1} of biochar, the amount of P increased by approximately 500%, and it can be inferred that biochar can be considered a source of total P (Table 4).

In Brazilian soils, Guerra et al. (1996), and Cunha et al. (2007) found a variation of 13 to 47% of the total P occurring in the form of Po and Duda (2000) reports from 7 to 83%, and this variation may be, according to

these authors, related to the contents of C and total soil phosphorus. Wang et al. (2012) describe that some raw materials and carbonization temperatures, between 250 °C and 550 °C, and which have high ash content generate high carbon content with higher P content and larger element recoverability, thus may be potential sources of P with high agronomic efficiency.

Table 4. Effect of different doses of biochar and P_2O_5 in Yellow Oxisol on total Phosphorus contents 365 days after the installation of the experiment

P_2O_5 (kg ha^{-1})	Biochar (t ha^{-1})				
	0	20	40	60	80
0	7.02 Bb	9.25 Db	15.10 Dab	23.00 Dab	34.6 Ba
100	27.10 B	32.87 C	37.55 C	40.97 CD	38.45 B
200	57.32 Ab	54.15 BCb	58.60 BCb	62.45 BCab	80.87 Aa
300	64.72 A	69.30 AB	73.30 B	79.55 B	82.82 A
400	62.32 Ad	89.40 Ac	122.47 Aa	112.55 Aab	97.27 Abc

Note. Averages followed by the same lowercase letters in horizontal do not differ statistically from each other. Averages followed by the same capital letters in vertical do not differ statistically from each other.

When comparing the available phosphorus and total phosphorus contents, as a function of the biochar doses, it is observed that for the total phosphorus, there was a significant increase even the material with P contents (0.6 g Kg), and 5.1 mg kg. Wang et al. (2013), showed that the effect of P availability by biochar can be influenced by the P content present in the material, however, it is available P, which in this experiment showed a slight increase.

Chintala et al. (2014) also found that P adsorption in biochar was significantly ($p < 0.0001$) affected by the initial concentration of P and biochar type. These results indicate that not all biochars can be used to increase retention of P fertilizers in soils. However, biochar's ability to increase P retention in soils is quite variable and varies with P concentration in the soil solution.

Jorio et al. (2012), report that in black earth coal grains were found, through microscopy techniques, P contents at the edges of the material, showing inert internal structure, similar to graphite external structure with some nutrients such as Ca, P and Al.

4. Conclusion

The biochar produced from shell of Brazil nut fruit presented high yield for biochar production.

The biochar produced from shell of Brazil nut fruit showed high potential to corrective action and also as a source of total phosphorus.

The increase of biochar levels promoted the increase of availability of phosphorus from mineral sources.

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Use of Amazon Fruits Barks as Source of Nutrients

I. F. Montero[1,2], S. A. M. Saravia[3], R. A. Santos[4], J. A. F. Marcia[5], S. M. Silva[6] & E. A. Chagas[2,7]

[1] Polytechnic School, Department of Organic and Inorganic Chemistry, University of Extremadura, Cáceres, Spain

[2] Postgraduate Program in Biodiversity and Biotecnology, State Coordination of Roraima, Federal University of Roraima, Campus Paricarana, Boa Vista, RR, Brazil

[3] Faculty of Earth Sciences and Conservation, National University of Agriculture, Catacamas, Olancho, Honduras

[4] Department of Food Science, Louisiana State University, USA

[5] Faculty of Technological Sciences, National University of Agriculture, Catacamas, Olancho, Honduras

[6] Graduate Program in Health Sciences, Federal University of Roraima, Campus Paricarana, Boa Vista, RR, Brazil

[7] Brazilian Agricultural Research Corporation (Embrapa), Boa Vista, Brazil

Correspondence: I. M. Fernández, Polytechnic School, Department of Organic and Inorganic Chemistry, University of Extremadura, Avenida de la Universidad s/n, Cáceres, Spain. E-mail: ismonterof@unex.es

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Abstract

The barks of fruits are usually discarded as organic waste; a valuable source of nutrients is obtained are used as a starting source in the preparation of functional foods. In this work, the physicochemical properties (pH, titrable acidity and soluble solids), mineral and bromatological analysis of nine Amazonian fruits were studied: *abiu*, *acerola*, *araçá*, *bacupari*, *biribá*, *camu-camu*, *fruta-do-conde*, *araçá* and *taperebá*. The most acidic values stand out for the different fruits, with the exception of the abiu bark (pH = 4.7). As for its nutritional contribution, it was the *araçá* barks that presented the highest energy value of 276.29 Kcal 100 g^{-1}. Among the macrominerals, the potassium concentration stands out, being the highest concentration for the *graviola* bark, 521.04 mg 100 g^{-1} followed by magnesium, where the concentration in the *biribá* was 64.21 mg 100 g^{-1}. On the other hand, the husks are rich in micronutrients, highlighting the concentration of zinc in the bark of *araçá*, 12.23 mg 100 g^{-1} and manganese in the bark of *abiu*, 6.84 mg 100 g^{-1}. The Pearson correlation coefficient presented a highly significant correlation for Fe-Al (0.96), P-Fe (0.94) and Fe-Zn (0.89). O bligpot of principal components (PCA) explains 56% of the cases, being the minerals Mg, Na, Co, K, S and Ca highly associated for the *graviola* and *bacuparí*.

Keywords: minerals, sustainability, bromatology, foods

1. Introduction

Brazil produces about 140 million tons of food per year, being the largest exporters of agricultural products, but at the same time there are problems with waste (Godim et al., 2005). From the foods wasted, 32 million tons are fruit (Maia et al., 2007). Among those industrial waste we have the part of the fruit husks, rich in nutrients, they are source of compounds with antioxidant activity (Montero et al., 2018). In addition, they have minerals both in high concentrations and in moth concentrations, as well as source of vitamins.

