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Grain Quality and Yield of Rice in the Main and Ratoon Harvests in the Southern U.S.

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Abstract

The ratoon rice system is an energy-saving, high-efficiency cultivation method. Harvests from a two-year field trial with a main crop (MC) and a ratoon crop (RC) were used to evaluate milled grain quality traits and yield performance. The results indicated that chalkiness was significantly lower in the RC than in the MC. Chalkiness ranged from 1.90 to 15.01%, with an average of 6.46%, in the MC and from 0.66 to 3.28%, with an average of 1.50%, in the RC across two years. In addition, nearly all of the RC of the test entries had lower white vitreous (higher translucency) than the MC of the same entry. In 6 of the 20 entries, the MC had longer or wider milled grain than the RC in 2017. The milled rice recovery for the MC was higher in both years, but there was no difference in head rice recovery within the same year. The average total yield (MC+RC) in the two years was 12.6 and 13.0 t/ha, and the two-year average RC yields were 47.5 and 37.3% those of the MC. Our results revealed that the RC milled grains showed better appearance quality than the MC grains, and several genotypes had comparable or even better milled grain quality and yield compared with the check entries that were suitable for the ratoon rice system.

Keywords: grain yield, milled rice, ratoon, grain quality

1. Introduction

As one of the most important staple foods, rice feeds nearly half of the population in the world. However, with increasing human population and decreasing arable land, rice yield must be doubled by 2050 to meet food demand (Nathaniel et al., 2012). There are two ways to improve crop yield: increase the yield per unit of land (Cassman et al., 2003) or harvest the existing cropland more frequently (Ray et al., 2013). For the former strategy, crop yields have experienced plateaus in recent years (Grassini et al., 2013), and for the latter, ratoon rice would be one of the choices (Chen et al., 2018). The ratoon crop (RC) is the second harvest from the tillers originating from the stubble of the previous crop, known as the main crop (MC); the growth duration of the RC is shorter, approximately 40% shorter than the 100-120 days required for the MC (Krishnamurthy et al., 1988). For this reason, it is suitable for areas where the annual accumulation of solar radiation and temperature is in excess of the requirements for a single season but not enough for a double season. In addition, a ratoon crop saves seed costs and approximately 50% of labor and 60% of water costs because it is free of land preparation, seeding or transplanting, unlike the MC (Flinn et al., 1988; Oad et al., 2002); thus, ratooning is the most suitable cropping system in areas where labor and water are in short supply. Due to its shorter cropping duration, fewer inputs, such as fertilizer and pesticides, are needed in RCs. For the merits mentioned above, ratooning is practiced increasingly in the USA, Southeast Asia and China (Nakano et al., 2007).

Studies have shown that attention should be given to achieving ratooning success. Variety characteristics are the major factor for ratoon success because varieties used in ratooning will be exposed to different day lengths,

temperatures and amounts of sunlight in the main season and ratoon season, so varieties with wider adaptability behave better in ratooning practice (Sun et al., 1988).

Low temperatures after the main season will prevent ratoon development, so earlier planting is another factor for ratoon success. In addition, it has been reported that the harvest mode of the main crop will also affect ratooning. First, the best harvest time for the main crop is before full maturity, while the culms are still greenish in color; late harvest will reduce the number of auxiliary buds sprouting and thus reduce productive tiller and ratoon yields (Ichii et al., 1981; Xiong et al., 1991). Second, the stubble is left with a 2-3 node or 20-40 cm height because panicles from the upper node contribute more to the ratoon yield than those from the lower node (Yi et al., 2009; He et al., 2014). Insect pests and disease control during the main crop also play key roles in ratooning. Unlike a rice-rice rotation system, ratooning prolonged the duration of rice serving as a host of diseases or insect pests, so these biotic stresses in the main crop would affect ratoon tiller production, thereby reducing ratoon yield. Therefore, varieties with high biotic stress resistance are very important for ratooning (Dela Cruz et al., 1988).

The grain quality of rice, including grain appearance, grain size, and grain milling quality, is genetically controlled by quantitative trait loci (QTLs) or genes such as *Chalky 5*, *qPGWC-7* and *UGPase1*, which control grain chalkiness (Woo et al., 2008; Zhou et al., 2009; Li et al., 2014); *Wx* and *DULL*, which affect grain translucency (Wan et al., 2007; Zeng et al., 2007); and *GW2*, *GS3* and *GW5*, which control grain size (Mao et al., 2010; Liu et al., 2017; Choi et al., 2018). Some QTLs control grain size also have an effect on grain chalkiness, such as upregulation of *GW7* expression is correlated with more slender grains and less chalky grains (Wang et al., 2015a); overexpression of *GL7* resulted in an increase in grain length and improve in grain chalkiness (Wang et al., 2015b). Head rice recovery is influenced by grain characteristics, such as chalkiness, grain shape, and grain moisture (Moldenhauer et al., 2004), and some QTLs related to milled rice recovery and head rice recovery have been mapped (Mei et al., 2002; Septiningsih et al., 2003).

Environmental conditions, especially temperature, also have a great influence on grain quality. It has been reported that high nighttime temperatures during the filling stage increase grain chalkiness (Cooper et al., 2008; Ishimaru et al., 2009) and decrease grain weight and grain translucency (Peng et al., 2004; Ishimaru et al., 2009). As mentioned above, the main crop and ratoon crop are genetically the same, but they are exposed to different environments during growth and development, so different grain quality could be expected between them. However, no detailed study has been conducted yet. In addition, variety identification is practiced in the United States, and the harvests from the MC are always mixed with the RC in a bin of the same variety to facilitate storage. However, it is not clear whether the quality difference in the MC and the RC affects the practice of identity preservation. Therefore, a detailed study comparing grain quality between the MC and the RC will be a helpful guide in ratoon rice production.

In this study, a two-year field trial was conducted using 18 elite tropical Japonica breeding lines and two check (CK) varieties to identify the grain quality differences between the MC and the RC, determine the potential effects of mixing MC and RC harvests and screen for elite breeding lines with high total yield for ratooning rice production.

2. Method

2.1 Rice Entries and Culture

The two-year field experiments were conducted at Eagle Lake (29°37′ N, 96°22′ W), Texas, USA. Each year, 18 elite tropical Japonica breeding lines with similar growth durations and two CK varieties, ANTONIO as a yield check and PRESIDIO as a grain quality check for MC/RC, were planted in plots arranged in a completely randomized block design with three replications. Each plot had 6-meter rows spaced 25 cm apart. Entries were directly seeded at a rate of 90 kg ha^{-1}. Fertilizer for the MC was applied in a 3-way split: preplanting at 50-50-50 kg NPK ha^{-1} using Triple 13, preflooding at 100 kg N ha^{-1} using urea and at panicle initiation at 90 kg N ha^{-1} using ammonium sulfate. A fertilizer rate of 90 kg N ha^{-1} using urea was applied immediately after the MC harvest as ratoon fertilizer. The Texas rice production guidelines were followed for the other cultural practices. A rice combine harvester was used in both the MC and RC harvests with cutting heights of approximately 38 and 25 cm, respectively.

Table 1. Comparison of main crop (MC) and ratoon crop (RC) mean chalkiness and translucency in tested breeding lines and two varieties

No.	Breeding Line/Variety	Chalkiness				White Vitreous			
		2016 MC	2016 RC	2017 MC	2017 RC	2016 MC	2016 RC	2017 MC	2017 RC
1	ANTONIO	11.37 a	3.17 b	9.48 a	1.52 b	129.26 a	113.27 a	127.31 a	127.23 a
2	RU0803147	14.70 a	1.11 c	8.86 b	0.95 c	129.15 a	127.23 a	128.93 a	126.55 a
3	RU0803153	12.06 a	1.43 c	8.29 b	1.29 c	129.71 a	128.58 a	129.33 a	126.70 b
4	RU1303138	6.96 a	1.77 b	6.43 a	1.24 b	128.57 a	128.63 a	127.88 a	126.27 b
5	RU1303153	7.79 a	1.92 b	7.87 a	1.20 b	128.72 a	128.59 a	128.36 a	126.85 b
6	RU1303181	5.07 a	1.78 b	3.52 a	1.43 b	129.19 a	128.50 a	127.44 a	127.18 a
7	RU1403138	4.23 b	1.58 b	7.27 a	2.38 b	129.78 a	129.25 a	128.99 ab	128.29 b
8	RU1403141	3.81 a	0.81 c	2.70 b	0.99 c	129.82 a	128.10 a	127.27 b	126.02 b
9	RU1503147	5.64 a	0.66 b	5.12 a	1.21 b	129.57 a	127.06 a	127.12 b	126.20 b
10	RU1503169	14.21 a	1.90 c	7.77 b	0.70 c	128.70 a	128.01 a	128.82 a	125.83 b
11	RU1503175	15.01 a	0.91 c	10.96 b	1.02 c	128.37 a	127.58 a	128.34 a	126.39 a
12	RU1603086	4.11 a	2.20 ab	3.87 a	1.19 b	129.72 a	128.90 a	128.50 a	128.04 a
13	RU1603089	10.29 a	1.51 c	5.79 b	1.26 c	129.00 a	128.66 a	127.97 a	128.08 a
14	RU1603113	3.48 a	1.51 a	3.40 a	1.44 a	129.01 a	128.42 a	128.21 a	127.53 a
15	RU1603116	2.76 a	1.24 b	1.90 ab	1.49 ab	130.54 a	128.40 a	128.29 b	128.15 b
16	RU1603138	9.18 a	1.15 b	4.81 b	1.27 b	130.70 a	128.48 a	128.58 ab	127.48 b
17	RU1603166	3.68 a	2.70 a	3.34 a	1.72 a	129.18 a	128.92 a	126.06 b	126.96 ab
18	RU1603178	5.00 a	3.28 a	3.84 a	1.71 a	131.31 a	129.62 a	129.48 a	129.11 a
19	RU1603187	4.33 a	1.82 b	3.95 a	1.08 b	129.18 a	128.50 a	126.43 a	127.09 a
20	PRESIDIO	2.50 a	0.90 b	2.92 a	1.51 b	131.69 a	127.61 a	129.91 b	127.84 c
Mean		7.31 a	1.67 c	5.60 b	1.33 d	129.56 a	128.37 b	128.16 b	127.19 c

Note. Data followed by different letters within one row denote a significant difference between the main crop and ratoon crop in a given year (difference was calculated for four data points over two years) for each test entry at the 5% level according to the LSD test. Means followed by bold letters within one row denote significant differences between the main crop and ratoon crop across two years for all test entries at the 5% level according to the LSD test.

2.2 Data Recorded

Four inner rows in a plot were harvested as they approached 20% grain moisture. Rough (unmilled) rice samples were dried to 12% moisture using an ambient-forced-air dryer. Rice milling was performed using a PAZ 1 Zaccaria mill (Zaccaria, USA), while an S21 Rice Statistical Analyzer (TKD Tecnologia, Brazil) was used to evaluate milled rice for chalkiness, translucency and grain size (length and width). Chalky grains were defined as grains with chalky areas of at least 50%.

2.3 Data Analysis

Data were analyzed using SPSS 22 (IBM Corp). The difference between the MC and the RC was determined using the least significance difference (LSD) test at the 0.05 probability level. Graphical representation of the data was made using Excel and CorelDRAW X8.

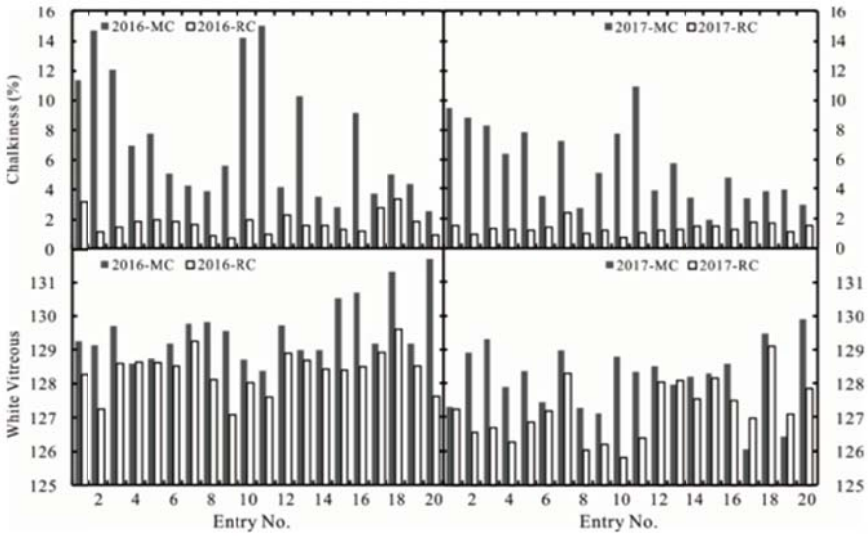

Figure 1. Yearly variation in main crop (MC) and ratoon crop (RC) chalkiness and translucency of tested breeding lines and two varieties

3. Results

3.1 Chalkiness and Translucency Performance in MC and RC Across Two Years

Across the two years, the MC chalkiness ranged from 1.90 to 15.01%, with a mean value of 6.46%, and the RC ranged from 0.66 to 3.28%, with a mean value of 1.50%. On average, the RC had significantly lower chalkiness than the MC for nearly all tested lines, and the average RC chalkiness was 5.64% and 4.27% lower than that in the MC in 2016 and 2017, respectively. In addition, the mean yearly chalkiness was higher in 2016 than in 2017 for both the MC and the RC (Table 1; Figure 1).

Table 2. F-values for three-way analysis of variance (ANOVA) for different traits

Source	Chalkiness	Translucency	Grain Length	Grain Width	Milled Rice Recovery	Head Rice Recovery	Yield
Year (Y)	43.87**	5.73*	200.93**	56.02**	76.01**	45.12**	3.72
Crop Type (CT)	1035.92**	14.63**	2.41	4.54*	31.17**	3.74	2173.68**
Entry (E)	22.25**	1.67	24.46**	49.60**	2.07**	1.78*	9.43**
Y × CT	19.74**	1.62	44.16**	29.86**	0.09	0.02	16.61**
Y × E	3.59**	1.02	3.12**	1.56	1.23	2.57**	1.85*
CT × E	24.52**	1.05	4.02**	12.23**	0.62	1.48	3.35**
Y × CT × E	2.87**	1.2	2.57**	2.29**	0.52	0.75	0.47

Note. *: $P < 0.05$; **: $P < 0.01$.

White vitreous (WV) readings are indicative of grain translucency. Higher values suggest lower (inferior) translucency. The majority of test entries showed that RC entries had lower WV (higher translucency) than the MC entries in each year, but only 25% of entries in 2016 or 2017 showed significant differences between MC and RC. The mean yearly WV in MC and RC in 2016 was significantly higher than that in 2017 (Table 1; Figure 1).

A three-way analysis of variance indicated that, for chalkiness, Year (Y), Crop Type (CT, MC or RC) and Entry (E) individually and in 2 or 3-way interactions affected chalkiness significantly, but CT had the most significant effect on chalkiness. For grain translucency, only CT and Y had significant effects on grain translucency (Table 2).

Table 3. Comparison of mean milled grain length and width in main crop (MC) and ratoon crop (RC) of tested breeding lines and two varieties

No.	Breeding Line/Variety	Grain length				Grain width			
		2016 MC	2016 RC	2017 MC	2017 RC	2016 MC	2016 RC	2017 MC	2017 RC
1	ANTONIO	6.37 a	6.47 a	6.42 a	6.27 a	1.84 a	1.94 a	1.89 a	1.88 a
2	RU0803147	6.49 a	6.45 a	6.39 a	6.24 b	1.92 ab	1.87 b	1.98 a	1.90 b
3	RU0803153	6.49 a	6.43 a	6.39 a	6.35 a	1.93 ab	1.92 ab	1.99 a	1.90 b
4	RU1303138	6.34 b	6.42 ab	6.47 a	6.34 b	1.91 a	1.89 a	1.97 a	1.92 a
5	RU1303153	6.24 a	6.40 a	6.37 a	6.28 a	1.90 b	1.91 b	1.97 a	1.89 b
6	RU1303181	6.70 a	6.59 a	6.59 a	6.42 b	1.9 ab	1.88 b	1.95 a	1.92 ab
7	RU1403138	6.51 a	6.45 a	6.28 a	6.25 a	2.04 b	1.95 c	2.10 a	1.94 c
8	RU1403141	6.43 ab	6.58 a	6.13 b	6.24 b	1.85 a	1.86 a	1.89 a	1.89 a
9	RU1503147	6.63 a	6.67 a	6.54 b	6.33 c	1.91 a	1.93 a	1.94 a	1.91 a
10	RU1503169	6.44 ab	6.51 a	6.35 b	6.20 c	1.89 a	1.88 a	1.95 a	1.84 a
11	RU1503175	6.44 a	6.48 a	6.33 a	6.29 a	1.89 b	1.89 b	1.96 a	1.90 b
12	RU1603086	6.5 ab	6.55 a	6.27 b	6.40 ab	1.83 b	1.86 b	1.86 b	1.90 a
13	RU1603089	6.56 a	6.59 a	6.64 a	6.31 a	1.87 b	1.87 b	1.95 a	1.87 b
14	RU1603113	6.59 a	6.44 a	6.36 a	6.29 a	1.91 ab	1.89 b	1.97 a	1.95 ab
15	RU1603116	6.86 a	6.90 a	6.94 a	6.53 b	1.78 bc	1.80 b	1.77 c	1.84 a
16	RU1603138	6.61 b	6.77 a	6.46 bc	6.38 c	1.89 a	1.86 a	1.91 a	1.90 a
17	RU1603166	6.39 b	6.48 a	6.34 b	6.35 b	1.75 b	1.85 a	1.74 b	1.83 a
18	RU1603178	6.34 a	6.45 a	6.16 a	6.28 a	1.86 b	1.89 ab	1.90 a	1.88 ab
19	RU1603187	6.41 a	6.49 a	6.28 a	6.24 a	1.74 b	1.80 ab	1.75 b	1.84 a
20	PRESIDIO	6.27 a	6.56 a	6.32 a	6.30 a	1.79 b	1.89 a	1.87 a	1.88 a
Mean		6.48 b	6.53 a	6.40 c	6.32 d	1.87 b	1.88 b	1.91 a	1.89 ab

Note. Data followed by different letters within one row denote significant differences between the main crop and ratoon crop in a given year for each test entry at the 5% level according to the LSD test. Means followed by bold letters within one row denote significant differences between the main crop and ratoon crop across two years for all test entries at the 5% level according to the LSD test.