In Brazil, an important amount of waste is generated in the processing of fruits, mainly composed of husks, barks and bagasse, whose common destination is being discarded or destined for the production of fertilizers, occupying a total volume of 40% of processed fruits (Silva, 2014; Ajila et al., 2007). Among the main industrial residues in Brazil are the residues of the wine industry with a high concentration of antioxidants (Rubilar et al., 2007), in fruits, for example the tomato (*Solanum lycopersicum*) (Correia et al., 2004), the *goiaba* (*Psidium guajava*) (Melo & Vilela, 2005) and malted barley (*Hordeum vulgare*) in the beer industry (Santos, 2005).

Due to the nutritional importance of fruit residues, in this study, the fruits of nine fruits were evaluated in northern Amazonia: *abiu* (*Pouteria caimito*), *acerola* (*Malpighia emarginata*), *araçá* (*Psidium cattleianum*), *bacuparí* (*Rheedia gardneriana*), *biribá* (*Rollinia mucosa*), *camu-camu* (*Myrciaria dubia*), *fruta-do-conde* (*Annona squamosa*), *graviola* (*Annona muricata*) and *taperebá* (*Spondias mombin* L.), the nutritional value,

macro and microminerals, as well as the physico-chemical properties (pH, titratable acidity, total soluble solids) and reducing and non-reducing sugars in order to be considered the use of the residues for the production of bioproducts.

2. Materials and Methods

2.1 Preparation of Samples

Samples (Table 1) were collected from fruit markets and producers in Roraima state, Brazil. Then, the collected fruits were taken to the Laboratory of the Agronomic Research Center, at the Agricultural Sciences Center, Cauamé campus, Federal University of Roraima, fruits with good appearance were selected, washed previously with distilled water and then with hypochlorite solution of sodium chloride and finally with distilled water again.

Table 1. Names and families of fruits cultivated in the Northern Amazon studied in this work

Scientific name	Family	Name in Brazil
Pouteria caimito	Sapotaceae	*Abiu*
Malpighia emarginata	Malpighiaceae	*Acerola*
Psidium cattleianum	Myrtaceae	*Araçá*
Rheedia gardneriana	Clusiaceae	*Bacuparí*
Rollinia mucosa	Annonaceae	*Biribá*
Myrciaria dúbia (Krunth) Mc Vaugh, Myrtaceae	Myrtaceae	*Camu-camu*
Annona squamosa	Annonaceae	*Fruta-do-conde*
Annona muricata	Annonaceae	*Graviola*
Spondias mombin L.	Anacardiaceae	*Taperebá*

The fruits were pulped, weighed and frozen in an ultra-freezer at -80 ^{o}C for further lyophilization in Liotop L101 lyophilizer for 48 hours, until complete drying. After drying, the samples were ground in a knife mill and sieved between 30-40 Mesh, and stored in hermetically sealed sachets and protected from light to perform nutritional.

2.2 Physical Chemistry Parameters: pH, Titratable Acidity and Soluble Solids

The pH was determined by potentiometry using a pH meter previously calibrated. The titratable acidity (AT) was determined by diluting 5 grams of lyophilized material, dissolved in 100 mL of distilled water with NaOH titration (0.1 M) until the phenolphthalein was turned (pH 8.1) and the results expressed as g citric acid in 100 g of pulp. Soluble solids (SS) were determined by refractometry with the fresh samples, expressed in ºBrix and lastly, the SS/TA ratio were determined by the ratio between soluble solids content and titratable acidity (IAL, 2008).

2.3 Nutritional Analysis

The physical parameters evaluated to determine the nutritional composition were the percentage of moisture and ash. The other nutritional parameters evaluated were the determination of total proteins, lipids and carbohydrates, to determine the total energy content (IAL, 2008).

2.3.1 Determination of Humidity

To determine moisture, 5 g of fresh samples were placed in porcelain capsules for 6 hours at 105 °C to constant mass, and then cooled in desiccator to room temperature (IAL, 2008).

$$\text{Humidity (g/100 g)} = [(P' - P'')/(P' - P)] \times 100 \quad (1)$$

Where, P = weight of porcelain capsule (g); P' = weight of the porcelain capsule + fresh sample (g); P'' = weight of the capsule + sample after the oven (g).

2.3.2 Determination of Ashes

To determine the ash in the samples, the methodology proposed for the food analysis (IAL, 2008) with modifications was used, where 5 grams of the lyophilized samples were weighed. These were placed in preheated porcelain crucibles in an oven at 110 °C for one hour, to remove moisture, and cool them in a desiccator to room temperature. The samples were incinerated at 600 °C in a FDG 3P-S EDG muffle for 16 hours, after which the samples were left in the desiccator until reaching room temperature.

$$\%\text{ashes} = (N \times 100)/M \quad (2)$$

Where, N = mass in grams of ash and M = mass of the sample in grams.

2.3.3 Determination of Total Proteins

Protein determination is performed from the total nitrogen analysis by Kjeldahl distillation, in which the existing organic matter is transformed into ammonia. The nitrogen content of the different proteins is approximately 16%, which introduces the empirical factor of 5.75 (conversion factor for vegetable protein), this will transform the number of grams of nitrogen, found with the number of grams of protein (IAL, 2008).

$$\%proteins = \%N \times 5.75 \quad (3)$$

2.3.4 Determination of Lipids

To determine the total amount of lipids, 20 g of each sample was weighed, and placed in the Soxhlet extractor apparatus with hexane as the solvent for six hours. The solvent was recovered in a rotary evaporator (IAL, 2008).

$$\%lipids = (N \times 100) \times m \quad (4)$$

Where, N = mass in grams of lipids and M = mass of the sample in grams.

2.3.5 Determination of Carbohydrates

The carbohydrate content is achieved by the difference of the value 100 subtracted from the sum of the already obtained values of moisture, ashes, lipids and proteins.

$$Carbohydrates = 100 - (\%moisture + \%ash + \%lipids + \%proteins) \quad (5)$$

2.3.6 Energetic Value

In order to quantify the energy value, it was necessary to use the protein (P), lipid (L) and carbohydrate (C) contents of each sample. The result should be expressed in kcal $100g^{-1}$ (Mendes-Filho, Carvalho & de Souza, 2014).

$$Energy\ value\ (kcal/100\ g) = (P \times 4) + (L \times 9) + (C \times 4) \quad (6)$$

Where, P = value of protein (%), L = lipid value (%), C = carbohydrate value (%), 4 = conversion factor in kcal determined in calorimetric pump for proteins and carbohydrates and 9 = conversion factor in kcal determined in a calorimetric pump for lipids.

2.4 Mineralogical Analysis

The extraction of the minerals into the epidermis was done according to the methodology described by (Embrapa, 2009) in which the perchloric nitric digestion (3:1) was used in TECNAL model TE 0079 digester block, washed with distilled water up to 25 mL for subsequent analysis.

Calcium (Ca), magnesium (Mg), iron (Fe), copper (Cu), zinc (Zn), manganese (Mn) and aluminum (Al) were determined by Flame Atomic Absorption Spectrophotometry (FAAS) Shimadzu AA-7000, coupled with ASC-7000 auto sample. Calibration was performed with standard solutions prepared from commercial standards of 1000 mg L^{-1} Qhemis High Purity PACU 1000-0125, according to the specific conditions of each element (Table 2).

Table 2. Analytical parameters of calibration

Element	Technique	(λ) nm	Correlation coefficient (r^2)	LOD (mg L^{-1})	LOQ (mg L^{-1})
Ca	FAAS	422.70	0.999	0.481	2.004
Mg	FAAS	285.21	0.997	0.571	1.992
P	UV-Vis spectroscopy	660.00	0.999	0.113	1.773
K	AES	766.50	0.993	0.571	1.754
S	UV-Vis spectroscopy	420.00	0.998	0.074	0.897
Fe	FAAS	248.33	0.996	0.002	0,011
Zn	FAAS	213.80	0.991	0.002	0.071
Mn	FAAS	279.48	0.999	0.001	0.603
Cu	FAAS	324.75	0.997	0.003	0.010
Na	AES	589.0	0.999	0.098	1.103
Al	FAAS	309.3	0.998	0.0008	0.078
B	UV-Vis spectroscopy	420.00	0.999	0.089	0.123
Co	FAAS	240.73	0.997	0.0005	0.0008

Note. FAAS = Flame Atomic Absorption Spectroscopy. AES = Flame Atomic emission Spectroscopy. LOD = detection limit. LOQ = Quantification limit.

As the ionization suppressor for the Ca and Mg elements, 0.1% of the lithium oxide solution (La_2O) was used. In the case of sodium (Na), it was determined in the same equipment, but in atomic emission mode. As for potassium (K), it was determined by means of flame photometry on the Digimed Flame Photometer DH-62, calibrated using a Digimed standard solution whose concentration range was 2-100 mg L^{-1}.

For the determination of the phosphorus (P), boron (B) and sulfur (S) elements, the ultraviolet molecular absorption spectrophotometry technique was used using a SHIMADZU UV-1800 model, according to the (Embrapa, 2009), by formation of the colorimetric reaction with ammonium molybdate ($(NH_4)_2MoO_4$). In the case of P, blue complex formed, where the readings were made at λ = 660 nm; in the case of B complex was formed with Azometine-H of yellow color and absorbs light at λ = 460 nm; and for the sulfur was precipitated with $BaCl_2$, calibrating with potassium sulphate, at λ = 420 nm.

Nitrogen determination was carried out by the distillation method followed by titration (Kjeldahl), where the ammonium ion produced in the digestion with sulfuric acid (H_2SO_4) is distilled in strongly alkaline medium in the Kjeldahl distiller model TECNAL TE-036/1, collected (0.01%) and methyl red (0.04%) and titrated with 0.01 mol L^{-1} HCl solution were added in 2% boric acid solution with a mixture of green bromocresol (0.01%) and methyl (Embrapa, 2009).

$$\%N \text{ total} = (V \times 0.028)/m \quad (7)$$

Where, V = difference in the titration volume of the sample blank; m = mass of the sample in grams; and the value 0.028 = milliequivalents grams of nitrogen multiplied by the concentration.

2.5 Statistical Analysis

Correlations between the amounts of the different minerals in the epidermis of the fruit were evaluated using the Pearson statistical test using INFOSTAT (Rienzo et al., 2016) for significance levels of 5%, 1% and 0.1% respectively, as well as the principal component analyzes (PCA) and Hierarchical component analysis (HCA).

3. Results and Discussion

3.1 Physicochemical Characterization

The parameters of physicochemical analysis studied in this work (pH, titratable acidity (TA), total soluble solids (SS) and SS/TA ratio) its serve to characterize the quality of the fruit, to potentiate or as a functional food (Canuto et al., 2010). In Table 3, the results of the physicochemical parameters for the different fruits are presented, with their standard deviation made for three repetitions using the value of the t-student for the 95% probability.

Table 3. Physicochemical parameters for the skin of different fruits

Fruit	pH	TA (g citric acid 100 g^{-1})	SS (ºBrix)	SS/TA
Abiu	4.7±0.1	5.6±0.1	2.7±0.2	0.48±0.1
Acerola	2.1±0.2	1.2±0.1	2.9±0.1	2.41±0.1
Araçá	4.3±0.1	0.4±0.1	4.5±0.2	11.25±0.2
Bacuparí	3.1±0.2	1.9±0.2	7.1±0.1	3.74±0.2
Biribá	3.0±0.2	2.4±0.2	8.1±0.1	3.38±0.1
Camu camu	2.4±0.1	1.7±0.2	4.3±0.2	2.52±0.2
Fruta-do-conde	2.9±0.1	2.7±0.1	8.7±0.1	3.22±0.1
Graviola	3.1±0.1	2.1±0.1	8.4±0.1	4.0±0.1
Taperebá	2.7±0.2	1.7±0.2	5.7±0.1	3.35±0.1

The pH value for the different fruits studied ranges from 2.1 for *acerola* bark, reaching 4.7 for the bark of the *abiu*. The titratable acidity expressed in mg of citric acid 100 g^{-1} presents values of 0.4±0.1 for the *araçá* bark up to 5.6±0.1 for the *abiu*. This parameter is important, since it indicates the maturity of the fruit, measuring the titratable hydrogens contained in the fruits of all the acids that constitute it until they are neutralized at a fixed pH value. It is expressed as the equivalent of citric acid since it is the predominant acid in fruits and according to Fernández et al. (2006), this parameter can not be less than 0.4. The value of the SS expressed in ºBrix varies between 2.7±0.2 for the abiu, reaching the value of 8.7±0.1 for the fruta-do-conde. Finally, the SS/TA ratio gave values between 0.48 for the *abiu* to values of 11.25 for the *araçá*. This parameter relates the quality of the fruit in terms of maturity and flavor (M. I. F. Chitarra & A. B. Chitarra, 2005).