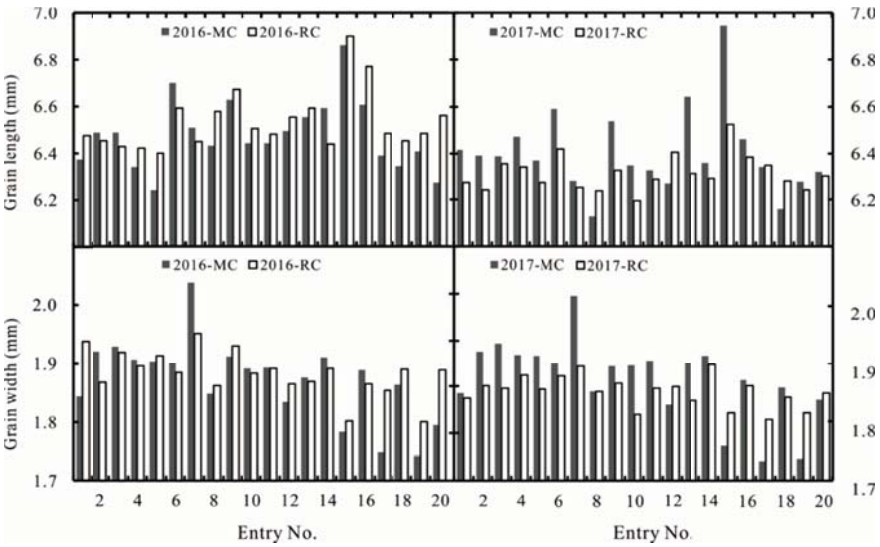

Figure 2. Yearly variation in grain length and width in main crop (MC) and ratoon crop (RC) of tested breeding lines and varieties

3.2 Grain Size Performance in the MC and RC Across Two Years

The mean grain length was significantly longer in 2016 than in 2017 for both the MC and the RC. RC grains were significantly longer than MC grains on average in 2016, but the reverse was true in 2017. The variation in grain width was relatively small, and no difference was observed between the MC and RC within the same year; only the MC in 2017 was wider than the MC and RC in 2016 (Table 3; Figure 2).

A three-way analysis of variance indicated that the Year effect was most significant on both grain length and width; in addition, they were affected by E, Y × CT, CT × E and Y × CT × E (Table 2).

Milled rice recovery and head rice recovery performance in the MC and RC.

Table 4. Comparison of mean milled rice recovery and head rice recovery in main crop (MC) and ratoon crop (RC) of tested breeding lines and two varieties

No.	Breeding Line/Variety	Milled Rice Recovery				Head Rice Recovery			
		2016 MC	2016 RC	2017 MC	2017 RC	2016 MC	2016 RC	2017 MC	2017 RC
1	ANTONIO	75.06 a	73.29 a	72.46 a	72.13 a	67.67 a	65.75 a	63.52 a	66.26 a
2	RU0803147	73.95 a	72.05 a	73.92 a	72.27 a	66.08 a	64.04 a	65.06 a	66.85 a
3	RU0803153	73.89 a	72.30 a	73.61 a	72.41 a	66.31 a	65.71 a	65.10 a	66.12 a
4	RU1303138	73.03 a	71.58 a	70.79 a	70.96 a	61.05 a	65.12 a	62.03 a	65.44 a
5	RU1303153	72.89 a	71.74 a	70.39 a	70.87 a	61.10 a	65.95 a	59.54 a	65.80 a
6	RU1303181	73.81 a	73.17 a	72.08 ab	70.17 b	68.15 a	67.14 ab	64.27 bc	62.56 c
7	RU1403138	75.18 a	73.50 ab	71.91 ab	70.25 b	70.20 a	67.86 a	60.58 a	60.54 a
8	RU1403141	75.08 a	73.36 a	73.75 a	70.52 b	69.08 a	67.82 a	69.08 a	62.77 b
9	RU1503147	74.39 a	73.31 a	73.37 a	71.36 b	64.63 a	66.31 a	59.29 b	64.40 a
10	RU1503169	74.79 a	72.54 ab	70.75 b	72.15 b	65.88 a	66.93 a	60.72 a	66.41 a
11	RU1503175	74.98 a	72.16 b	74.12 ab	72.92 ab	65.50 a	64.81 a	65.32 a	67.21 a
12	RU1603086	74.01 a	73.17 a	71.81 a	71.47 a	67.79 a	67.67 a	63.77 a	64.05 a
13	RU1603089	74.31 a	73.99 a	72.96 a	71.55 a	61.66 a	68.63 a	63.68 a	64.41 a
14	RU1603113	74.31 a	73.42 ab	70.47 b	70.43 b	68.21 a	68.45 a	59.48 b	62.47 ab
15	RU1603116	73.06 a	72.93 a	70.19 b	70.20 b	62.90 b	67.31 a	59.76 c	60.56 bc
16	RU1603138	74.50 a	72.44 ab	73.18 a	70.06 b	65.13 ab	65.89 ab	67.02 a	61.62 b
17	RU1603166	74.75 a	73.30 a	73.11 a	69.17 b	67.90 a	66.30 ab	62.01 ab	57.60 b
18	RU1603178	72.71 a	72.28 a	69.45 b	68.92 b	66.37 a	65.49 ab	57.54 c	59.39 bc
19	RU1603187	75.84 a	74.24 ab	72.16 bc	70.88 c	68.99 a	70.01 a	61.21 b	62.93 b
20	PRESIDIO	73.09 a	72.70 a	73.01 a	71.24 a	60.11 a	63.54 a	63.24 a	63.88 a
Mean		74.18 a	72.87 b	72.17 c	71.00 d	65.73 a	66.54 a	62.61 b	63.56 b

Note. Data followed by different letters within one row denote significant differences between the main crop and ratoon crop in a given year for each test entry at the 5% level according to the LSD test. Means followed by bold letters within one row denote significant differences between the main crop and ratoon crop across two years for all test entries at the 5% level according to the LSD test.

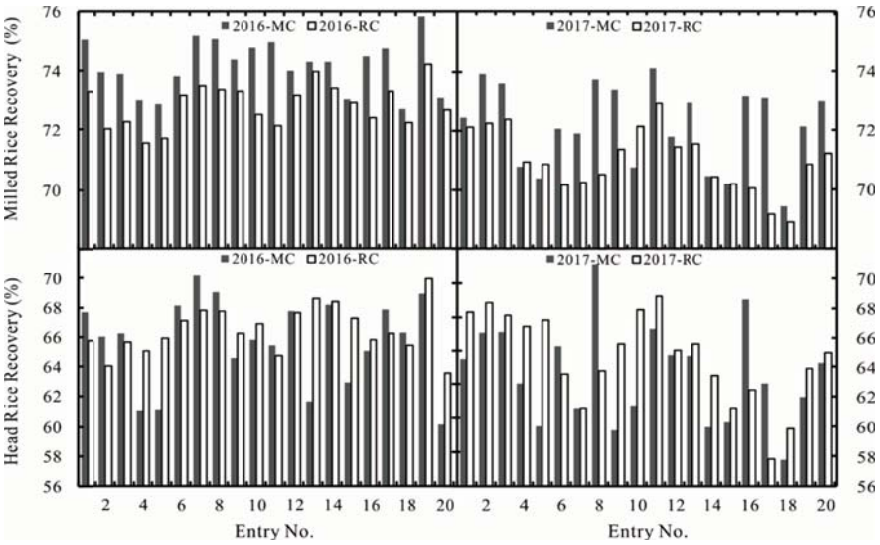

Figure 3. Yearly variation in milled rice recovery and head rice recovery in main crop (MC) and ratoon crop (RC) of tested breeding lines and varieties

3.3 Milled Rice Recovery and Head Rice Recovery Performance in the MC and RC Across Two Years

The milled rice recovery for the MC was 1.8% higher than that of the RC in 2016 and 1.65% higher than that of the RC in 2017. For head rice recovery, there was no significant difference between MC and RC within the same year, but both were higher in 2016 than in 2017, and head rice recovery was 4.83% higher in 2016 than in 2017 on average for both MC and RC (Table 4; Figure 3). Year had the most significant effect on both milled rice recovery and head rice recovery. Crop Type and Entry also had significant effects on milled rice recovery, and Entry and Y × E had significant effects on head rice recovery (Table 2).

Table 5. Comparison of main crop (MC), ratoon crop (RC) and total (MC+RC) mean yield of tested breeding lines and two CK varieties

No.	Breeding Line/Variety	Yield (t/ha)					
		2016 MC	2016 RC	2016 Total (MC+RC)	2017 MC	2017 RC	2017 Total (MC+RC)
1	ANTONIO	8.92 c	4.48 d	13.41 a	10.14 b	3.24 e	13.39 a
2	RU0803147	9.39 b	3.69 c	13.08 a	9.77 b	3.22 d	12.99 a
3	RU0803153	9.37 b	3.17 c	12.54 a	10.40 b	3.08 c	13.48 a
4	RU1303138	8.49 b	6.97 c	15.47 a	9.64 b	5.59 c	15.24 a
5	RU1303153	7.39 b	6.29 d	13.68 a	9.02 c	5.37 d	14.38 a
6	RU1303181	8.53 c	3.95 d	12.48 a	10.31 b	3.33 d	13.64 a
7	RU1403138	8.24 c	3.64 d	11.88 b	11.20 b	3.59 a	14.79 a
8	RU1403141	9.24 b	4.54 c	13.78 a	9.43 b	3.79 c	13.22 a
9	RU1503147	8.77 b	4.94 c	13.71 a	9.90 b	4.11 c	14.01 a
10	RU1503169	9.40 b	3.16 c	12.55 a	8.92 b	2.64 c	11.55 a
11	RU1503175	9.46 b	3.25 c	12.71 a	9.62 b	2.98 c	12.60 a
12	RU1603086	7.89 c	4.39 d	12.28 a	9.72 b	2.73 d	12.45 a
13	RU1603089	8.92 b	2.17 c	11.10 a	9.88 b	2.59 c	12.48 a
14	RU1603113	8.51 b	3.95 c	12.46 a	9.50 b	3.35 c	12.85 a
15	RU1603116	9.52 c	5.19 d	14.72 a	9.79 b	4.35 d	14.14 a
16	RU1603138	8.37 b	3.34 c	11.71 a	9.72 b	3.13 c	12.85 a
17	RU1603166	8.07 b	2.67 c	9.86 a	8.18 b	2.37 c	10.55 a
18	RU1603178	6.39 bc	5.48 c	11.87 a	8.62 b	4.98 c	13.60 a
19	RU1603187	8.42 b	3.39 c	10.68 a	8.11 b	2.49 c	10.60 a
20	PRESIDIO	7.91 b	4.72 c	12.63 a	7.57 b	3.70 c	11.26 a
Mean		8.56 c	4.07 d	12.63 a	9.47 b	3.53 e	13.00 a

Note. Data followed by different letters within one row denote a significant difference in grain yield among the main crop, ratoon crop and total yield across two years for each entry at the 5% level according to the LSD test. Means followed by bold letters within one row denote a significant difference in grain yield among the main crop, ratoon crop and total yield across two years for all test entries at the 5% level according to the LSD test.

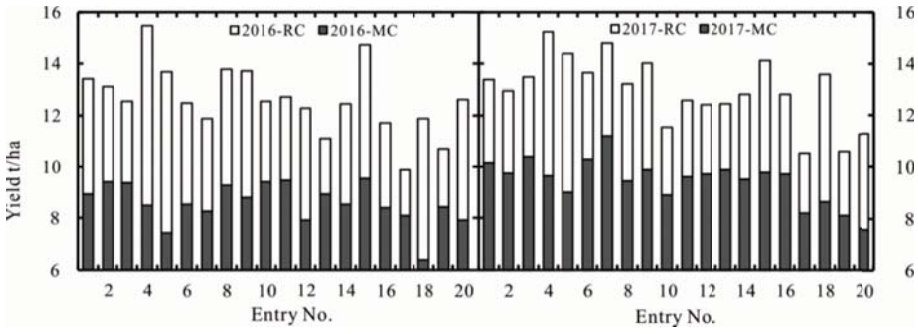

Figure 4. Yearly variation in main crop (MC) yield, ratoon crop (RC) yield and total yield of tested breeding lines and two varieties

3.4 Yield Performance in the MC and RC Across Two Years

The MC in 2017 (with an average of 9.47 t/ha) yielded 10.6% more than the MC in 2016 (with an average of 8.56 t/ha). However, the RC in 2017 (with an average of 3.53 t/ha) yielded 13.30% lower than the RC in 2016 (with an average of 4.07 t/ha); as a result, no significant difference was detected for total yield (MC + RC) between these two years, with values ranging from 9.86 to 15.47 t/ha and an average yield of 12.63 and 13.00 t/ha, respectively. The RC yield was approximately half of the MC yield in both years, with an average ratio of 47.5 and 37.3% (Table 5; Figure 4). The total yield in the two years was statistically similar, but it varied with entry and crop type. All two-way interactions were found to be significant, but three-way interactions were not significant (Table 2).

Table 6. Correlation coefficients of all evaluated traits in main crop and ratoon crop

	MC-CY	MC-WV	MC-GL	MC-GW	MC-MRR	MC-HRR	MC-YD	RC-CY	RC-WV	RC-GL	RC-GW	RC-MRR	RC-HRR
MC-WV	0												
MC-GL	-0.05	0.12											
MC-GW	0.32*	-0.02	-0.09										
MC-MRR	0.31*	0.25	0.12	-0.17									
MC-HRR	0.1	0.23	0.11	-0.08	0.78**								
MC-YD	0.24	-0.39*	0.2	0.48**	-0.18	-0.14							
RC-CY	-0.02	0.19	-0.12	-0.16	0.1	0.18	-0.35*						
RC-WV	-0.11	0.55**	0.16	-0.22	0.22	0.23	-0.46**	0.62**					
RC-GL	-0.02	0.57**	0.62**	-0.39*	0.44**	0.28	-0.22	0.05	0.48**				
RC-GW	0.18	0.01	-0.22	0.73**	-0.07	-0.01	0.22	0.1	-0.05	-0.28			
RC-MRR	0.25	0.51**	0.26	-0.13	0.74**	0.58**	-0.25	0.08	0.29	0.53**	-0.13		
RC-HRR	0.27	0.38*	0.23	0.03	0.58**	0.52**	-0.18	0	0.18	0.37*	-0.09	0.91**	
RC-YD	-0.11	0.29	0.01	0.12	-0.25	-0.24	-0.26	0.12	0.17	0.19	0.25	-0.09	0.01

Note. The numbers in the table indicate the correlation coefficient between different traits in the main crop and ratoon crop. MC, main crop; RC, ratoon crop; CY, chalkiness; WV, white vitreous; GL, grain length; GW, grain width; MRR, milled rice recovery; HRR, head rice recovery; YD, Yield. *: $P < 0.05$; **: $P < 0.01$.

3.5 Correlation Analysis of All Evaluated Traits in the Main Crop and Ratoon Crop

The correlation coefficients for the relationships of all quality-related traits and yield in MC and RC are shown in Table 6. Grain appearance, including grain chalkiness and translucency, correlated with grain size, *e.g.*, MC chalkiness was positively correlated with MC grain width (0.32), and RC white vitreous was positively correlated with RC grain length (0.48). In addition, increased chalkiness in the RC resulted in increased white vitreous (0.62). Milled rice recovery and head rice recovery were positively correlated in the MC and RC, and grain length was correlated with milled rice recovery and head rice recovery in the RC.

Yield was also correlated with grain size or grain quality traits, *e.g.*, increasing grain width in the MC was accompanied by increasing MC yield (0.48), and increasing MC yield resulted in decreasing MC white vitreous (-0.4). In addition, MC yield was negatively correlated with chalkiness and white vitreous in the RC, with correlation coefficients of -0.35 and -0.46, respectively, but no correlation was observed for RC yield.

4. Discussion

The ratoon rice system, as an energy-saving crop production method, has gradually become more common worldwide, but little is known about the grain quality difference between the MC and RC within the same rice varieties. Our results suggested that ratoon rice had a better appearance, with 4.96% lower chalkiness than main crop rice and higher translucency for most test entries in the two years. Additionally, some breeding lines, such as RU1603166 and RU1603178, had comparable quality traits with quality CK varieties, with consistently low chalkiness in both the MC and RC for two years. Although the milled rice recovery in the MC was higher than in the RC in both years, there was no difference in head rice recovery between the MC and RC in the same year.