There are few data in the literature about the physicochemical parameters in the skin of these Amazonian fruits, being limited to the study of pulps. In the case of *camu-camu*, studies carried out by Maeda et al. (2006), the physicochemical parameters for the pulp of the *camu-camu*, being the pH of the pulp of 2.64 slightly higher than that of the bark, the solids solids also, with 6.20 ºBrix and the titratable acidity is also higher for the pulp (3.40 g 100 g^{-1} of citric acid). In the case of the fruit-do-count, Bonfim et al. (2014) study said fruit in different stages of fruit maturity, finding values in the mature state of soluble solids between (17.25-20.22 ºBrix) values higher than those found for the skin and potential acidity value between (0.18-0.23%) also higher than those found for the skin in this work.

There is a work developed by Carlone et al. (2016), where they prepare a flour of bacuparí made from the pulp and the barks finding pH values of 3.18 similar to those determined in this work and titulable acidity of 7.82 g 100 g^{-1} of citric acid, greater than those presented here, since in this work not only the skin of the fruit is being evaluated, but also the pulp is being evaluated.

For the *graviola*, if studies were found that evaluate the bark of the same, made by Silva (2016), where the pH value determined is approximately one unit lower than the one determined in this work and for the titratable acidity, it finds a value of 3.70 g 100 g^{-1} of citric acid, somewhat higher than what we determine, since acidity influences the degree of ripeness of the fruit. On the other hand, Sacramento et al. (2003), study the pulp of the graviola, where the determined value of pH is 3.44 being approximate to the one we determined in this work for the husk and acidity titulable in the case of the pulp it was approximately 1 g 100 g^{-1} of citric acid less than that determined in the bark.

In the case of *taperebá*, no results have been found for physicochemical analysis in the bark, only for the pulp being determined by Freitas (2017), pH values for the pulp between 2.60-2.95, being within the range of pH determined for the bark in this work. The titratable acidity is slightly lower (0.60-1.40 g 100 g^{-1}) of citric acid to the one determined for the skin and the soluble solids in the pulp are slightly larger (9.96-11.30).

The *araçá* presents studies for the pulp, whose pH values vary between 3.0-4.0, titratable acidity between 1.80-1.87 g 100 g^{-1} of citric acid and soluble solids between 4.5-11 ºBrix. (Andrade et al., 1993; Canuto et al., 2010). The pH values determined for the pulps are close but slightly lower than those of the bark of the fruits evaluated in this work, the titratable acidity is much lower for the skin and in the case of the SS, these are close to the determined by Canuto et al. (2010) for the pulp.

In the case of the abiu, the pH of the skin is close to that of the pulp determined by Canuto et al. (2010), who obtains a pH value of 5.0 The titratable acidity is lower for the pulps than for the barks but with very close value found by the same author 5.9 g 100 g^{-1} of citric acid and for soluble acids these are greater for the pulp (3.8) according to the same author as for the skin. For *acerola* Godoy et al. (2008) and Canuto et al. (2010), the pH value for pulp (2.8-3.4) is slightly higher than that found for the skin in this work, the potential acidity for the

skin is within the range determined by these authors (0.92-1.90) g 100 g^{-1} of citric acid and the soluble solids for the bark of this work are lower than those found by the previous authors for the pulp of these fruits with values of (3.5-8.24) ºBrix.

3.2 Bromatological Analysis From Bark of Amazon Fruits

Table 4 presents the nutritional analysis values for the bark of the different Amazonian fruits studied.

Of all the parameters that make up the bromatological analysis, moisture is the majority in the barks of the fruits studied compared to the other parameters, ranging from 32.12% for yellow *araçá* bark to 88.99% for *acerola*. The content of ashes in the fruit exocarp does not reach 1%, with the lowest concentration for the *taperebá* with 0.24% and 0.89% for the *fruta-do-conde*.

The content of lipids in the husk is low, in relation to other parts of the fruit, being in lower concentration for the *taperebá* with a percentage of 0.12% and the highest concentration for the *bacuparí* with 1.41%. The carbohydrate content varies according to the fruit in a high percentage range, determining a percentage of 9.62% for the *biribá* to 65.58% for the *araçá*.

The proteins are another one of the nutrients that are in low concentration in the bark of the fruits, being only in concentration of 0.04% for the acerola, reaching values of 0.41% for the abiu. The energy contribution of the bark varies from 48.06 kcal 100 g^{-1} for *acerola* to 276.29 kcal 100 g^{-1} for *araçá*.

Table 4. Nutritional composition in bark of Amazonian fruit

Fruit	Nutritional Contribution					
	Moisture	**Ashes**	**Lipids**	**Carbohydrates**	**Proteins**	**Energetic Value**
	----------% ----------					--- Kcal 100 g^{-1} ---
Abiu	82.49±0.09	0.41±0.02	1.27±0.02	15.42±0.01	0.41±0.02	**74.75±0.01**
Acerola	88.89±0.05	0.27±0.03	0.94±0.02	9.86±0.01	0.04±0.00	**48.06±0.01**
Araçá	32.12±0.12	0.52±0.09	1.37±0.13	65.58±0.01	0.41±0.02	**276.29±0.01**
Bacuparí	84.37±0.21	0.38±0.11	1.41±0.03	13.52±0.02	0.32±0.02	**68.05±0.01**
Biribá	88.32±0.07	0.77±0.17	1.12±0.06	9.62±0.02	0.17±0.02	**49.24±0.02**
Camu-camu	83.12±0.09	0.31±0.09	1.12±0.04	15.37±0.01	0.08±0.00	**71.88±0.01**
Fruta-do-conde	85.43±0.02	0.89±0.03	1.27±0.01	12.30±0.02	0.11±0.01	**61.07±0.03**
Graviola	74.16±0.08	0.72±0.04	1.04±0.04	23.91±0.02	0.17±0.03	**105.68±0.05**
Taperebá	73.21±0.13	0.24±0.12	0.12±0.04	26.32±0.02	0.11±0.01	**106.80±0.02**

Note. Analyzes performed in triplicate and using as a standard deviation the value of the t-student for 95%.