Because of the shorter growth stage for the RC, approximately half that of the MC, the RC yield was approximately half that of the MC on average. However, some elite breeding lines had better ratooning ability,

e.g., RU1303138 and RU1303153 had similar MC and RC yields and higher total yields than CK ANTONIO, so these lines could be candidate donor varieties for ratoon production.

Rice chalkiness not only affects grain appearance and milling properties but also cooking quality, so it is a key determinant of the commercial value of milled rice. It has been well documented that chalkiness is controlled by polygenes (Wan et al., 2007; Woo et al., 2008; Zhou et al., 2009; Li et al., 2014; Gong et al., 2017), resulting from loose packing of starch granules in the grain endosperm (Zhang et al., 2013; Lu et al., 2018). However, chalkiness has also been reported to be sensitive to the environment, especially high temperatures during the grain filling stage, which accelerate ripening and result in loose packing of the starch granules, thereby greatly increasing the proportion of chalky grains (Cooper et al., 2008; Ishimaru et al., 2009; Chen et al., 2013). Tashiro et al. (1991) reported that a temperature of over 26 °C during the filling stage would significantly enhance chalkiness, and Laenoi et al. (2017) reported that a variety with the chalkiest grain in the hot season had nearly no chalkiness in the cool season. Ratoon rice is a different cultivation system. In contrast to the main crop, the temperature during the RC total growth period decreased gradually. Considering the weather data during the growth period of the MC and RC in two years, a comparison was made of the average maximum and minimum temperatures of the MC and the RC from heading to maturity covering most of the test entries. The average maximum and minimum temperatures for the MC were 30 °C and 16 °C in 2016 and 30 °C and 19 °C in 2017. These values were much higher than the maximum and minimum temperatures for the RC, of 12 °C and 0 °C in 2016 and 10 °C and 4 °C in 2017. The low temperature for the MC was nearly the highest temperature for the RC. The RC, therefore, was exposed to much lower maximum and minimum temperatures compared with the MC during the grain filling stage, which may be the main reason for the lower chalkiness of the RC.

A three-way ANOVA indicated that crop type had the most significant effect on chalkiness, so temperature difference may have played the key role in determining the chalkiness of the MC and RC milled grains. The yearly difference in average temperature for both the MC and the RC further affected the degree of chalkiness. As expected, genotype is a factor in grain chalkiness, which could be due to the QTLs that were reported. However, the interaction of genotype with crop type and year and the three-way interaction obtained in this study support the complexity of chalkiness as a trait to focus on in breeding programs. These complex interactions could be the reason for slow progress in breeding for low chalk in rice. The stability of low chalk across years and crop type is a very desirable trait, and achieving it could be challenging. It can be noted, however, that some entries, such as RU1603166 and RU1603178, were stable over the two years and had nearly the same chalkiness in both the MC and the RC; thus, they could be potential donors to a breeding program for low chalk.

It was reported that endosperm translucency had the highest correlation with chalkiness (Li et al., 2003b; Hao et al., 2009), and its formation was also similar to chalkiness, in that endosperm with tightly packed starch granules and no air space is more translucent than endosperm with loosely packed starch granules and more air space (Lu et al., 2018); thus, those genes that are related to starch synthesis and packaging affect grain translucency (Wan et al., 2007; Zeng et al., 2007). Our results suggested the same; RC chalkiness had the highest correlation with RC white vitreous with a correlation coefficient of 0.62, but no correlation was observed between chalkiness and white vitreous in the MC. As in chalkiness, crop type had the most significant effect on translucency. Similarly, the year affected translucency but not the entry or genotype. No interactions were observed, indicating that this trait is less complex than chalkiness. Based on these results, it can be concluded that translucency was determined mainly by the environment.

Head rice recovery is a high-priority objective for breeders because the market value of head grain is twice that of broken grain. Many QTLs related to head rice recovery have been mapped, and some of them overlapped with QTLs that control grain length because long grains tend to break easily (Tan et al., 2001; Nelson et al., 2011). However, our results showed that head rice recovery in RC was positively correlated with grain length.

Recent studies have identified several key QTLs and genes controlling grain size (Mao et al., 2010; Liu et al., 2017; Choi et al., 2018), and the molecular mechanisms of grain size regulation are gradually becoming clearer (Li et al., 2018). Aside from genetic control, it has been reported that environment has an effect on grain size; Funaba et al. (2006) reported that low temperature augmented grain weight for the extended grain maturity period. Our results indicated, although with lower average temperature and longer growth duration from heading to maturity, that some RC entries had smaller grain size (grain length or width) than the MC in 2017, but not in 2016. This may be due to environmental differences in different years and the shorter total growth period for RC. A three-way ANOVA also indicated that year had the most significant effect on both grain length and width, and crop type, but not entry, also affected grain width.

Grain size was also correlated with other quality traits and yield, and our results showed that wider grain width resulted in higher chalkiness in the MC but not in the RC. A similar result was also reported by Song et al. (2007), that the *GW2* allele for increasing grain width had a negative effect on grain chalkiness. In addition, the RC grain length had a positive correlation with white vitreous readings in MC and RC, indicating that increasing grain length had a negative effect on grain translucency.

Rice yield is a complex trait determined by its three components: grain weight, the number of grains per panicle and the number of panicles; many QTLs for these traits have been mapped, cloned or functionally characterized, *e.g.*, *OsNPF7.2* and *MOC1* control tiller number in rice (Li et al., 2003a; Wang et al., 2018); *GS3* and *GW2* control grain size or weight (Mao et al., 2010; Choi et al., 2018); and *Gn1a* and *PROG1* control grain number (Ashikari et al., 2005; Jin et al., 2008). In addition, yield always had a positive correlation with growth duration (Ying et al., 1998), so the RC yield was only approximately half that of the MC because the total growth duration of the RC was only approximately 60% that of MC. However, with the aid of different component-of-yield-related genes or QTLs in each entry, those with better ratooning ability could be selected as candidate donors for ratoon production. For example, RU1303138 had the highest total grain yield, and its RC yield was 82.10 and 58.00% of MC in 2016 and 2017, respectively. In addition, several others also yielded much better than the yield CK, ANTONIO.

A three-way ANOVA indicated that yield was a complex trait affected by many factors. Crop type had the most significant effect on yield, followed by entry; in addition, two-way interactions among years, crop type and entries were also observed. Yield was also correlated with quality traits, such as higher yield resulting in lower white vitreous (better translucency) in MC and RC.

A common practice in the U.S. while ratooning is to combine both MC and RC harvests of a variety in a bin for convenient storage and to maintain the varietal identity. Based on our results, we concluded that ratoon grain could help improve the quality of the mixture because of its good appearance (lower chalkiness and higher translucency). However, the instability of the grain size between the MC and the RC should be considered to minimize the nonuniformity of the grain size of the mixture. Currently, in China, rice grain is classified into four types according to its origin: ratoon grain, late-season grain, middle-season grain and early-season grain. The price decreases from the first classification to the fourth. The price of the RC grain is nearly two times that of the MC grain because, generally speaking, the ratoon crop, due to the low temperatures during its grain filling stage, has the best grain quality. Thus, for higher profit, the MC and RC could be stored and sold separately, which would be a good choice for U.S. rice growers.

5. Conclusion

A comparison of milled grain quality and yield from the MC and RC was conducted in this study, it was shown that RC grains had better appearance quality, including lower chalkiness and higher translucency, than the MC grains; in addition, some MC entries behaved larger grain size in one or two years. Therefore, RC harvests can be mixed with their corresponding MC harvests to improved total grain quality of a variety when variety identification is being practiced. For better uniformity in grain size of the mixture of MC and RC, data on grain length and width should be considered during this practice. Some breeding lines had better and near stable ratooning ability and yield more than CKs, so they could be donors in breeding for ratoon rice production.

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Market Integration in the International Market of Soybeans: Are GM Soy and Non-GM Soy Markets Integrated?

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Abstract

This paper aims to study market integration in the international trade of soybeans from 1999 to 2019. The hypothesis is that the market remained integrated between genetically modified (GM) and non-GM soy, even after stringent regulations against GM soy in major importers starting in 1999. Using FOB prices from major exporters of GM soy (USA and Argentina) and non-GM soy (Brazil), I test for market integration with cointegration analysis and Granger causality tests. All tests show that the market between all three exporters remained integrated throughout the sample period. Furthermore, Granger causality tests show that USA remains the sole price leader. Short run elasticities for reactions to American price changes in Brazil and Argentina are 0.33 and 0.25, respectively. The results validate the Law of One Price and inform policy decisions and forecasts efforts in this valuable commodity.

Keywords: market integration, soybeans, cointegration, granger causality, structural breaks

1. Introduction

The purpose of this paper is to study the horizontal spatial transmission for the international market of soybeans over the period 1999-2019, in order to check for market integration under the Law of One Price (LOP), as well as report the elasticities and the directionality of the price transmission.

The LOP is the basic foundational notion in economics that the prices of equal goods in different locations within an open market will tend to equalize, as market agents make use of arbitrage opportunities to close existing gaps. When markets share a price under LOP they are said to be integrated. Product differentiation can cause deviations from LOP (Pippenger & Phillips, 2008). Such disruptions to trade have occurred in soybeans due to the development of genetically modified (GM) soy, where differences regarding the legal status of GM soy in major exporters and importers has led to the possibility of a rift between a GM soy and a non-GM soy markets.

First developed in 1996, GM soy quickly became the dominant form in major producers and exporters, including USA, Argentina, and Paraguay, where by 2016 it accounted for 94%, 100%, and 96% of total production, respectively. However, Brazil, the single biggest exporter, still produces a majority non-GM crop, with 32% GM cultivation the same year (ISAAA).

Moreover, the EU, who collectively make up the second largest importer, has banned trade of GM soy varieties, starting in 1999 with a moratorium on trade of GM food, fixed in place by stringent new regulations in 2003, and then formalized in bans across numerous member countries. Also China, currently the biggest importer by a large margin, has placed stringent regulations on GM soy trade, including prohibition of direct consumption, all imports are exclusively for industrial use, and long delays in approving individual GM varieties. Because each new GM event requires each separate country's approval before it can be freely traded, this causes further trade disruptions as specific new events are approved in exporting countries but not yet in the importers.

By testing for market integration between major exporters Brazil, USA, and Argentina, who altogether account for nearly 90% total world supply, price transmission between GM and non-GM soy prices can be tested, i.e. market integration between GM and non-GM soy.

Market integration under LOP assumptions will be checked for through cointegration analysis. Then, if spatial transmission is found, the dynamics of price leadership will be studied through Granger causality. This provides further proof of market integration (Ravallion, 1986). The hypothesis is that the international market for soybeans is highly integrated even through the period of highest volatility and the growing trend in GM bans. In addition, I expect USA to appear as price leader, given the traditional role of that the prices in the Chicago Board of Trade (CBOT) have played as reference prices for the market. However, I also expect the possibility that Brazil might have assumed price leadership as well, given their growing importance in the market, surpassing USA as main exporter in 2012. This study was done using STATA 15.

The chosen period involves a time of significant price volatility, as American FOB prices went from a minimum of 152 USD near the start of the period in October 2001, to experience several dramatic peaks starting in 2007 with a maximum of 628 USD in August, 2012, and finish at the range of low 300s USD by the end of the period. This volatility and shifts in trend and levels might surface in the form of a structural break in the time series analysis.

The results aim to validate the LOP, and do so in the context of an exported commodity, a market heavily distorted by trade regulation, and over a period of wide price fluctuation. Furthermore, given the relevance of this commodity for the world economy, I hope to provide useful information on the dynamics of price transmission for agricultural commodities, that might assist forecast efforts and policy design.

2. Literature Review

The transmission of international commodity prices has traditionally been studied under the theoretical framework of the LOP, which states that markets that trade tend to show equal prices for equal products, within a certain rate measured by the efficiency of the transmission (for a full review, see Fackler & Goodwin, 2001). It was originally tested for with lineal correlations, however, when innovations related to cointegration analysis came about, the LOP came under intense criticism because many studies found no supporting evidence for it. Ardeni (1989) found that under cointegration the LOP did not stand empirical tests, as permanent deviations to it were apparent and widespread. Later studies have salvaged the LOP by introducing conditions that need to be checked for, in order to ensure methodological robustness. Pippenger and Phillips (2008) identify such conditions, one of them being using identical products, which has been broken in the international soybeans market by the advent of GM soy.

For price transmission, there is a large literature focused on horizontal spatial transmission, mostly in areas such as international financial markets and commodity trade, in agriculture and energy. A key concept in horizontal spatial transmission is that of market integration. Markets are said to be integrated when co-movements of prices are observed in them, that is, price information is transmitted between them. Since the influential work of Ardeni (1989), the academic literature has taken to the presence of cointegration as sufficient proof that market integration exists (Listorti & Esposti, 2012). This is because a stable (nonstationary) long-run relationship is both a requisite for the validity of LOP as well as the presence of cointegration (Ardeni, 1989). Ravallion (1986) and Garbade and Silver (1978) have provided alternative methods for establishing market integration, based on Granger causality and weak exogeneity tests.

2.1 Studies on Soybeans International Market Integration

Uri, Chomo, Hoskin, and Hyberg (1993) tested for market integration in soybeans, soy meal, and soy oil international markets, using Granger causality. They test the prices of American, Brazilian, and EU products pairwise, and establish market integration in all cases. However, it should be noted this study was done before the development of GM soy and its possible effects on market integration.

For price transmission, Machado and Margarido (2001) study the Argentinian, American and Brazilian FOB prices as well as Rotterdam (EU) CIF price series using ARIMA deseasoning and Granger Causality on a vector autoregression (VAR) on log-levels, and find that both Brazilian and Argentinian prices are Granger-caused by European CIF prices, whereas American prices are not related to any of the other series. In a similar later study (Machado & Margarido, 2004) they find evidence for transmission from USA to Argentina and Brazil, albeit weaker than the responses to EU prices. It is worth noting that these studies were done before the EU banned the trade on GM soybeans and before the dramatic rise of Chinese imports, so the EU was back then still the most important destination for Argentinian and Brazilian soybeans. In addition, these studies were conducted before it

was widely understood that Granger Causality is not robust to the presence of orders of integration as well as cointegration in the underlying VAR when using the true order of lags (Toda & Phillips, 1994).

Margarido, Turolla, and Bueno (2004) used cointegration, Granger Causality, and other methods to characterize the seasonal international price transmission for soybeans using Argentinian, American and Brazilian FOB prices as well as Rotterdam (EU) CIF prices. They find that shocks in American and European prices transmit to Brazilian prices, whereas Argentinians only respond to American ones, and American prices are only weakly affected by Brazilians. Overall the short-run reactions appear to be slow. They offered the absence of trade due to EU regulations on GM as the explanation for the lack of Argentinian response to EU shocks, and the seasonal symmetry between USA and Brazil as the reason behind the small reactions observed in USA to Brazilian prices.

More recent studies of price leadership have tended to focus in futures markets. Christofoletti, Silva, and Mattos (2012) studied market integration for both spot and future soybeans prices between USA, China, Brazil, and Argentina, using cointegration analysis on daily prices from 2002 to 2011, split into subperiods to control for structural breaks. Their results show that the relationship has changed over time, interestingly, both USA and Argentina are shown to no longer be integrated to the other prices during and after the food crisis. These results could indicate a rift between GM soy and non-GM soy markets.

Han, Liang, and Tang (2013) used cointegration to study price discovery between CBOT and the Dalian Commodity Exchange (DCE) in China from 2002 to 2011. They found bidirectional effects, lead by CBOT who has the greater impact. Li and Hayes (2016) studied future prices in China, USA, and Brazil, from 2005 to 2015, using non-linear cointegration and weak exogeneity, as well as sample splitting. They found instead that CBOT prices retain single leadership over the futures prices market, although there is a weakening trend in the link, and in addition it is affected by seasonal variations with Brazil adopting temporal leadership during its marketing season. Liu et al. (2015) used the generalized autoregressive conditional heteroskedasticity-generalized error distribution (GARCH-GED) model to study future prices volatility transmission between CBOT and DCE from 2006 to 2012, with sample splitting to account for the 2008 subprime crisis, and found CBOT prices no longer lead DCE future prices for non-GM soy after the break. Finally, Lee, Lin, and Liao (2013) used a cointegration representation with GARCH and sample splitting for structural breaks to study price leadership between CBOT and DCE in several commodities futures prices, including soybeans, from 2002 to 2011. For soybeans, they found CBOT leading DCE in long-run adjustments, however for short-run transmissions the opposite is true.

It can be seen that the existing literature on this subjects, except futures prices, is quite dated, for market integration preceding the development of GM soy and for price transmission being before the advent of stringent GM soy regulation and market changes in the last 15 years. Regarding price leadership in this market, the consensus among the literature reviewed is that USA is the traditional price maker, thanks to the leading role played by CBOT prices, and Brazil and Argentina are price takers.

However, the development of GM soy and the subsequent regulations on its trade, the growing role of Brazil in export trade, as well as findings on futures prices about a weakening trend in American price leadership, all prompt the need to update the study of market integration and price transmission in the international market for soybeans, with more recent data that might reflect the changing trade patterns in the last 20 years.

3. Data

The price series to be compared are the spot prices of Brazil, USA, and Argentina soybeans export prices. These three exporters altogether account for close to 90% of total international supply during the chosen period. The price series start in October, 1999, and end in February, 2019, with a total of 233 observations. Their natural logarithms are taken, to provide of ease of interpretation of some of the results.

The price series used were as provided in Table 29 of the publication *Oilseeds: World Market and Trade* over many numbers (1999-2019), published by the United States Department of Agriculture (USDA). For Brazil, Brazil Paranagua FOB price is used; for USA, U.S. NO.1 Yellow Cash Central Illinois; and for Argentina, the Argentina Up River FOB. All prices are measured in US dollars.

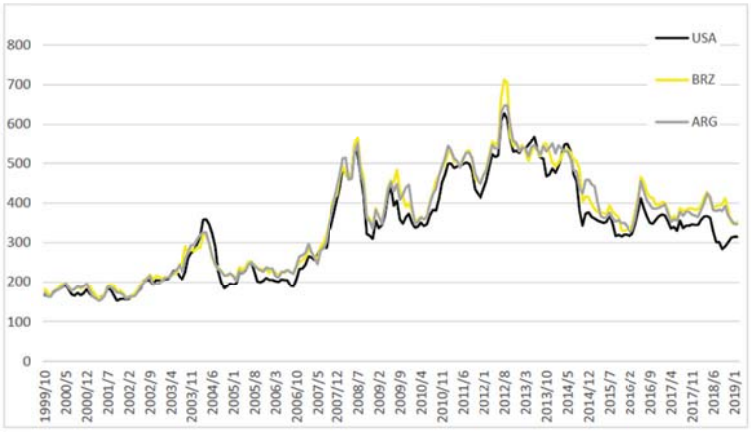

Figure 1. Argentinian, American and Brazilian FOB prices for soybeans, in USD (1999-2019)

The period 1999-2019 saw agricultural commodity prices experience great volatility, with two distinct spikes in the years 2008 and 2012, as part of a general worldwide food-items price inflation. A new development by the end of the period was the growing spread between the export prices, as a large temporary malus to the American soybeans export price appeared as a result of trade frictions with China. American prices are, on average, the cheapest of the three main exporters throughout the whole period, however, during the second semester of 2018 this spread was momentarily much more pronounced than in the rest of the period under study.

4. Methodology

Market integration will be tested for using Johansen's method for cointegration. Cointegration analysis begins by testing the selected variables for unit roots using an Augmented Dickey-Fuller (ADF) test (Said & Dickey, 1984), in order to test a null hypothesis of nonstationarity against an alternative hypothesis of stationarity or trend-stationarity. This further involves a test to their first level difference in order to clarify the exact order of integration, if the first test fails to reject the null hypothesis but the test to their first differences does not, then the series is taken to be nonstationary with first level differences stationarity, that is, I(1). If the second test fails too, then the series must be an explosive process (higher than first order integration), and would require transformation.

The following step is to use the widely known Johansen procedure (Johansen, 1995) in order to sequentally test the null hypothesis of no cointegration starting from 0 and increasing until the null hypothesis is rejected, or the amount of variables is reached. If at any point before that the null hypothesis is rejected, then cointegration is validated and the amount of cointegrating vectors shown. This way, the procedure tests for both the presence and number of cointegrating vectors between the variables.

If, as expected per previous studies, the time series for the selected prices turn out to be I(1) per ADF results, and a cointegrating relationship is detected by the Johansen test, then the variables can be fitted into a Vector Error Correction Model (VECM) that follows the form:

$$\Delta p_t = c + \Pi ECM_{t\text{-}1} + \sum_{j=1}^{l} \Gamma_j \Delta p_{t\text{-}j} + \varepsilon_t \quad (1)$$

Where, Π and Γ are matrixes, and p, c, and ε are nx1 vectors, l is the autoregressive order such that $l \geq 1$, and ε residuals are assumed independently and identically distributed (iid).

The ECM term represents a lagged error correction term, measuring the stationary linear combination of the cointegrated variables, that reintroduces the long term dynamics that were lost by differencing the variables.

$$ECM_{t-1} = p_{1t-1} - \beta_0 - \beta_1 p_{2t-1} \quad (2)$$

In fact, it is simply the residuals from a linear regression of the terms, however, by the properties of cointegration, it should not carry the nonstationarity of the original level variables. Its lag is set at 1 per model definition (Johansen, 1995).

When estimating the VECM representation, coefficients for the lagged differences of the variables then provide the short-run relations between them. If the variables are in log-levels, then the significant coefficients can be read directly as elasticities. The coefficients of the error correction term, in turn, provide the long-run adjustment of the variables to equilibrium, implied by the cointegration.

The final step of the study will be running Granger Causality tests under the Toda-Yamamoto procedure (Toda & Yamamoto, 1995), to look at directionality of the horizontal price transmission and provide further proof of market integration. The null hypothesis implies that lagged coefficients for a variable do not add power to explaining the variation of another variable solely based on its own lagged value. On the other hand, rejecting the null, i.e. declaring Granger causality, means that knowing and including the lagged values of the Granger-causing variable can enhance our forecasting models for the Granger-caused variable. This is because there is a tendency for the variation on the Granger-causing variable to precede similar variation in the Granger-caused one.

In the context of this study, it is critical to note that existing literature has proven that it is best not to run Granger causality tests on coefficients obtained through a VECM model. This is because in these cases the pretesting for unit roots and cointegration causes the distribution of the test statistics not to properly converge to a chi-squared distribution under the null. As a result, estimates are unaffected but critical values are unreliable, and there is a severe increase to Type I error probabilities (Toda & Phillips, 1994; Dolado & Lütkepohl, 1996).

In order to address these issues, Toda and Yamamoto (1995) developed a method for testing Granger Causality over VAR models that may be integrated or cointegrated. It does not require pretesting for integration and cointegration, and thus bypasses the above-mentioned problems related to unit root and cointegration.

Instead, it relies on intentionally overfitting a VAR on levels model with as many extra lags as orders of integration the variables may possibly have. However, the coefficients of the extra lags are not used as part of the Wald test in the following step. The equations for Granger Causality thus remain the same. They prove that such a model provides a robust basis for Wald-testing the resulting coefficients, other than those of the extra lags which are not included in the test and assumed to be zero.

The authors realize that this method suffers from inefficiency due to the intentional overfitting in lag order, however they believe this inneficiency is preferable to the problems that arise with alternative methods. Both Zapata and Rambaldi (1997), and Clarke and Mirza (2006), when directly comparing the properties of the likelihood ratio test as used under Johansen's method, to the Toda-Yamamoto method with extra lags augmented-Walt tests, agree that the latter is far preferable to the former, unless the sample size is very small (less than 50 observations). Additionally, they prove the Toda-Yamamoto procedure is robust to a variety of situations such as stability conditions, cointegration rank, and many other forms of underlying model misspecification.

4.1 Identification of Structural Break

Johansen's method suffers from very low power when faced with time series whose true underlying model have an unknown structural break in levels (constant) or regime (constant and slope) of the cointegrating vectors or the trend component, as shown by Gregory and Hansen (1996, 2009).

They propose instead three possible residuals-based tests with a null of no cointegration and an alternate hypothesis of cointegration with a single structural break point of unknown timing. These tests are multivariate generalizations of the modified ADF, Za and Zt tests (Zivot & Andrews, 1992). The purpose of the Gregory-Hansen tests is to find the break point under cointegration by repeatedly running ADF, Za or Zt tests for all points and choosing the one that minimizes the modified statistics. They provide the asymptotic distributions and critical values for up to four regressors.

However, it is worth noting that the Gregory-Hansen tests are powerful against the alternate of cointegration without a structural break as well, just like the unmodified ADF (Gregory & Hansen, 2009). If the purpose is to test for structural break under cointegration alone, then only the case where ADF fails to reject the null while Gregory-Hansen does reject it, would provide proof of the presence of a structural break. Otherwise, Gregory-Hansen does not provide conclusive proof that the true model is one of cointegration with a structural break.

If structural breaks have been identified, dummy variables will be used to control for their effects (Dawson, Sanjuan, & White, 2006; Dawson & Sanjuan, 2006).

5. Results and Discussion

The ADF results for all three series shows they are all I(1) nonstationary, since the tests fail to reject the null of nonstationarity at the levels, but do reject it at the first difference.

Table 1. ADF results

Variable	Test Stat.	p-value
ARG	-1.723	0.4193
ΔARG	-11.397	0.0000*
USA	-1.753	0.4038
ΔUSA	-10.279	0.0000*
BRZ	-1.658	0.4527
ΔBRZ	-11.800	0.0000*

Note. ADF results at log-levels and first differences. (*) is for rejections of the null of unit root.

Since the variables are all I(1), the next step is to test for cointegration, in order to decide if the better fit is a VECM or a VAR in differences. I use Gregory-Hansen tests for the null of no cointegration against a null of cointegration with a single structural break, with 2 optimal lags chosen through AIC (Akaike Information Criteria). The result of the test based on ADF was failure to reject the null of cointegration with a regime break in July, 2009. The date corresponds to a second short peak after the initial rise and drop in the 2007-2008 period.

Table 2. Gregory-Hansen modified ADF test result

	Breakpoint	Test Stat.	1% Crit. Value	5% Crit. Value	10% Cr. Value
ADF	118	-7.29*	-5.97	-5.90	-5.23

Note. (*) is for rejection of the null of no cointegration.

In order to further test the break point, Johansen tests were run on two models, where one was fit with a dummy variable to account for the break and one without it, and then compare the results. Dawson, Sanjuan, and White (2006) and Dawson and Sanjuan (2006) have proven the validity of this method. The results, listed in Table 3, show that per our expectations, Johansen's procedure fails to detect cointegration without the break, whereas two cointegrating vectors were found with the break placed inside the model.

Table 3. Johansen method results

Rank	Critical-value	Without Break		Including Break	
		Eigenvalue	Trace	Eigenvalue	Trace
0	29.68		95.6576		96.1373
1	15.41	0.28121	19.3861	0.28142	19.7953
2	3.76	0.06431	4.0320	0.06762	3.6224 *
3		0.01730		0.01556	

Note. Johansen test for cointegration on two models, one that includes a structural break at July 2009 and one that ommits it. Both tests used 2 lags and the same sample. (*) is for first non-rejection of the null

When including the dummy for structural break according to the results of the Gregory-Hansen test, the resulting model has two cointegrating vectors, the maximum possible in a three variable model, and this is an indication of the stability and integration of the long-run relationship between the price series, i.e. the international market of soybeans (Listorti & Esposti, 2012).

The next step of this study is to fit a VECM model to capture the short run and long run dynamics as well as provide information for the Granger Causality tests in later steps, since it reinforces the causal relationship found in those tests. The model includes two dummy variables, one for seasonal variations and one for the structural break in July, 2009, as identified by the Gregory-Hansen test.

Table 4 lists the results of the VECM model for the equation. The single relevant short run elasticities are those for lagged differences of American prices. They show a 1% increase in the American FOB price will cause a 0.33% increase in Brazilian price and a lower 0.25% increase for Argentinian prices within one month.

Table 4. VECM results

ΔARGt	Coef.	Std. Error	z	P>z	95% Confidence Interval	
ce1	-0.3088	0.1105	-2.79	0.005*	-0.5254	-0.0922
ce2	0.0066	0.0649	0.10	0.918	-0.1206	0.1339
ΔARG t-1	0.2430	0.1405	1.73	0.084	-0.0324	0.5185
ΔUSA t-1	0.2531	0.1037	2.44	0.015*	0.0498	0.4564
ΔBRZ t-1	0.2122	0.1336	-1.59	0.112	-0.4741	0.0497
season	0.0050	0.0083	0.60	0.546	-0.0112	0.0213
break	-0.0139	0.0093	-1.49	0.137	-0.0322	0.0044
const.	0.0009	0.0090	0.913	0.913	-0.0166	0.0186
ΔUSA t	Coef.	Std. Error	z	P>z	95% Confidence Interval	
ce1	-0.3481	0.1067	-3.26	0.001*	-0.5573	-0.1389
ce2	0.1934	0.2040	0.95	0.343	-0.2064	0.5933
ΔARG t-1	-0.2394	0.2560	-1.59	0.350	-0.7413	0.2625
ΔUSA t-1	0.6957	0.1486	4.68	0.000*	0.4044	0.9871
ΔBRZ t-1	-0.0663	0.2326	-0.29	0.775	-0.5223	0.3896
season	-0.0047	0.0137	-0.35	0.729	-0.0316	0.0221
break	-0.0199	0.0170	-1.17	0.240	-0.0533	0.0133
const.	0.0064	0.0089	0.72	0.472	-0.0111	0.0240
ΔBRZ t	Coef.	Std. Error	z	P>z	95% Confidence Interval	
ce1	-0.2104	0.0970	-2.17	0.030*	-0.4007	-0.0200
ce2	-0.2798	0.1856	-1.51	0.132	-0.6437	0.0839
ΔARG t-1	-0.0989	0.2329	-0.42	0.671	-0.5556	0.3576
ΔUSA t-1	0.3380	0.1352	2.50	0.012*	0.0730	0.6031
ΔBRZ t-1	-0.0712	0.2116	-0.34	0.737	-0.4861	0.3436
season	0.0268	0.0124	2.15	0.031*	0.0024	0.0513
break	-0.0163	0.0154	-1.05	0.292	-0.0466	0.0140
const.	-0.0042	0.0081	-0.52	0.603	-0.0202	0.0117
ce1 = 0.2086 + ARG + USA – 1.038BRZ				ce2 = 0.5438 + USA – 1.104BRZ		

Note. VECM results for Argentinian, American, and Brazilian prices, in that order. Cointegrating equations included at the bottom. (*) for p-values significant under 5% level of confidence.

The last step of this study is to run the Toda-Yamamoto procedure for the Granger Causality test of the cointegrated variables. AIC produced lag order 2 for the underlying VAR model, however an extra lag was required in order to address autocorrelation in the residuals, resulting in a VAR(3+1) underlying VAR model.

The results of the Granger Causality test show that both Argentina and Brazil are unidirectionally Granger-caused by United States, at 5% confidence level, per my expectations according to relevant literature. This reflects the traditional role as price maker that USA has had on the international soybeans market, with the CBOT prices acting as the reference prices for the market, as already identified by several studies (Machado & Margarido, 2004; Margarido, Turolla, & Bueno, 2004; Li & Hayes, 2016).

An additional possible source of Granger causality is found for Argentina from Brazil, which must have surfaced in the period under study. This could be explained by the dynamics of seasonal variation (Margarido, Turolla, & Bueno, 2007). However, that result only arises at 10% confidence level, so it is clear that despite becoming the main exporter since 2012, Brazil has not become a price leader in the market.

Table 5. Granger Causality tests results

Variable	Excluded	p-value
Argentina	Brazil	0.065
Argentina	USA	0.046*
Brazil	Argentina	0.716
Brazil	USA	0.044*
USA	Argentina	0.421
USA	Brazil	0.387

Note. Granger Causality test results obtained through Toda-Yamamoto method. (*) for p-values significant under 5% confidence level of confidence.

6. Conclusion

This study seeks to characterize the international price transmission of exports of soybeans, in order to check for market integration between GM and non-GM soy varieties. I first used cointegration analysis which proved the international market for soybeans has been well integrated between the three major exporters Brazil, USA, and Argentina over the last two decades. This shows that GM and non-GM soy still share price information and there has not yet been a split between these two markets. This has wide implications for price forecasts and policies. It shows, for example, that market analysis and forecasts for exclusively GM soy exporters like USA and Argentina should still track developments ocurring in exclusively non-GM markets like the EU.

It also showed Argentinian and Brazilian prices are significantly affected in the short-run by American prices, with 0.33% elasticity over a one month lag for Brazil, which is higher than the 0.25% for Argentinian prices. As a result Brazilian prices are more sensitive in the short run to American price changes, than Argentinian prices are.

Then the Toda-Yamamoto procedure for Granger Causality, showed that USA has retained price leadership throughout the period. Against expectations Brazil has largely remained a price taker, despite it becoming the main exporter in 2012 by a widening advantage. It seems that despite the changes in the market in the last 20 years, CBOT prices are still the undisputed reference for traders in both GM and non-GM varieties.

From a theorical point of view, this study provides further proof on the continued validity of the Law of One Price and methodologically for the use of lineal cointegration to analyze horizontal spatial transmission and market integration in the context of international commodity markets. It also provides an example of robust econometrical analysis in controlling structural breaks under cointegration.

Further research is required on the dynamics of price leadership, in order to provide empirical proof on the reasons behind the observed results. In addition, the use of recent innovations in econometrical studies, such as threshold models that reveal arbitrage prices and assymetrical transmission, could provide further proof and insight into the results found here. Finally, applying the methodology used here to higher-frequency data samples, *i.e.* daily or weekly prices, can help improve on the robustness of results for cointegration analysis.

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Germination and Seedling Growth of Genotypes *Crambe abyssinica* Submitted to Water Deficit

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Abstract

Determining drought tolerance in plants is an increasingly important feature due to the reduction of water resources, since water stress is one of the main environmental factors that limit agricultural growth and productivity. The objective of this study was to evaluate the tolerance of crambe (*Crambe abyssinica* Hochst) genotypes submitted to water stress induced by polyethylene glycol during germination and early growth of seedlings. A randomized block experimental design was used in a factorial scheme consisting of five crambe genotypes (FMS Brilhante, FMS CR 1203, 1307, 1312 and 1326) and five levels of osmotic potential [0.0 (control), -0.2, -0.4, -0.5 and -0.6 MPa] in five replicates of 40 seeds. Germination rate (%), normal seedling development (%), germination speed index, root and shoot length, total fresh matter, and water content of seedlings (%) were analyzed. Physiological quality of seeds and initial development of crambe genotypes was improved in the group submitted to Ψw = -0.2 MPa. Germination and vigor index of crambe seeds were hampered by reduction of the potential to -0.4 MPa. The genotype FMS CR 1203 was the most tolerant to water stress, whereas FMS CR 1307 and 1312 were the most sensitive, as corroborated by PCA.