3.3 Mineral Analysis

In the Table 5 and the Table 6, the values of macronutrients and micronutrients are presented for the different barks studied. Among the macronutrients detected in the barks of different fruits, potassium stands out as the majority, being in the barks of the *graviola* where it is in the highest concentration 521 mg 100 g^{-1}, and in lower concentration for the bark of *taperebá* with concentration of 111.34 mg 100 g^{-1}. These values of K in the edible fraction are in agreement with those established by Almeida et al. (2009), where they establish that K levels in the edible fraction of fruits ranges between 143.67-790.11 mg 100 g^{-1}. Elcinto (2000), notes that this element is found in high concentrations in fresh fruits and vegetables, especially in the bark and stem of edible fruits. Its importance in the organism is in the maintenance of the hydroelectric balance with sodium, the concentrations of these elements being regulated inside and outside the cell Cuppari and Bazanelli (2010). The concentrations of sodium in the fruits studied are lower than the potassium concentrations, with the highest concentration for the *camu-camu* husk being 18.26 mg 100 g^{-1} and the lowest concentration for the *abiu* with 0.36 mg 100 g^{-1}. Studies of this mineral in plants of the Annonaceae family, determined Na concentration for the *graviola* of 3.11 mg 100 g^{-1}, slightly higher than that found in this work and for the *fruta-do-conde* of 9.94 mg 100 g^{-1} also superior to the one found in this work (Bramont et al., 2008).

Unlike what happens with potassium, calcium in the barks of the fruit is of great importance, since it is the element that gives it firmness, being associated with high levels of calcium to a good quality of the fruit (Johnston et al., 2002; Poovaiah et al., 1988). In this study, Ca is the second element in abundance after K, with the highest concentration in the skin of *camu-camu* with 52.21 mg 100 g^{-1} and the lowest concentration for *abiu* with 22.11 mg 100 g^{-1}. The next element in abundance in the fruits studied is the Mg, being the skin of *biribá* who presents a higher concentration of Mg, even superior to that of Ca with a concentration of 64.21 mg 100 g^{-1},

with a lower concentration of the skin of the *abiu* again with 13.21 mg 100 g^{-1}. Berto et al. (2015) determined Mg concentrations of 69.07 mg 100 g^{-1} for *biribá*, a value close to that determined in this work.

The importance of phosphorus in the organism lies in its involvement in metabolic functions such as the synthesis of ATP, synthesis of carbohydrates, nucleic acids and coenzymes (Epstein & Bloom, 2006), being found in the body between 0.8-1.1% (Monteiro & Vannucchi , 2010). The highest concentration of phosphorus was in *araçá* with 43.47 mg 100 g^{-1} and the lowest concentration of *abiu* husk with 4.3 mg 100 g^{-1}. The concentration of minerals in *Biribá* fruits was studied by Berto et al., (2015) who determined P concentrations in this fruit of 25.32 mg 100 g^{-1}, close to the value found in this work.

Sulfur is within the macrominerals, found in a wide range of values for the fruits studied, from 3.14 mg 100 g^{-1} for the *bacuparí*, to 37.22 mg 100 g^{-1}, in the bark of *acerola*. This element is necessary for the human body since it is part of amino acids such as cysteine and methionine present in hair and nails, being found in the body in concentrations of 140 g of this element (Lisboa, 2015).

The last element to consider in this work is nitrogen, being a constituent in several components of plants and the sea in the form of amino acids, nucleic acids and chlorophyll, as well as part of numerous microbiological reactions (Novais et al., 2007), In the fruits studied they are stored in the bark of the *biribá*, whose concentration is 8.56 mg 100 g^{-1}.

Table 5. Macronutrients analyzed in bark fruit in the northern Amazon

Fruit	Macronutrients						
	Calcium (Ca)	Magnesium (Mg)	Phosphorous (P)	Potassium (K)	Sodium (Na)	Sulfur (S)	Nitrogen (N)
	---------- mg 100 g^{-1} ----------						------ % ------
Abiu (*Pouteria caimito*)	22.11±0.14	13.21±0.15	4.31±0.11	242.11±0.14	0.36±0.02	15.44±0.12	0.07±0.02
Acerola (*Malpighia emarginata*)	27.12±0.13	37.21±0.05	7.21±0.11	121.33±0.22	17.81±0.04	37.22±0.14	$6.96.10^{-3}$±0.00
Araçá (*Psidium cattleianum*)	27.23±0.01	15.27±0.11	43.47±0.04	123.11±0.07	6.83±0.11	7.31±0.02	0.07±0.02
Bacuparí (*Rheedia gardneriana* Planch & Triana)	44.12±0.10	35.55±0.12	6.21±0.09	411.08±0.07	7.13±0.01	3.14±0.08	0.06±0.01
Biribá (*Rollinia mucosa*)	47.91±0.12	64.21±0.11	20.22±0.01	441.12±0.12	17.13±0,31	19.14±0.14	8.56±0.02
Camu-camu (*Myrciaria dúbia* (Kunth) Mc Vaugh)	52.21±0.13	32.12±0.09	17.30±0.12	431.21±0.17	18.26±0.11	27.78±0.13	0.21±0.04
Fruta-do-conde (*Annona squamosa*)	50.11±0.04	28.07±0.12	15.11±0.01	417.09±0.11	3.48±0.07	23.12±0.09	0.02±0.00
Graviola (*Annona muricata*)	33.12±0.04	21.08±0.09	16.11±0.21	521.04±0.15	2.16±0.08	13.11±0.05	0.03±0.00
Taperebá (*Spondias mombin* L.)	45.21±0.02	28.11±0.04	16.22±0.08	111.34±0.04	6.56±0.07	3.21±0.03	0.02±0.00

Note. Analyzes performed in triplicate and using as a standard deviation the value of the t-student for 95%.