Keywords: abiotic stress, FMS Brilhante, osmotic potential, polyethylene glycol, seed

1. Introduction

Water deficit is one of the most important and complex environmental factors limiting the germination of seeds (Viçosi et al., 2017) and development of seedlings (Machado et al., 2017). In seeds, water deficit reduces the turgor pressure, negatively affecting the expansion and growth of cells (Bewley & Black, 1994), reducing the availability of oxygen, gas exchanges and synthesis of enzymes and hormones for digestion, translocation and assimilation of reserves (Marcos-Filho, 2005), resulting in a reduced germination speed index (GSI) (Kader & Jutzi, 2002). This exposes the seeds to the action of pathogens and attacks by insects and other pests (Machado et al., 2017). Water deficit also causes an increase in the dry weight of embryos, due to the need for osmotic adjustment, associated with the accumulation of compatible solutes (Gill et al., 2003). According to Patanè et al. (2013), water deficit leads to more concentrated root tissue and lower water content in the roots. Furthermore, in genotypes more sensitive the metabolic signaling that regulates gene expression during water deficit can be reduced (Coelho et al., 2010), consequently stunting the growth of the hypocotyl and radicle (Viçosi et al., 2017).

Plants have developed many strategies to maintain growth when water availability is restricted or inconsistent (Silva et al., 2016), such as ionic homeostasis and activation of the enzyme antioxidant system, to promote cell detoxification and growth regulation (Zhu, 2001; Silva et al., 2017). However, these responses are generally more pronounced in genotypes that are tolerant to water deficit. According to Kappes et al. (2010), experiments involving germination of seeds under different osmotic potentials are important for selection of genotypes that are tolerant or susceptible to water deficit.

Several studies have investigated the germination of seeds submitted to different osmotic potentials, to screen for genotypes that are tolerant to water deficit (Machado et al., 2017; Viçosi et al., 2017; Paiva et al., 2018). These studies have the objective of improving the establishment of crops in the field. Machado et al. (2017) observed a higher germination rate, germination first count, fresh and dry matter in *Crambe abyssinica* (Hochst.) FMS CR 1101 genotype submitted to different osmotic potentials induced by polyethylene glycol 6000 (PEG 6000), which was attributed to higher tolerance to water deficit compared with the FMS Brilhante genotype. The water stress tolerance levels of seeds of cowpea [(*Vigna unguiculata* L. (Walp)] and the arabica coffee (*Coffea arabica* L.) varieties Red Bourbon and Mundo Novo compared to cultivar BA-10 utilizing PEG 6000 were evaluated (Paiva et al., 2018; Almeida et al., 2018). In these studies, it was possible to differentiate between drought-resistant and drought-sensitive cultivars by observing the level of seed germination and early development of seedlings. PEG 6000 is a chemically inert and nontoxic product that simulates low water potentials without being absorbed by seeds, due to the large size of its molecules (Villela et al., 1991). The application of PEG 6000 is one of the most widely used methods to identify genotypes that are tolerant to water deficiency by osmotic stress.

Crambe (*Crambe abyssinica* Hochst.) is an annual plant belonging to the family Brassicaceae, grown for industrial purposes as an oilseed crop. The oil is highly valuable and has multiple uses, such as to make plastics, lubricants and biodiesel (Carlsson et al., 2007). The oil content of the seeds ranges from 36 to 38% (Pitol et al., 2010), higher than that of soybeans (Faria, 2014). Because it does not compete with oilseed crops used to obtain edible oils, its cultivation is expanding in Brazil, particularly to produce vegetable insulating oil (Oliveira et al., 2015).

According to Pitol et al. (2012), new varieties are being tested to expand its cultivation and improve yield in Brazil. At present, FMS Brilhante is the only genotype registered in Brazil. However, the genotypes FMS CR 1312 and 1307 are considered to be candidate materials for pre-launch, and FMS CR 1213 and 1326 are still being tested by Brazil (Oliveira et al., 2015). The identification of genetic materials with high germinability and good development under environmental stress conditions like water deficit is necessary to improve productivity and expand the culture to regions characterized by low precipitation. Therefore, this study aimed to evaluate the tolerance of five crambe genotypes submitted to water stress during germination and early growth of seedlings.

2. Methods

The experiment was carried out at the Laboratory of Plant Ecophysiology of Federal University of Espírito Santo (UFES), in São Mateus, ES, Brazil, using seeds of five crambe genotypes (*Crambe abyssinica* Hochst., FMS Brilhante, FMS CR 1203, 1307, 1312 and 1326), obtained from the Mato Grosso do Sul Foundation (MS Foundation), an agency for research and diffusion of agricultural technologies.

Water deficit was induced by polyethylene glycol (PEG 6000) treatments. Four solutions with Ψw = -0.2, -0.4, -0.5, and -0.6 MPa were applied (distilled water was used as the control treatment), according to the levels established by Villela et al. (1991). To avoid hypoxia by flooding the seeds, which strongly inhibits germination, special care was taken during application of the solutions. The seed moisture level was determined by the oven-drying method, at 105±3 ºC for 24 hours (Brasil, 2009).

The crambe seeds were sterilized with solutions of 70% ethanol for 2 minutes, 1% (v/v) sodium hypochlorite for 20 min. and the fungicide Ridomil© for 10 min., followed by triple washing with autoclaved distilled water. The seeds were then planted in a gerbox box (11 × 11 × 3 cm) containing washed sand moistened to 60% of retention capacity (Brasil, 2009) and placed in a growth room at 25 ºC with photoperiod of 16 h light and 8 h dark.

The germination rate (%G) was determined according to the primary root emergence criterion. The percentage of normal plants (%NP) and germination speed index (GSI) were measured on the seventh day after sowing according to Brasil (2009), following Equations 1 and 2, as described by Maguire (1962).

$$\%G \text{ or } \%NP = (\Sigma ni/N) \times 100 \quad (1)$$

$$GSI = (G1/N1) + (G2/N2) + (Gn/n) \quad (2)$$

Where, %G or %NP: percentage of germination by radicle emission or percentage of normal plants, respectively; Σni: total number of germinated seeds; N: number of seeds placed to germinate; GSI: germination speed index; G1, G2, Gn: number of seeds germinated on the first, second and last day and N1, N2 and Nn: days since sowing on the first, second and last day.

At the end of the germination test, the primary root and hypocotyl of normal seedlings of each replicate were measured using a centimeter ruler, and the results were expressed in cm seedling^{-1}. Furthermore, to determine

shoot and root dry matter, the seedlings were cut and placed in paper bags, dried in a forced-air oven at 65 ºC for 72 h and weighed on an analytical scale (0.0001 g). The results were expressed in g seedling^{-1}.

Finally, the water content of the seedlings was determined using the fresh and dry weight values, according to Equation 3.

$$WC = [(Wi - Wf/Wi)] \times 100 \quad (3)$$

where, WC: water content of the seedlings (%); Wi: initial weight (fresh) and Wf: final weight (dry).

The experiment was carried out in randomized block design with five replicates containing 40 seeds each. The factors were five water potential levels [control (distilled water), -0.2, -0.4, -0.5, and -0.6 MPa] and five crambe genotypes. The data were submitted to analysis of variance (ANOVA) and the means of the factors (genotypes and osmotic potential levels) were compared using the Tukey test ($p < 0.05$ or $p < 0.001$), calculated by the Sisvar® program (Ferreira, 2011). Principal component analysis (PCA) were performed to visualize the data globally and in order to identify the correlations between the osmotic potential treatments and the genotypes variation using R software (R Core Team, 2018). The data obtained in the evaluation of each treatment and genotype were initially standardized and PCA was conducted using the Factor Mine R package (Le et al., 2008).

3. Results and Discussion

The seed moisture content varied significantly ($p \leq 0.001$) among the crambe genotypes evaluated (Table 1). The lowest seed moisture values were observed for the FMS Brilhante and FMS CR 1203 genotypes (5.65 and 5.69, respectively). In contrast, the highest values were recorded for FMS CR 1307 (Table 1). According to Marco-Filho (2005), the seed moisture content is related to seed vigor, since free water in the tissues increases various reactions, including those involved in seed deterioration, such as increased respiration, inducing the synthesis of adenosine triphosphate (ATP) (Kibinza et al., 2006) and malondialdehyde (MDA), in turn reducing cell membrane protection and increasing lipid peroxidation (Zhang et al., 2018), which occurs through the cascade of superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) (Noctor & Foyer, 1998; Zhang et al., 2018). In this study, although all crambe genotypes showed adequate seed moisture values (*e.g.*, values < 9 b.u., see Table 1), the higher seed moisture values reported for FMS CR 1307 may indicate increased biochemical reactions, which consequently increases the chances of seed deterioration (Cardoso et al., 2012).

The interaction between crambe genotypes and osmotic potential levels was significant ($p \leq 0.001$) for germination rate (%), normal seedling percentage (%), germination speed index (GSI), radicle and hypocotyl length and fresh matter (Figures 1-4). Total dry matter (DM) of the seedlings was significantly influenced ($p \leq 0.001$) by the isolated factors, genotypes and osmotic potential levels (Table 2).

Overall, the crambe seeds germinated at all the osmotic potentials tested. Higher germination occurred when seeds were treated at -0.2 MPa (Figure 1A). Under Ψ_s higher than -0.4 MPa, significant decreases in germination occurred for all genotypes and reached minimums of 17.5%, 22%, 12%, 15% and 9.5% for FMS Brilhante, FMS CR 1203, 1307, 1312 and 1326, respectively, at -0.6 MPa (Figure 1A). Furthermore, in the control group (0.0 MPa), the germination declined by about 22.7%, 82.1%, 77.5% and 36.2% in the FMS Brilhante, FMS CR 1307, 1312 and 1326 genotypes, respectively.

Table 1. Degree of seed moisture of different genotypes *Crambe abyssinica* Hochst

Genotypes *Crambe abyssinica*	Degree of seed moisture (%)
FMS Brilhante	5.65±0.078 C
FMS CR 1203	5.69±0.076 C
FMS CR 1307	6.36±0.064 A
FMS CR 1312	5.99±0.073 B
FMS CR 1326	6.11±0.085 AB
CV (%)	1.13

Note. Means followed by the same letter in the column do not differ by Tukey test at 0.001 probability level (±SD).

At the start of the germination process, the seeds are water dependent (phases I, II and II) (Bewley, 1997). During phase III, which is characterized by cell elongation, radicle emission occurs and the rate of seed

imbibition tends to decelerate (Bewley, 1997; Bove et al., 2001). In this study, the reduction of germination reported for the FMS Brilhante, FMS CR 1307, 1312 and 1326 genotypes in the control group (Ψs = 0 MPa) may have occurred due to fast seed imbibition, which can cause damages to the embryo, as reported by Bewley and Black (1994). Furthermore, the reduction of germination from -0.4 MPa on ward suggests osmotic interference in enzymatic activity, delaying meristematic development and retarding root emergence (Bewley et al., 2013). According to Marco-Filho (2015), low germination rates are related to membrane disorganization, followed by tissue death in different parts of seeds, especially meristematic tissues. In this study, the seed germination of all crambe genotypes was inhibited at -0.4 MPa, except for FMS CR 1307. Under in not adequate water potential, inhibition of seed imbibition capacity occurs, which limits the activation of the main metabolic pathways that act directly or indirectly on seed germination (Marcos-Filho, 2005).

The percentage of normal plants was zero for both FMS CR 1307 and 1312 genotypes when the seeds were treated at -0.6 MPa. Therefore, the seeds of FMS CR 1307 and 1312 that showed some germination (denoted by radicle emission) (Figure 1A) did not generate normal seedlings (Figure 1B). The formation of abnormal seedlings of these crambe genotypes treated at -0.6 MPa suggests dysfunction and/or damage to the biomembrane system, caused by the progressive loss of protoplasmic turgor and increased concentration of cellular solutes (Bruni & Leopold, 1992). The lower values of germination and normal plants (Figure 1A and B) reported for both crambe genotypes may indicate greater susceptibility to water deficit caused by PEG.

Reductions in GSI occurred in all genotypes with the reduction of water potential (Figure 1C). According to Dell'Aquila (1992), reduction in GSI values is a common response to water deficit and can be attributed to the impaired synthesis of proteins in embryonic tissues due the low hydration. The FMS CR 1203 genotype showed higher values of GSI when submitted to 0.0 and -0.4 MPa (14.4, 3.2, respectively). In contrast, lower GSI values were reported at 0.0 and -0.2 MPa (7.2 and 7.9, respectively) for FMS CR 1307 and -0.4 MPa (1.5) for FMS CR 1312. Rapid germination generally corresponds to seed vigor, leading to faster emergence of seedlings in the field (Marcos-Filho, 2015). In this study, the higher GSI observed for the FMS CR 1203 genotype suggests higher probability that the seeds reached the next phase of the biocycle (Oliveira et al., 2015), because an increase in the GSI under water deficit conditions indicates less susceptibility to pathogens, insects and other pests (Machado et al., 2017), increasing the success of seedling development.

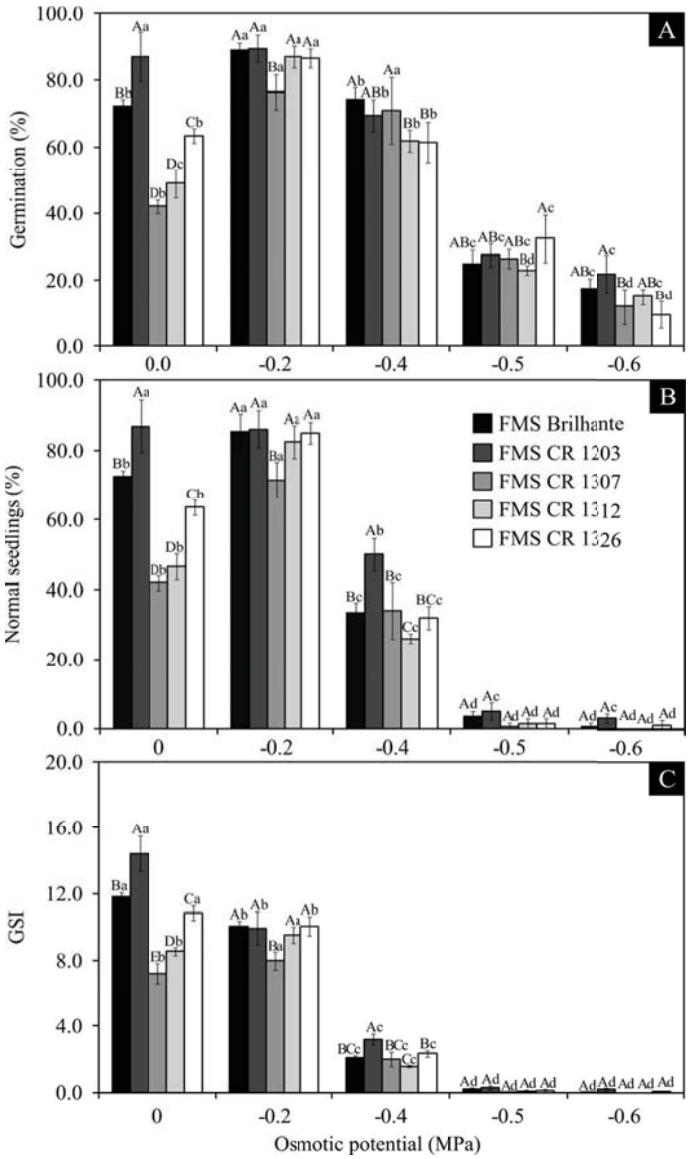

Figure 1. Gemination (A), normal seedlings (B) and germination speed index-GSI (C) of *Crambe abyssinica* seeds, genotypes FMS Brilhante, FMS CR 1203, 1307, 1312 and 1326, under different osmotic potentials. SD is shown, the same letters mean no significant difference (small letter: osmotic potentials, capital letter: different genotypes) ($p \leq 0.001$)

The crambe seedlings showed different growth patterns when submitted to different osmotic potentials (Figure 2 and 3). However, up to the potential of -0.5 MPa, all seedlings had long and thin primary roots, coated with numerous root hairs, thin and elongated hypocotyl and green or greenish cotyledons (Figure 2). At Ψs = -0.6 MPa, only FMS CR 1307 and 1312 genotypes produced seedlings classified as abnormal according to Brasil (2009). Seedlings of FMS CR 1307 had underdeveloped and yellowish cotyledons. Furthermore, seedlings of FMS CR 1312 showed under developed hypocotyl and poor root growth.

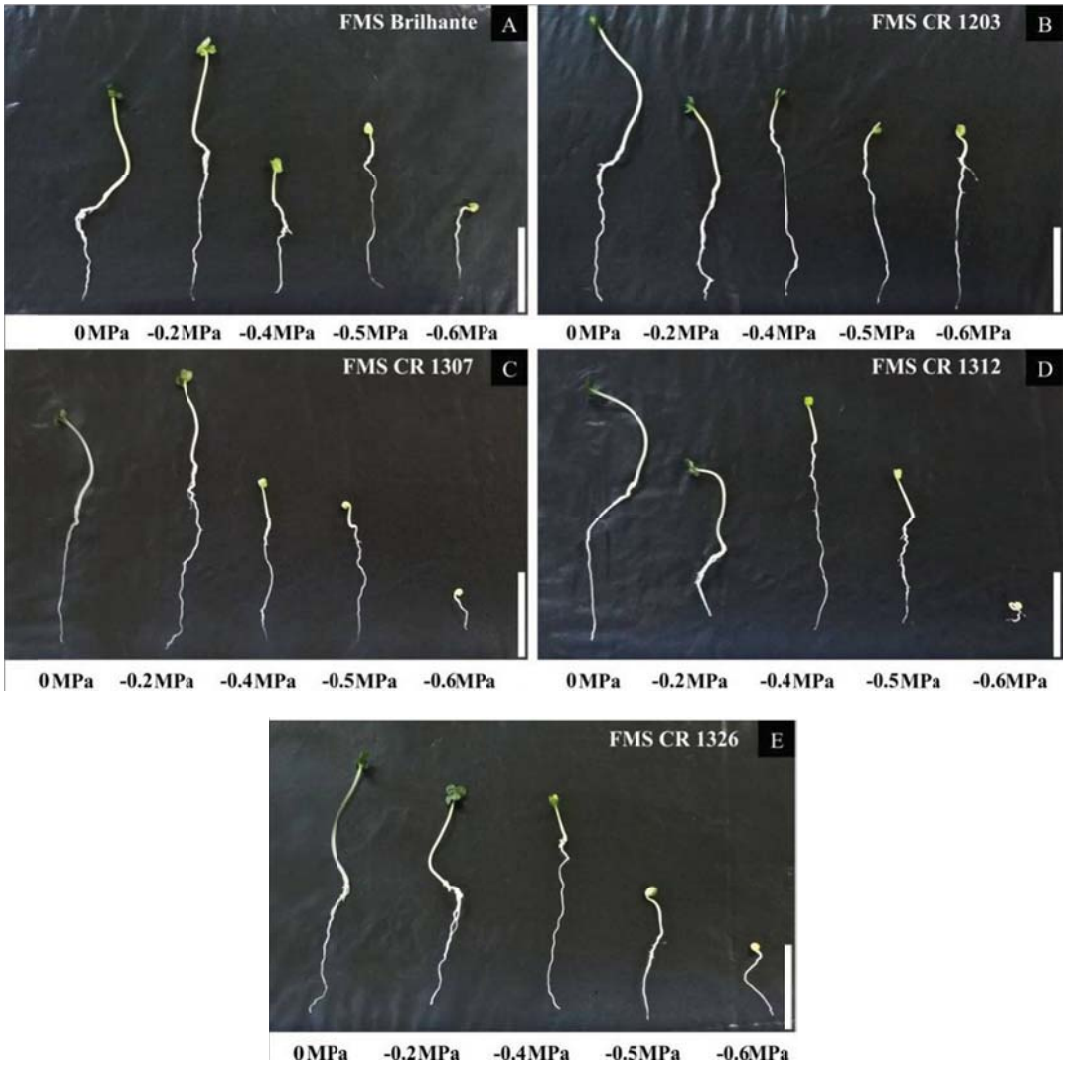

Figure 2. Seedlings of different genotypes of crambe (FMS Brilhante, FMS CR 1203, 1307, 1312 and 1326) under different osmotic potentials (0, -0.2, -0.4, -0.5 and -0. 6 MPa)

When the seeds were treated at 0.0 and -0.2 MPa, no difference in root length was noted among the crambe genotypes (Figure 3A). However, osmotic potential values equal or greater than -0.4 MPa resulted in longer root length for FMS CR 1203. Although the increase of root length immature plants submitted to water deficit is a common morphological change (Pimentel, 2004), in seedlings this response varies according to the species/genotype, as reported by Zhu et al. (2006), Paiva et al. (2018) and Almeida et al. (2018), studying the germination and early growth of *Pinus sylvestris* var. mongolica, *Vigna unguiculata* L. Walp and *Coffea arabica* L., respectively. In this study, higher root length was obtained for the FMS CR 1203 genotype treated at -0.6 MPa compared to the other genotypes, which was similar to the values obtained under control conditions. According to Echer et al. (2010), seedlings with the ability to grow roots under water stress conditions can maintain the hydration of tissues through osmotic adjustment. These results indicate higher growth capacity of the root system in order to improve water absorption (Ávila et al., 2007).

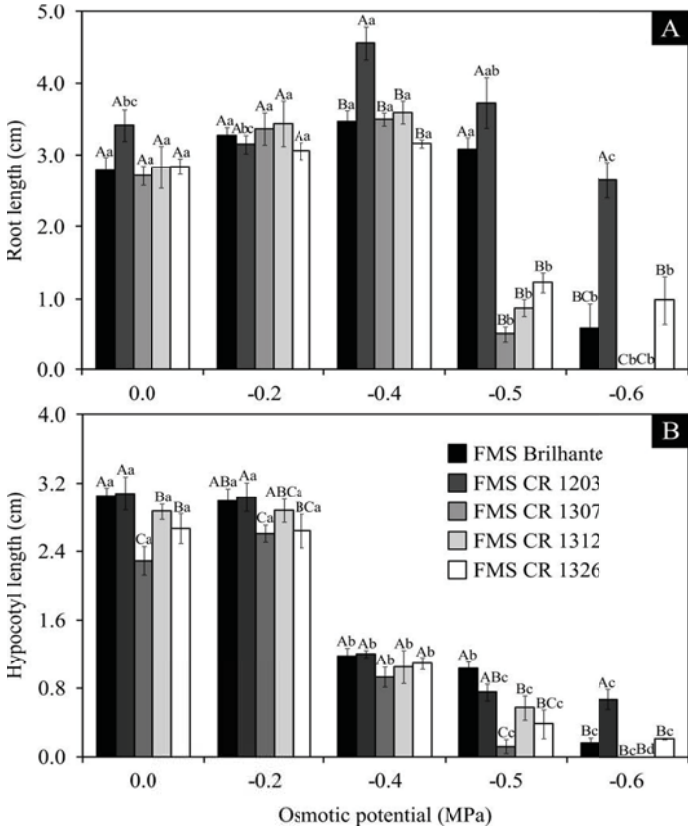

Figure 3. Root length (A) and hypocotyl length (B) of *Crambe abyssinica* Hochst. seedlings, genotypes FMS Brilhante, FMS CR 1203, 1307, 1312 and 1326, under different osmotic potentials. SD is shown, the same letters mean no significant difference (small letter: osmotic potentials, capital letter: different genotypes) ($p \leq 0.001$)

In this study, hypocotyl length was also affected by water deficit (Figure 3B). The FMS CR 1307 and 1312 genotypes showed no hypocotyl growth when the seeds were treated at -0.6 MPa. These results were consistent with those obtained for the percentage of normal plants (Figure 1B). Reductions in hypocotyl length have also been reported for all these crambe genotypes subjected to osmotic potential values equal to or greater than -0.4 MPa, suggesting a reduction in the water potential of plant cells and, however, a decrease in the pressure, expansion and cell growth, which limits the development of the seedlings (Jaleel et al., 2009). In contrast, the FMS CR 1203 genotype showed greatest hypocotyl length at the osmotic potential of -0.6 MPa. This result can be attributed to the higher values of root growth reported for this genotype under water deficit (Figure 3A), indicating higher hydration of the tissue through osmotic adjustment, as reported by Echer et al. (2010).

The total fresh matter values decreased when the seedlings were treated at osmotic potential values equal to or greater than -0.4 MPa for all genotypes (Figure 4A). FMS CR 1307 and 1312 showed the lowest total fresh matter values in all treatments, except for -0.2 and -0.4 MPa. This result was corroborated by the lower dry mass accumulation observed for these genotypes (Table 2). In contrast, at Ψ_s -0.6 MPa, significantly higher total fresh matter values were obtained for FMS CR 1203 compared to the other genotypes, which also was evidenced by the higher dry mass accumulation (Table 2).

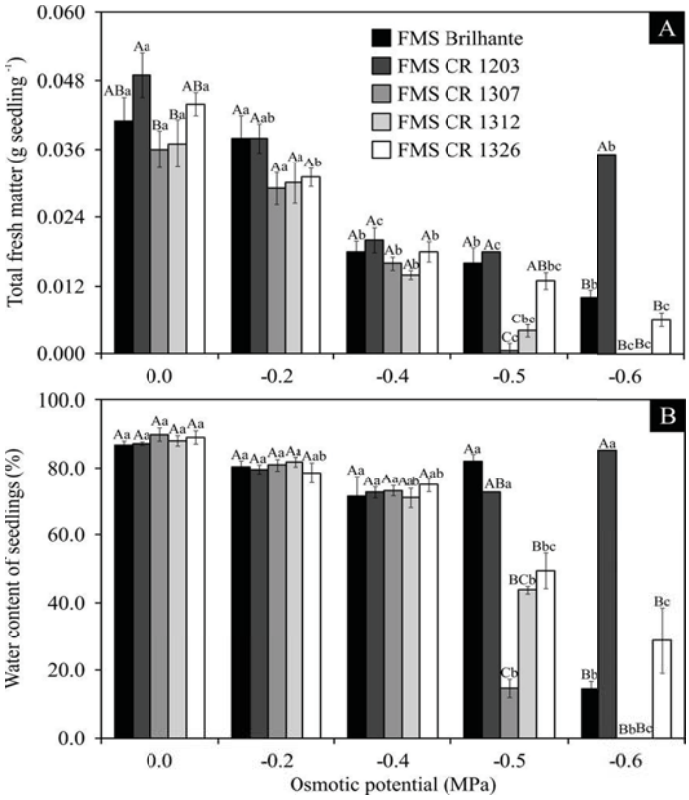

Figure 4. Total frech matter (A) and water content of seedlings (B) of *Crambe abyssinica* Hochst. seedlings, genotypes FMS Brilhante, FMS CR 1203, 1307, 1312 and 1326, under different osmotic potentials. SD is shown, the same letters mean no significant difference (small letter: osmotic potentials, capital letter: different genotypes) ($p \leq 0.001$)

According to Coelho et al. (2010), plant species have the ability to signal and regulate the protein expression when subjected to water stress, and consequently activate antioxidant enzymes such as peroxidase, catalase and ascorbate peroxidase and reduce the activity of superoxide dismutase, resulting in better reactive oxygen species elimination capacity under water deficit in seedlings, as observed by Ali et al. (2017), studying the effects of water deficit on germination and growth of bean seedlings (*Vigna radiata* L., cultivars NM-2006 and 8005). According to the authors, the better growth of seedlings is associated with a more efficient mechanism to eliminate reactive oxygen species, which is associated with both activity and content of antioxidant enzymes.

Table 2. Total dry matter of different genotypes *Crambe abyssinica* Hochst. under different osmotic potentials

Osmotic potential (MPa)	Shoot dry matter (g seedling^{-1})
0	0.0048±0.0010 B
-0.2	0.0065±0.0011 A
-0.4	0.0048±0.0007 B
-0.5	0.0023±0.0020 C
-0.6	0.0019±0.0034 C
CV (%)	36.9
Genotypes *Crambe abyssinica*	Shoot dry matter (g seedling $^{-1}$)
FMS Brilhante	0.0047±0.0031 AB
FMS CR 1203	0.0059±0.0010 A
FMS CR 1307	0.0027±0.0023 D
FMS CR 1312	0.0030±0.0022 CD
FMS CR 1326	0.0040±0.0022 BC
CV (%)	36.9

Note. Means followed by the same letter in the column do not differ by Tukey test at 0.001 probability level (±SD).

Under osmotic potential equal to or greater than -0.5 MPa, reductions of water content were observed for all crambe genotypes except FMS Brilhante and FMS CR 1203 (Figure 4B). At Ψs = -0.6 MPa, higher water content values were obtained for FMS CR 1203, suggesting production and accumulation of several compounds, reducing the internal osmotic potential in order to maintain water uptake by seedlings (Manishankar et al., 2018), as well as the activation of enzymes for cell detoxification, as reported by Coelho et al. (2010) and Gupta and Huang (2014). In contrast, the reduction of water content indicates poor osmotic regulation. Osmotic adjustment maintains turgor and reduces growth sensitivity under water deficit conditions, causing low growth rate under stress (Meyer & Boyer, 1981). Higher values of total dry matter were obtained at the osmotic potential of -0.2 MPa (Table 2). According to Carpiski et al. (2013), *C. abyssinica* Hochst is a species considered tolerant to water deficit and intolerant to excess moisture, indicating that osmotic potentials below -0.2 MPa are not adequate for germination of the studied genotypes. FMS CR 1203 showed the highest values of total dry matter, while FMS CR 1307 had the lowest values (-54.2% compared to the highest dry matter value of FMS CR 1203).

Principal component analysis (PCA) was employed to assess the responses of each osmotic potential treatment and all the genotypes tested in all the data. PCA is a powerful method that converts the number of high-dimensional variables into a few numbers of principal components (PCs), representing the original data (Jolliffe & Cadima, 2016). The results revealed that two principal components explained 91.5% of the total variance in the dataset, 72.9% in the first principal component (PC1), and 18.6% in the second (PC2) of the observed variability (Figure 5). Overall, the PCA revealed distinct clusters formed among the crambe genotypes proportional to their water potential, indicating the existence of a response pattern of seeds/seedlings of different crambe genotypes to variation in water potential conditions. Under low Ψ_w (0.0 and -0.2 MPa), the genotypes were clearly grouped in two clusters, in which the greatest difference was observed between FMS CR 1203 and FMS CR 1307. However, under high Ψ_w (-0.5 and -0.6 MPa), the FMS CR 1203 was grouped with all other genotypes submitted to Ψ_w = -0.4 MPa. Establishing principal components made it possible to show differences among the genotypes response to drought stress and under high Ψ_w FMS CR 1203 demonstrates a better performance to tolerate these conditions.

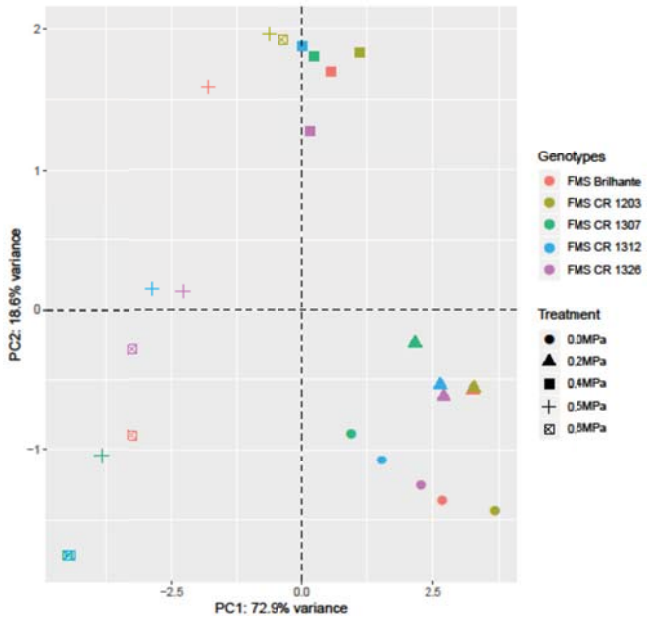

Figure 5. PCA-Principal component analysis generation plot considering all osmotic potentials treatments and the genotypes of *Crambe abyssinica*, FMS Brilhante, FMS CR 1203, 1307, 1312 and 1326

The results obtained in this study suggest the existence of significant genetic differences among the crambe genotypes studied in response to water deficit induced by polyethylene glycol. All genotypes evaluated were obtained through natural selection, so FMS CR 1203, 1307, 1312 and 1326 are natural crosses of different genotypes from the genotype FMS Brilhante. According to Kappes et al. (2010), the seed germination at different Ψs values depends on the genetic material used, whereby genotype is a determinant factor for tolerance or susceptibility to water deficit under field conditions. Attention is necessary to choose genotypes that are

tolerant to water deficit (Machado et al., 2017). Thus, the results obtained in this study are relevant to evaluate the behavior of crambe genotypes under critical osmotic potentials for germination and early growth of seedlings. Finally, the PCA analysis corroborated the higher stress tolerance previously observed for FMS CR 1203, because even when increasing the intensity of water deficit, no differences in germination and seedling growth were noted. Furthermore, the grouping of FMS CR 1307 and 1312 corroborates the lower tolerance to water deficit.

4. Conclusions

The physiological quality of seeds and initial development of crambe genotypes are improved by Ψw = -0.2 MPa and germination and vigor index of crambe seeds are hampered by the reduction in the potential to -0.4 MPa. The genotype FMS CR 1203 is the most tolerant to water stress, whereas FMS CR 1307 and 1312 are the most sensitive.