Table 6. Micronutrients analyzed in bark fruits in the northern Amazon

Fruit	Iron (Fe)	Zinc (Zn)	Manganese (Mn)	Copper (Cu)	Aluminum (Al)	Boron (B)	Cobalt (Co)
	---------- mg 100 g^{-1} ----------						
Abiu (*Pouteria caimito*)	0.07±0.00	3.04±0.01	6.84±0.11	1.24±0.05	0.08±0.01	0.74±0.03	N.D.
Acerola (*Malpighia emarginata*)	0.44±0.02	0.04±0.01	0.78±0.07	0.09±0.02	0.16±0.04	0.22±0.04	N.D.
Araçá (*Psidium cattleianum*)	4.41±0.03	12.23±0.02	0.31±0.03	3.38±0.02	0.03±0.00	0.19±0.01	N.D.
Bacuparí (*Rheedia gardneriana* Planch & Triana)	0.32±0.04	2.94±0.09	0.50±0.07	0.82±0.06	0.22±0.04	0.12±0.03	0.031±0,006
Biribá (*Rollinia mucosa*)	1.32±0,12	0.94±0.09	0.57±0.07	0.87±0.06	0.27±0.04	0.22±0.04	0.011±0,006
Camu-camu (*Myrciaria dúbia* (Kunth) Mc Vaugh, Myrtaceae	0.21±0.08	0.71±0.03	1.07±0.07	0.72±0.02	0.04±0.01	0.23±0.02	0.061±0.002
Fruta-do-conde (*Annona squamosa*)	0.23±0.07	0.19±0.01	2.55±0.01	2.48±0.04	0.18±0.03	0.29±0.17	N.D.
Graviola (*Annona muricata*)	0.81±0.04	0.32±0.01	0,64±0.05	0.39±0.02	0.38±0.01	0.37±0.03	0.010±0.001
Taperebá (*Spondias mombin* L.)	0.45±0.08	0.07±0.01	0.87±0,05	1.03±0.07	0.14±0.02	0.79±0.06	N.D.

Note. N.D. not detected. Analyzes performed in triplicate and using as a standard deviation the value of the t-student for 95%.

Analyzes of microminerals in Amazonian fruit barks are scarce or nonexistent. In Table 6, the values of micronutrient concentrations for the nine fruits studied are presented.

Zinc is one of the important micronutrients since among other functions it is present in the liver mobilization of vitamin A, sexual maturation, fertility and reproduction as well as participating in more than 300 metalloenzymes (Manganaro, 2008; Cominetti, 2009) being found in low concentrations in the fruits studied with the exception of araçá where the concentration of this element in the bark is 12.23 mg 100 g^{-1} followed by *abiu* bark with Zn concentrations of 3.04 mg 100 g^{-1} and the lowest concentrations of This element was obtained in the barks of acerola with concentrations of 0.04 mg 100 g^{-1}. Studies conducted by Berto et al. (2015) on barks of different Amazonian fruits obtain values of concentration of Zn for the skin of the *biribá* of 1.04 mg 100 g^{-1} being a value practically similar to that obtained in this work. The recommendations of this element according to the DRI (2001) for adulthood are of 9.4 mg dia^{-1} for men and 11 mg dia^{-1} for women.

Another of the micronutrients found in the highest concentration in some fruits is the Mn, being the barks of the *abiu*, which presents a higher concentration with 6.84 mg 100 g^{-1}, followed by the fruta-do-conde with concentrations of 2.55 mg 100 g^{-1}. This mineral presents lower concentrations for the *araçá* barks with only 0.31 mg 100 g^{-1}. Berto et al. (2015) studied micronutrients in the barks of different Amazonian fruits, obtaining for the *biribá* concentrations of 0.47 mg 100 g^{-1}, close to the concentration determined in this work (Table 6). For the *taperebá*, Sena et al. (2014), study the composition of micronutrients in flour obtained from residues of fruit processing, with concentrations of Mn of 0.04 mg 100 g^{-1}, concentration lower than that found in its shell pure. This element is interesting for its implication with diverse metabolic reactions in the organisms of immune response, synthesis of ATP and as cofactor in metalloenzymes (Burton & Guillarte, 2009) being the recommendations of this element for adults of 2.3 mg dia^{-1} for men and 1.8 mg dia^{-1} for women (DRI, 2001).

Iron is another essential micronutrient whose recommendations according to DRI (2001) for adults are 8 mg dia^{-1} and from the age of fifty, these amounts are reduced to 8 mg dia^{-1} being found in the human body at concentrations of 3-5 grams (Fantisi et al., 2008). As with Zn, it is the *araçá* that has a higher concentration in the barks of fruits studied whose values are 12.23 mg 100 g^{-1}, being the concentration of this element excessively low in the abiu shell whose concentration is of 0.07 mg 100 g^{-1}.

Copper is another microelement of interest in the bark of the fruits studied, being found in a higher concentration in the *araçá* bark with a value of 3.38 mg 100 g^{-1} and in a lower concentration in the bark of *acerola* with a concentration of 0.09 mg 100 g^{-1}. The deficiency of this element, has important series implications for the organism as is the case of the diseases of Wilson and Menkes (Amancio, 2017) being the recommendations of Cu in adulthood according to the DRI (2011) of 700 μg day^{-1}.

Boron is another essential element for man, related to maintaining the integrity of the plasma membrane and involved with bone metabolism (Brown et al., 2002). According to the DRI (2001), the recommendations of B for adults are of 20 mg dia^{-1}. The highest concentrations of B found in the fruit bark studied in this study are in the bark of *taperebá* whose concentration is 0.79 mg 100 g^{-1} and for *abiu* with concentrations of 0.74 mg 100 g^{-1}. The lowest concentrations of this element are in the *bacuparí* barks whose concentration is of 0.12 mg 100 g^{-1}. Ribeiro et al. (2016) studied the concentrations of B in dry *camu-camu* fruits and obtained values of 1.7-1.8 mg 100 g^{-1}, values higher than only for the isolated skin (Table 6).