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Evaluation of the Impacts of Regional Climate Factors and Crop Management on Corn Yields in Different Climate Regimes of China Using the DayCent Model

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Abstract

Corn is one of most important agricultural products in China. Understanding impacts of regional climate change, as well as agricultural management practices, on corn yields is critical for maintaining stable corn production. Using the DayCent model and observed climatic data in Sichuan province (a humid and hot environment) and Hebei province (a cold and dry environment) in China, corn yields in 1948-2010 were simulated. The spatial variations of simulated corn yields and the relationship between regional climate variability and warming with corn yields in these two environments were analyzed. The results demonstrated that: (1) corn yields in Zhangjiakou of Hebei and most regions of Sichuan decreased significantly after 2000 compared to other regions; (2) relative humidity and precipitation exhibit a significant negative correlation with observed crop yields in the growing season in Hebei province; (3) air temperature from 23.33 °C to 29 °C constitutes the ideal range influencing the increase of corn yields in Sichuan; (4) the planting of the large amount of silage maize in Sichuan compensated the negative impact of the rising air temperature on corn yields; (5) sensitivity tests for different fertilization levels and OMAD suggest that an increasing fertilization level significantly affects corn yields in Hebei province, a cold and dry environment, while a decreasing fertilization level has a significant negative effect in Sichuan province, a hot and humid environment. The overarching goal of these analyses is to provide the theoretical basic for maintaining stable corn production under regional climate warming and different agricultural management practices.

Keywords: corn yields, DayCent model, climate variability and change, fertilization level, organic matter additions

1. Introduction

Corn is one of the most important crops globally, and its production has increased continuously from 2008 to 2015 in China, reaching 22 trillion kg in 2016. This increase of corn production has been attributed to enlargement of cultivated areas and advancements in production technology (B. Chen & G. Chen, 2007; Peng, Tang, & Zou, 2009; Yu, Huang, & Zhang, 2012; Bryan, King, & Zhao, 2014; Nendel, Kersebaum, Mirschel, & Wenkel, 2014). Effects of climate factors on corn yields have also been widely studied. Some extant literature has investigated the effect of air temperature (Wolfram & Michael, 2009; Yin et al., 2016; Lobell & Field, 2007; Basche et al., 2016; S. Chen, X. G. Chen, & J. T. Xu, 2016; Lee & Durmaz, 2016; Meng, Carew, Florkowski, & Klepacka, 2016), while others have focused on the effect of other climatic factors, such as precipitation and wind (Yin et al., 2016; Lee & Durmaz, 2016; Wang, Bocoling, & Cherkauer, 2016). A number of studies found that rising air temperature in the growing season, especially in July or in the seeding and maturity phases, increased corn yields (Wolfram & Michael, 2009; Yin et al., 2016). However, scholars have also reported that climate change has resulted in the reduction of corn yield over several countries in the past few decades (Wolfram & Michael, 2009; Lobell & Field, 2007; Basche et al., 2016; S. Chen, X. G. Chen, & J. T. Xu, 2016; Lee &

Durmaz, 2016; Almarza, Mabood, Zhou, Gregorich, & Smith, 2008). In fact, Chen et al. (2016) reported that corn yield is projected to decline by 3-12% by 2100 in China due to rising air temperature. In the U.S., it was found that after the average growing-season air temperature exceeded 29 °C, corn yield would decrease sharply (Wolfram & Michael, 2009). Warming since 1981 has resulted in annual combined losses of crops representing roughly 40 Mt or USD $5 billion per year, as of 2002 (Lobell & Field, 2007). A 1% increase in the growing season air temperature reduces corn yield per acre by 9% (Lee & Durmaz, 2016). Increases in minimum and maximum air temperatures were attributed to reduced yields of 1.6-2.7% by decade (Basche et al., 2016). Although different climate characteristics among the study areas explain some temperature responses in corn yields, there is a general lack of comprehensive research on the effect of air temperature on crop yield. In this study, we thoroughly investigate this issue by studying the relationship between air temperature and corn growth in two different regions in China.

To analyze the impact of climate on corn yield, various models have been utilized, such as empirical frameworks (S. Chen, X. G. Chen, & J. T. Xu, 2016), regression models (Wolfram & Michael, 2009), statistical models (Basche et al., 2016; Almarza, Mabood, Zhou, Gregorich, & Smith, 2008), climatic models (IHadRM3, C4I, REMO-MPI, ETHZ, CNRM, DMI-HIRHAM, KNMI, SMHI) (Voloudakis et al., 2015), the Agricultural Production Systems sIMulator (APSIM) (Basche et al., 2016), crop simulation models (Morell et al., 2016), and crop growth models (Silva, Reidsma, Laborte, & van Ittersum, 2017). These approaches possess respective strengths and weaknesses. For example, although the DNDC model considers the cropping system, fertilizer and straw return in its crop simulations, it only employs a few key management practices (Morell et al., 2016). A crop growth model (ORYZA v3) was able to simulate rice yield relatively well, but tended to overestimate other crops' yields (Silva, Reidsma, Laborte, & van Ittersum, 2017). APSIM and HERMES address the agricultural management practice factor in analyzing the impact of climate change on corn yield, but only limited numbers of management factors were considered. In this study, we employ the DayCent model (Parton, Hartman, Ojima, & Schimel, 1998; Parton et al., 2001) to test the effects of key climatic factors, warming air temperatures, and various agricultural management practices on crop yield.

DayCent is a process-based biogeochemical model, and is a useful tool to predict yields as it integrates crop growth, carbon and nutrient dynamics, hydrology, management, and climate. Many studies have utilized DayCent to simulate changes in soil C, soil N, and greenhouse gas emissions (Wieder, Bonan, & Allison, 2014; Sheehan et al., 2013; Frey, Lee, Melillo, & Six, 2015; Cheng, Ogle, Parton, & Pan, 2014; Robertson, Grace, Izaurralde, Parton, & Zhang, 2014; Rafique, Fieneu, Parkin, & Anex, 2013; Reay et al., 2012; Mangalassery et al., 2014). Only a few previous studies have examined grain yield change under different climatic conditions and agricultural management practices using this model in China (Campbell et al., 2014; Lee, De, & Six, 2011). Cheng et al. (2014) used the DayCent model to successfully simulate crop yields in China, but focused on analyzing greenhouse gas mitigation potential and not the response of crop yields to future climatic change. In this study, variability in corn yields within each study region and the response to future climatic change were analyzed.

We selected Hebei province (a dry and cold environment) and Sichuan province (a warm and humid environment) in China as study areas, which possess quite different climate backgrounds (Figure 1). The spatial variation of simulated corn yields and the relationship between regional climate change, regional warming, and corn yield are reported in this study. The sensitivity of agricultural management practices in simulating corn yields by DayCent is also discussed.

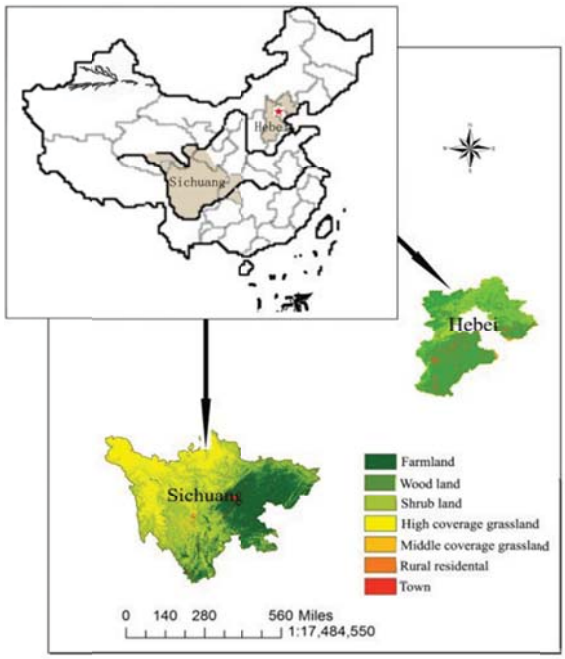

Figure 1. The geographic locations of the study area in China

2. Methods

2.1 Study Area

The geographic areas in this study, Hebei (E 113°04′ to 119°53′, N 36°01′ to 42°37′) and Sichuan (E97°21′ to 108°33′, N26°03′ to 34°19′) provinces in China are located in the northeast and southwest of China, respectively (Figure 1). Although they exhibit different climate characteristics, both have high corn acreage and production. Hebei has a temperate monsoon climate/warm temperate, humid-semi–arid continental monsoon climate, with an annual mean air temperature of 4-13 °C and an annual mean precipitation of 400-800 mm/yr (based on 63 years (1948-2010) of data). Climate is characterized by cold winters with little snow, hot summers with substantial rainfall, and windy springs and autumns with less rainfall. Climate in Sichuan is characterized by warm winters, dry springs, hot summers, rainy autumns, substantial fog, and less sunshine in the east, and by cold and long winters, short summers, abundant sunshine, and concentrated rainfall in the west.

Hebei is located in northern China, with an area of 188 thousand km^2. It encompasses 11 prefecture-level cities, 22 county-level cities, 108 counties, and six autonomous counties. The province's terrain slopes upward from the northwest to southeast. Hills, mountains and plateaus are in the northwest, with an average altitude of 1200-1500 m; plains are in central and southeast China, with an average altitude of less than 50 m.

Sichuan is located in the upper reaches of Changjiang River in southwest China, with an area of 99 thousand km^2. It is covered by plateaus in the west and basins in the east. The terrain is higher in the west with an altitude of 4000-5000 m, and lower in the east.

2.2 Selection of Study Locations

In Sichuan, we selected 18 counties (Figure 2a), including Abazhou, Bazhong, Chengdu, Dazhou, Deyang, Guangan, Guangyuan, Leshan, Xiaojin, Luzhou, Meishan, Mianyang, Nanchong, Neijiang, Yaan, Yibin, Zigong and Ziyang as study areas, which constitute the primary agricultural regions in the province. In Hebei, we selected 17 grid locations (Figure 2b) that were uniformly distributed within the boundary ranges of latitude and latitude covered by Hebei province and were not based on counties. This method ensured the broadest range of surface conditions and crop yields within the province, since conditions within individual counties are quite variable. Some locations in the boundary and out of the boundary were discarded because they were not representative of farmland. The latitude or longitude of the interval between adjacent two grid locations is 1° and are marked with letters "a" to "q", respectively, in Figure 2b.

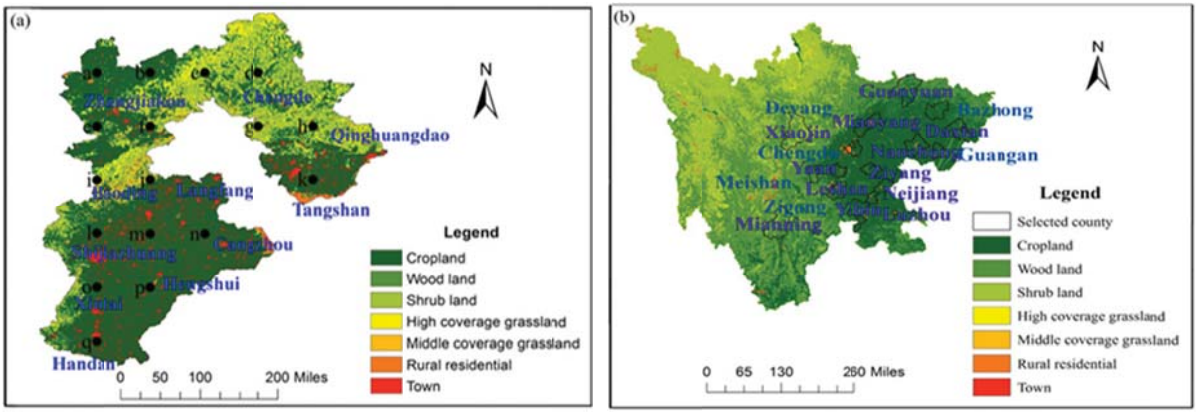

Figure 2. Land cover and selected locations in Hebei (a) and selected counties in Sichuan (b) in 2013. In selected locations of Hebei, a, b, c, e, and f represents Zhangjiakou. d, g, and h represent Chengde. k represents Tangshan. i represents Baoding. j represents langfang. l and m represent Shijiazhuang. n represents Cangzhou. o represents Xintai. p represents Hengshui. q represents Handan

2.3 DayCent Model

DayCent is a daily time-step version of the Century model that simulates plant production, trace gas fluxes (N_2O, NOx, CH_4), and the dynamics of carbon and nitrogen in grassland, forest, savanna, and agricultural systems (Wieder, Bonan, & Allison, 2014; Sheehan et al., 2013; Frey, Lee, Melillo, & Six, 2015; Cheng, Ogle, Parton, & Pan, 2014; Robertson, Grace, Izaurralde, Parton, & Zhang, 2014). The model has been widely utilized to simulate the impact of environmental changes (elevated CO_2 and climatic changes, management practices) on production and yield in agro-ecosystems globally (Cheng et al., 2014; Robertson, Grace, Izaurralde, Parton, & Zhang, 2014; Reay et al., 2012; Mangalassery et al., 2014).

2.3.1 Model Inputs and Data Collection

Model inputs are daily weather data, soil properties, and agricultural management practices (e.g., crop type, cultivation/planting schedules, amount and timing of nutrient amendments, irrigation, and tillage). The crop-specific parameter PRDX(1), a coefficient for calculating potential aboveground daily production as a function of solar radiation outside the atmosphere, is a critical important parameter. The values of PRDX(1) for the types of corn (C1, C2, C3, C4, C5, and C6) increased in value according to the various corn productivity levels and were different for each province. This set of corn parameters for Hebei and Sichuan provinces was the same for all simulations, respectively. Measured corn yields from 1978 to 2010 were obtained from the Hebei Economic Year Book (2008-2011) and the Sichuan Statistical Year Book (2000-2011).

(1) *Weather data*: Daily weather data, which include daily maximum and minimum air temperature (T_{min} and T_{max}), daily precipitation (Prcp), daily solar radiation (Dswrf), daily relative humidity (RH), and daily wind speed (Wind), are necessary forces to drive ecosystem processes in DayCent. The weather data from 1948 to 2010 for this study were derived from the Princeton University Hydroclimatology Group Bias Corrected Meteorological Forcing Dataset (Sheffield, Goteti, & Wood, 2006). The units of these data were converted according to the format required by DayCent.

(2) *Soil data*: Soil property data, specified by soil layer, comprised bulk density (g/cm^3), field capacity, wilting point, evaporation coefficient, fraction of roots, fraction of sand and clay, saturated hydraulic conductivity, and pH. All of these data were obtained by field survey and sample measurement in the study areas in 2016. Specifically, three plots of corn planted in 2016 in each county or location were selected in 18 counties of Sichuan province and 17 locations of Hebei province from August to September. Soil samples in each corn plot were collected using plastic bags. Fresh weight was determined at that time, and the samples were brought to the laboratory and dried at 105 ºC. Dry weight was then obtained, and then soil water content was calculated.

(3) *Crop management data*: Agricultural management options, which are specified in the DayCent schedule file, include crop types, irrigation intensity, cultivation practices, residue removal during harvest, organic matter additions, inorganic fertilizer additions, and planting and harvest dates (Cheng et al., 2014). Corn growth in Hebei province is mainly dependent on irrigation, while corn growth in Sichuan province relies on irrigation and rainfall. Assuming ample water for irrigation, corn production is not limited by water availability in these two provinces. We define five periods according to production technology levels during the periods of 1948-2010 in

China: 1948-1965, 1966-1975, 1976-1985, 1986-1995, and 1996-2010. The management options for these five periods were obtained through reports from local farmers and relevant literature (Liang, 2015; Liang et al., 2008; Luo et al., 2016; Liu et al., 1998; Chen, 2013).

2.3.2 Evaluation Method

Performance of the DayCent model was assessed using several quantitative methods (Janssen & Heuberger, 1995). First, the main difference between the simulated and observed values was assessed by calculating the root mean square error (RMSE):

$$\mathrm{RMSE} = \sqrt{\sum_{i=1}^{n} \left(S_i - O_i\right)^2 / n} \tag{1}$$

where, O_i and S_i denote the observed and simulated values, respectively; and n is the number of measurements. Second, the accuracy of the simulations was evaluated based on modeling efficiency (EF):

$$\mathrm{EF} = \frac{\sum_{i=1}^{n}(O_i - \bar{O})^2 - \sum_{i=1}^{n}(S_i - O_i)^2}{\sum_{i=1}^{n}(O_i - \bar{O})^2} \tag{2}$$

where, $\bar{O}$ denotes the mean of the observed data; and O_i and S_i denote the observed and simulated values, respectively. A positive value of EF indicates good modeling efficiency, in which the closer the value is to +1, the better is the modeling efficiency. Negative values of EF indicate poor modeling efficiency, in which the farther the value is from +1, the worse is the modeling efficiency. Third, bias constitutes the main difference between the simulated and observed values, and was determined by calculating the relative error (E):

$$\mathrm{E} = \frac{100}{n}\sum_{i=1}^{n}\left(O_i - S_i\right)^2 / O_i \tag{3}$$

An *E* value closer to 0 suggests a better model fit to the measurements. When both measurements and observations are positive numbers, a positive *E* value indicates that the model underestimates the observations, and a negative E value indicates that it overestimates the observations.

We also analyzed the spatial variation and temporal correlation between simulated and observed corn yields from 1978-2010 by the Pearson correlation analysis method using SPSS 13.0 software.