The Cobalt is the element found in ultra-trace concentrations in the barks of the studied fruits, being only detected in *bacuparí*, *biribá*, *camu-camu* and *graviola* whose values oscillate between 10 μg 100 g^{-1} for the skin of the *graviola* being the highest concentration of this element in the bark of *camu-camu* in concentration of 61 μg 100 g^{-1}. These values are lower than those recommended by FAO/WHO, which should be ingested 0.58 mg kg^{-1} as a function of the individual's body size (FAO, 2013). This element in higher concentrations can cause toxicity as is the case of cardiomyopathy, as well as linked to other nervous and blood clotting problems (Seghizzi et al., 1994).

Finally, the aluminum was also identified in the fruit skin studied, being one of the metals that must be found in low concentrations in foods since this metal being a neurotoxic substance, is involved with Alzehimer's disease (Armstrong, 2002). The concentrations of Al in this work are low, being the highest value for the *graviola* bark with Al concentration of 0.38 mg 100 g^{-1} and the lowest concentration of Al for *camu-camu* bark with concentration of 0.03 mg 100 g^{-1}.

3.4 Statistic Analysis

3.4.1 Pearson Correlation Coefficient

Table 7 presents the Pearson correlation matrix between the different elements for the bark of the different fruits.

Table 7. Pearson correlation matrix between the different elements for the bark of Amazonian fruits

	Ca	Mg	P	K	S	N	Fe	Zn	Mn	Cu	Na	Al	B	Co
Ca	1													
Mg	0.66*	1												
P	0.00ns	-0.10ns	1											
K	0.55ns	0.41ns	-0.19ns	1										
S	0.05ns	0.38ns	-0.25ns	0.09ns	1									
N	0.39ns	0.82**	0.14ns	0.36ns	0.17ns	1								
Fe	-0.10ns	-0.08ns	0.94**	-0.21ns	-0.16ns	0.11ns	1							
Zn	-0.26ns	-0.32ns	0.76*	-0.28ns	-0.30ns	-0.12ns	0.89**	1						
Mn	-0.28ns	-0.38ns	-0.38ns	-0.05ns	0.13ns	-0.19ns	-0.30ns	0.00ns	1					
Cu	0.12ns	-0.29ns	0.73*	-0.13ns	-0.19ns	-0.12ns	0.69*	0.75*	0.13ns	1				
Na	-0.19ns	0.44ns	0.01ns	-0.34ns	0.23ns	0.44ns	-0.01ns	-0.20ns	-0.51ns	-0.38ns	1			
Al	0.00ns	-0.09ns	0.88**	0.00ns	-0.14ns	0.02ns	0.96**	0.85**	-0.33ns	0.65*	-0.19ns	1		
B	-0.03ns	-0.30ns	-0.25ns	-0.24ns	-0.16ns	-0.20ns	-0.31ns	-0.22ns	0.58ns	-0.08ns	-0.49ns	-0.34ns	1	
Co	-0.36ns	-0.17ns	-0.18ns	-0.06ns	-0.49ns	-0.03ns	-0.26ns	-0.17ns	-0.26ns	-0.32ns	0.46ns	-0.32ns	-0.39ns	1

Note. ns (not significant) p >0.05, * p < 0.05, ** p < 0.01.

Table 7 presents the Pearson interaction values for the different fruit constituents in the bark of the fruit, where highly significant interactions are found at a significance level of 1% for nitrogen systems with magnesium (0.82), aluminum with phosphorus (0.88), iron with phosphorus (0.94), aluminum with iron (0.96), zinc with iron (0.89) and aluminum with zinc (0.85). On the other hand, there are significant interactions at the significance level of 5% for the magnesium systems with calcium (0.66), zinc with phosphorus (0.76), copper with phosphorus (0.73), copper with iron (0, 69), copper with zinc (0.75) and aluminum with copper (0.65). For the remaining elements there is no significant interaction.

3.4.2 Principal Component Analysis (PCA)

The analyzes of main components were carried out jointly for the evaluated systems (*abiu*, *bacupari*, *acerola*, *graviola*, *camu-camu*, *araçá*, *biribá* and *taperebá*), independently for bark of the fruit, in order to find a new set of variables (main components), uncorrelated, that explain the structure of the variation, being represented the weight of each variable analyzed in each component (axes).

In the blipot (Figure 1), the results of the analysis of the main components (PCA) for the bark of the different fruits are represented, being explained the 56.0% of the original variability of the data retained in these components. These results indicate that CP1 allowed to distinguish the fruits that are associated to the minerals in the barks, being the fruits biribá, fruta-do-conde, camu-camu and araçá who were associated.

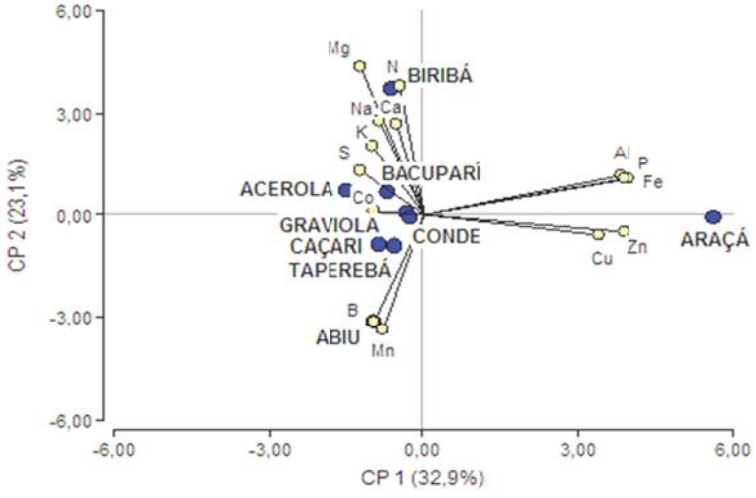

Figure 1. Distribution of the original variables among the different fruits for the barks on the first and second main component (CP1 and CP2)

The arrangement of the sequence in Figure 1, shows that the systems can be grouped into two sets, the first major component (CP1), contributed 32.9% of the total variance explained, however most of the minerals that were strongly affected, between (P), iron (Fe), zinc (Zn) and copper (Cu) contributing positively to CP1 and inverse with elements nitrogen (N), magnesium (Mg), calcium (Ca), sodium (Na), potassium (K), sulfur (S), cobalt (Co), boron (B) and manganese (Mn).

These results indicate that CP1 allowed to distinguish the fruits that are associated to the minerals in the part of the bark, being only the *araçá* who is associated with these minerals.

The second major component (CP2) accounted for 23.1% of the total data, nitrogen (N), magnesium (Mg), calcium (Ca), sodium (Na), potassium (K), sulfur (S), cobalt (Co), boron (B) and manganese (Mn).

3.4.3 Hierarchical Grouping Analysis (HCA)

Through the HCA, data can be displayed in a two-dimensional space in order to emphasize their natural groupings and patterns, relating the samples so that the most similar are related to each other, presenting the samples in dendogram, grouping the samples and variables according to with its similarity.

In Figure 2 the dendogram for the HCA analyzes of the different fruit bark studied is presented.

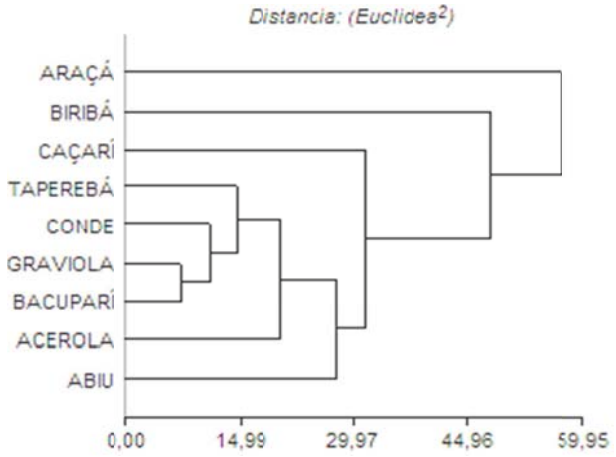

Figure 2. Dendogram by HCA, Euclidean distance and incremental connection technique for the minerals present in the fruit bark studied

For the production of tested fruits, the trends observed through or analysis of principal components, observed through HCA, observing that either *taperebá*, *fruta-do-conde, graviola, bacupari* and *abiu* are not grouped between them, and for distance. 29.98, sendo or value of metada gives maximum distance, or *araçá* e biribá separated rest.

4. Conclusions

Given the values obtained from nutritional intake and minerals for the barks of the fruits studied, which in some cases are superior to those of the edible parts, the husks could be used as an alternative source of nutrients, thus avoiding the waste of food, taking advantage of the source of nutrients and at the same time, other products can be prepared from these samples such as jellies, sweets and flours.

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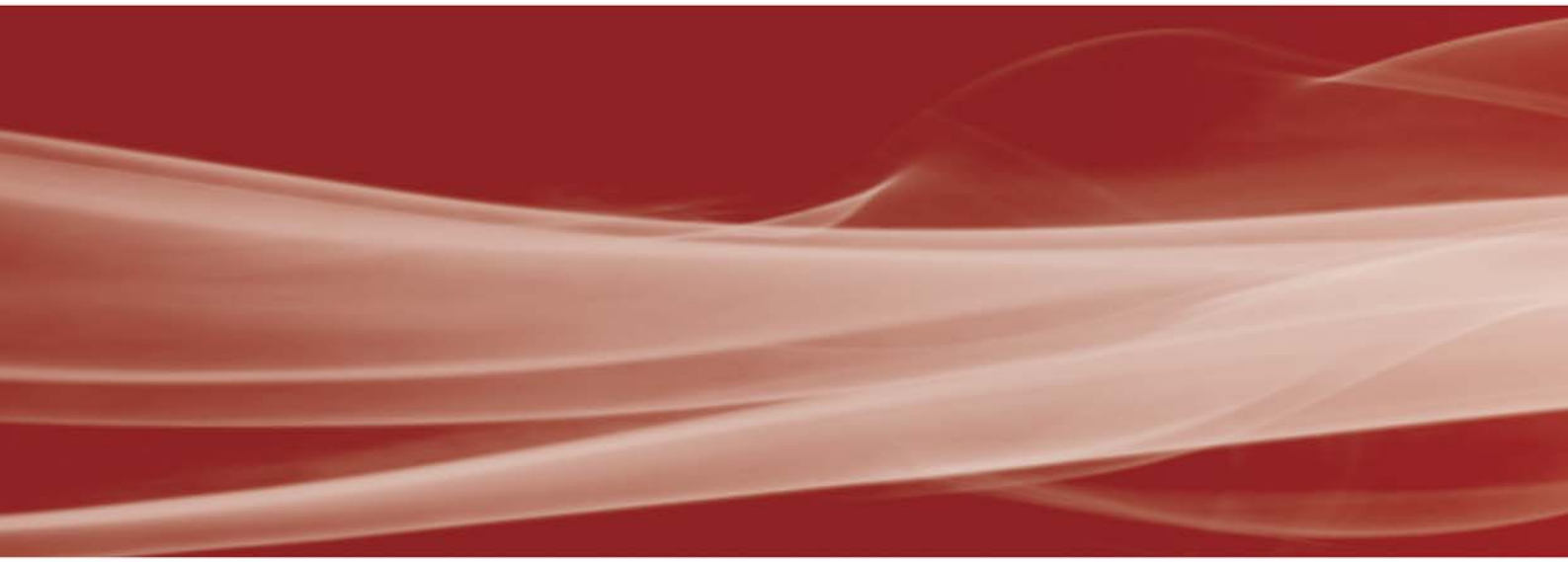

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Address 9140 Leslie St. Suite 110, Beaver Creek, Ontario, L4B 0A9, Canada
Telephone 1-416-642-2606
Fax 1-416-642-2608
E-mail jas@ccsenet.org
Website jas.ccsenet.org

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