2.4 Factors that Affect Corn Yields and Experimental Designs

Simulated corn yield was affected by numerous factors, including climate forcing and specified agricultural management practices. In this paper, we analyze the general relationship between climate variables and crop yields based on observation and model simulation. We also present the sensitivity of crop yields to management. Since we possessed no detailed information regarding crop management, we performed several sensitivity studies to test how each factor affects corn yield simulation. In the sensitivity tests, one factor at a time was changed (Figure 3). The sensitivity tests were designed as follows:

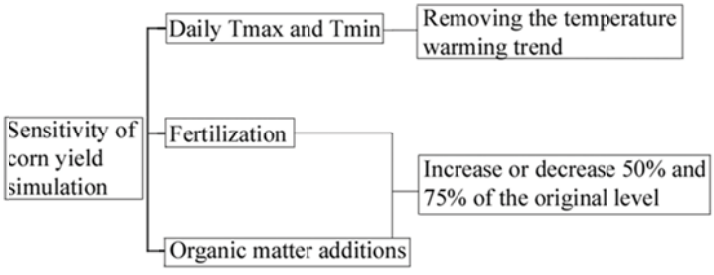

Figure 3. Schematic diagram of sensitivity analysis. (A) factors and (B) methods

(1) *Sensitivity to air temperature*. In the sensitivity study, we removed the trend of elevated air temperature from 1978 to 2010 in Hebei and Sichuan provinces (*i.e.*, de-trending) by subtracting the annual mean values, which were derived from original daily T_{max} and T_{min}. Comparisons between the actual and detrended mean growing season temperatures for each province are briefly presented in Figure 4. From Figure 4, it can be seen that the mean growing season T_{max} in Hebei and Sichuan decreased by 0.08 °C and 0.43 °C, respectively, after detrending; The mean growing season T_{min} in Hebei and Sichuan decreased by 0.09 °C and 0.44 °C, respectively, after detrending. All of these findings indicate that the mean growing season T_{max} and T_{min} in Hebei increased slightly in the past 23 years, but greatly in Sichuan.

(2) *Sensitivity to fertilizer and organic matter additions*. Fertilizer management includes different fertilization timing and levels. In this paper, only fertilizer level was considered. The application of organic matter (OMAD) in the field after harvest maintains soil fertility for the following year. In the sensitivity tests, we added or decreased fertilization and OMAD level by 50% and 75% compared with the original setting. These fertilizer or OMAD amounts did not vary spatially in Hebei or Sichuan in this experiment. Organic matter (*e.g.*, manure) is widely available in China so that applications can be increased (Zhao et al., 2017).

To assess sensitivity, we calculated the average simulated corn yields in the sensitivity simulation minus the average of the control simulated corn yields in the study period.

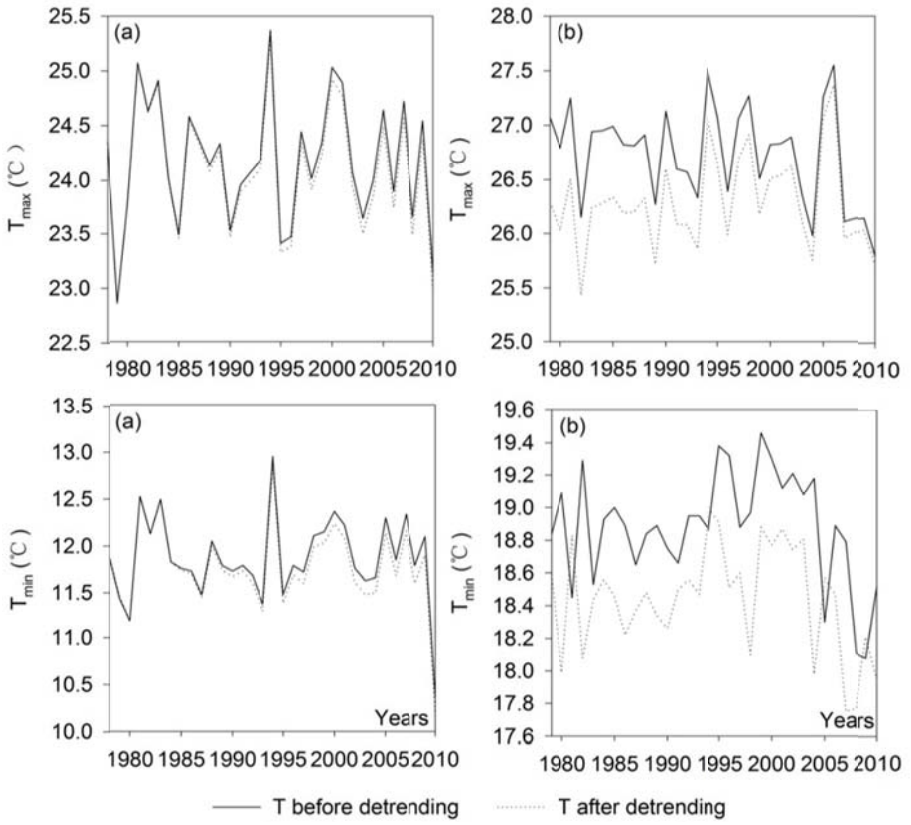

Figure 4. Change in mean growing season T_{max} and T_{min} before and after detrending in 1978-2010 in the province of (a) Hebei and (b) Sichuan

2.5 Data Availability

Weather data derived from the Princeton University Hydroclimatology Group Bias Corrected Meteorological Forcing Dataset. Soil data were obtained by field work in the study area. Crop management data were provided by the local farmers and Statistic Yearbook of the Sichuan and Hebei provinces.

3. Results

3.1 Relationship Between Climate Variables and Corn Yields

In this paper, corn yield only refers to grain yield, and does not include corn stalk. The simulated results are compared with measured corn yields for 1978-2010 (Figure 5). A strong correlation was found between mean simulated and observed corn yields, with R^2 values of 0.842 in Hebei and 0.751 in Sichuan. These results are in agreement with Cheng et al. (2014), who also reported that the DayCent model was reasonably accurate in simulating crop yields with R^2 values ranging from 0.71 to 0.85 in 350 cropland experiments in China. The values of RMSE, EF, and E are 875.12, -0.76, and 3.39 in Hebei, and 1173.28, -0.24, and 0.22 in Sichuan, respectively. RMSE in Hebei is lower than in Sichuan. The value of EF in Sichuan is closer to 1 than in Hebei. Moreover, the relative error, E, in Sichuan is lower and closer to 0 than in Hebei, which reflects the better simulation efficiency in Sichuan.

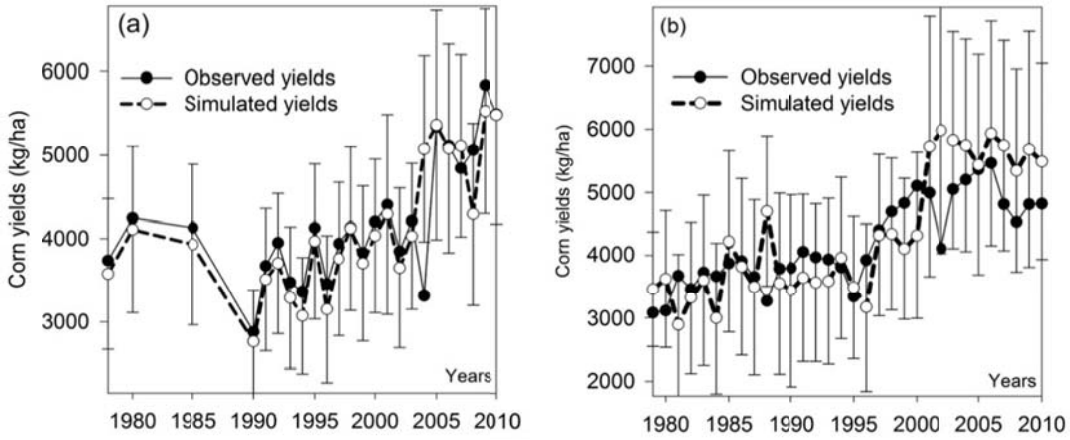

Figure 5. Mean observed and simulated corn yields in the province of (a) Hebei and (b) Sichuan. The bars represent plus and minus one standard deviation for simulated value

Figure 6 presents the variation of six meteorological variables (T_{max}, T_{min}, Dswrf, RH, Wind, and Prcp) over the past 33 years (1978-2010) in these two provinces. T_{max} and T_{min} increased at a rate of 0.22 °C/10 yr and 0.23 °C/10 yr (the method: estimation of linear trend (Fu et al., 2013) in Sichuan, respectively, and 0.34 °C/10 yr and 0.55 °C/10 yr in Hebei, respectively).

Downward shortwave radiation (Dswrf) increased at a rate of 1.92 (langleys/day)/10 yr in Sichuan, and decreased at a rate of -0.82 (langleys/day)/10 yr in Hebei. RH decreased at a rate of -0.06%/10 yr in Sichuan, and increased at a rate of -0.45%/10 yr in Hebei. Wind decreased at a rate of -0.11 (miles/hour)/10 yr in Sichuan, and increased at a rate of 0.08 (miles/hour)/10 yr in Hebei. Precipitation decreased at a rate of -0.01 cm/10 yr in both Sichuan and Hebei. The Z values (the method: Mann-Kendall nonparametric statistical test (Kahya & Kalayci, 2004) of the change in six meteorological variables were all more than 1.78 ($\alpha < 0.05$), indicating significant change trends of six climatic factors over the past 33 years.

To elucidate how each of the climatic factors affected corn yield, we analyzed the relations of six meteorological variables averaged over the growing season (April-September) from 1948-2010 with observed and simulated corn yields. Table 1 shows the correlation coefficients. In general, simulated and observed correlations are consistent with each other. However, in a few cases, the observations show significant results that were not confirmed by the simulations. In Hebei province, RH and Prcp exhibited a significant negative correlation with observed crop yields in the growing season. This is likely because the continuous rainfall in the pollination period seriously impacted the growth of maize (Liu, 2008). In Sichuan province, only T_{min} was negatively correlated with observed crop yields. There was no impact for T_{max} on observed corn yield in Sichuan. This is probably because the mean air temperature in Sichuan is relatively high, which usually met the needs of corn growth. The rising of T_{min} in Sichuan could cause the seeding time and the end of the growing season appear earlier and shorten the growing season of corn (Liu, Qin, Ge, Dai, & Chen, 2017), thus decreasing corn production.

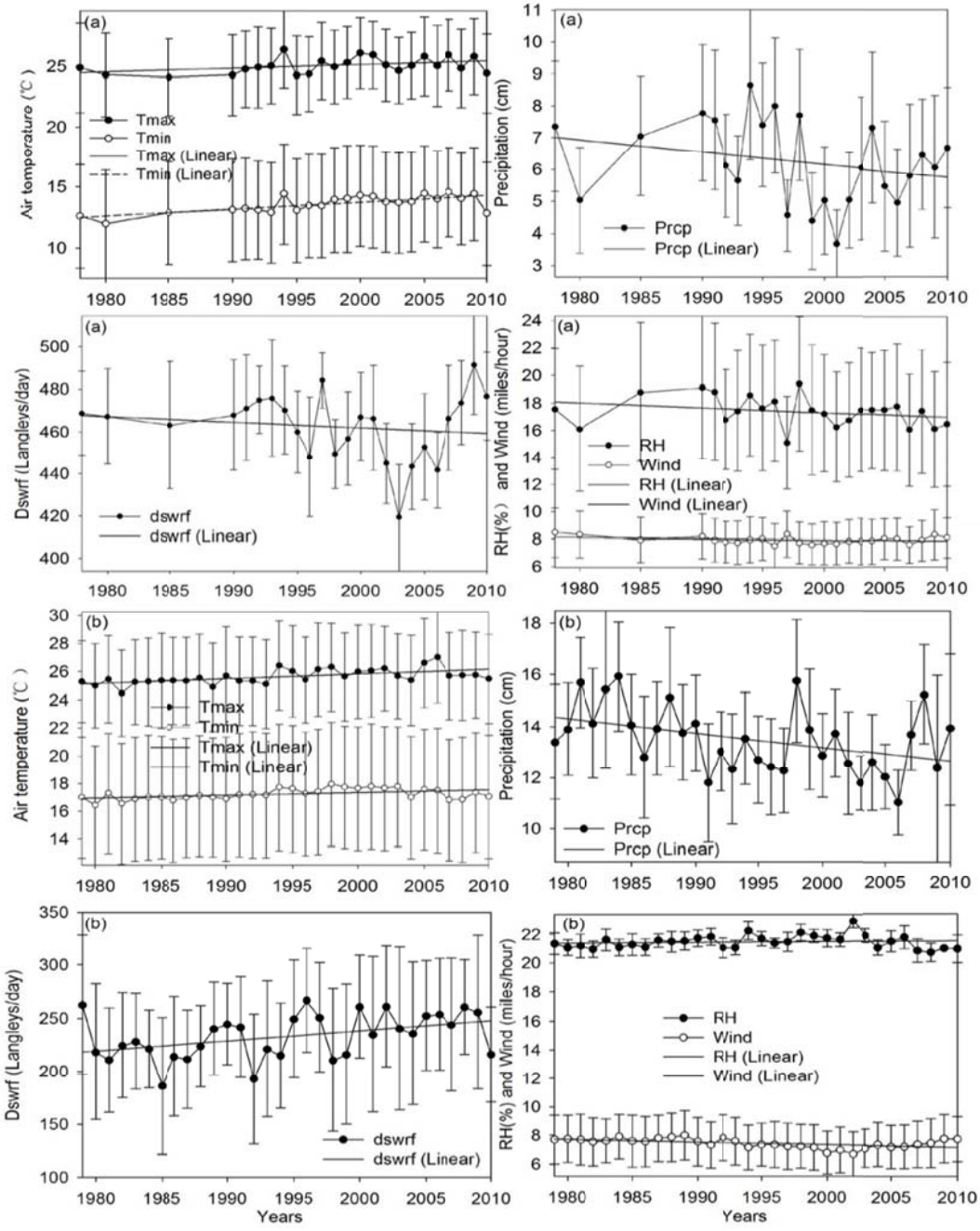

Figure 6. The annual change of six climatic factors (a) from 1978 to 2010 in Hebei and (b) from 1979 to 2010 in Sichuan. The bars represent plus and minus one standard deviation

Table 1. Pearson correlation between the average values of six meteorological factors from April to September, and crop yields in 1978-2010 in Hebei and in 1979-2010 in Sichuan provinces

Province	Variables		April-September		Province	Variables		April-September	
			Simulated	Observed				Simulated	Observed
Hebei	T_{max}	Pearson Correlation	0.172	-0.060	Sichuan	T_{max}	Pearson Correlation	-0.258	-0.133
		Sig.(2-tailed)	0.421	0.781			Sig.(2-tailed)	0.153	0.469
		N	24	24			N	33	33
	T_{min}	Pearson Correlation	0.299	0.059		T_{min}	Pearson Coefficient	-0.363**	-0.508**
		Sig.(2-tailed)	0.156	0.299			Sig.(2-tailed)	0.041	0.003
		N	24	24			N	33	33
	dswrf	Pearson Correlation	-0.010	0.105		dswrf	Pearson Coefficient	-0.166	-0.027
		Sig.(2-tailed)	0.961	0.625			Sig.(2-tailed)	0.364	0.884
		N	24	24			N	33	33
	RH	Pearson Correlation	-0.603**	-0.590**		RH	Pearson Coefficient	-0.056	0.073
		Sig.(2-tailed)	0.002	0.002			Sig.(2-tailed)	0.760	0.692
		N	24	24			N	33	33
	Wind	Pearson Correlation	0.323	0.303		Wind	Pearson Coefficient	0.024	0.181
		Sig.(2-tailed)	0.124	0.151			Sig.(2-tailed)	0.897	0.321
		N	24	24			N	33	33
	Prcp	Pearson Correlation	-0.373	-0.450*		Prcp	Pearson Coefficient	--0.379*	-0.295
		Sig.(2-tailed)	0.073	0.028			Sig.(2-tailed)	0.032	0.102
		N	24	24			N	33	33

Note. *. Correlation is significant at the 0.05 level (2-tailed); **. Correlation is significant at the 0.01 level (2-tailed).

3.2 Spatial Variations of Corn Yield Simulation

The spatial distribution of simulated corn yields in 1948-2010 in Hebei revealed that corn yields in central and north Hebei were more than yields in the south (Figure 7a). Corn yields in Chengde, Baoding and Shijiazhuang were the greatest in the whole province, while corn yields in Tangshan, Hengshui, Langfang, and Xintai were the least. Corn yields in the entire province increased from 1948 to 2010. North of Zhangjiakou, yields increased from 1948 to 1999, but decreased substantially after 2000 compared to other regions. Additionally, corn yields in the north of Chengde decreased substantially compared to other regions from 1948 to 2010. Reduction of corn production that occurred after 2000 was observed in Zhangjiakou and Chengde, which could be due to continuous rainfall in the pollination period and drought in the grain formation-mature period (Liu, 2008; Zhang, 2014; Liu, 2013).

In Sichuan province (Figure 7b), high-yield corn is mainly located east of Sichuan, especially in the northeastern and southeastern regions of the province, which is associated with topographic features and agriculture distribution. Corn yields in Guangyuan, Bazhong, Deyang, Ziyang and Daxian were the greatest in the whole province, while those in Xiaojin, Yaan, and Mianning distributed in the central of Sichuan were the least. After 2000, corn yields in Bazhong, Daxian, Ziyang, Nanchong, Guangan, Yibin, Luzhou, Neijiang, and Zigong decreased substantially compared to other regions (Figure 8). Zhu and Yang (2007) found that corn yields have continued to decline since 2000 due to large-scale adjustment of planting areas of maize under the policy of returning farmland to forest in Sichuan. This simulated yield reflected the declination of corn yields, in agreement with Zhu and Yang (Zhu & Yang, 2007).

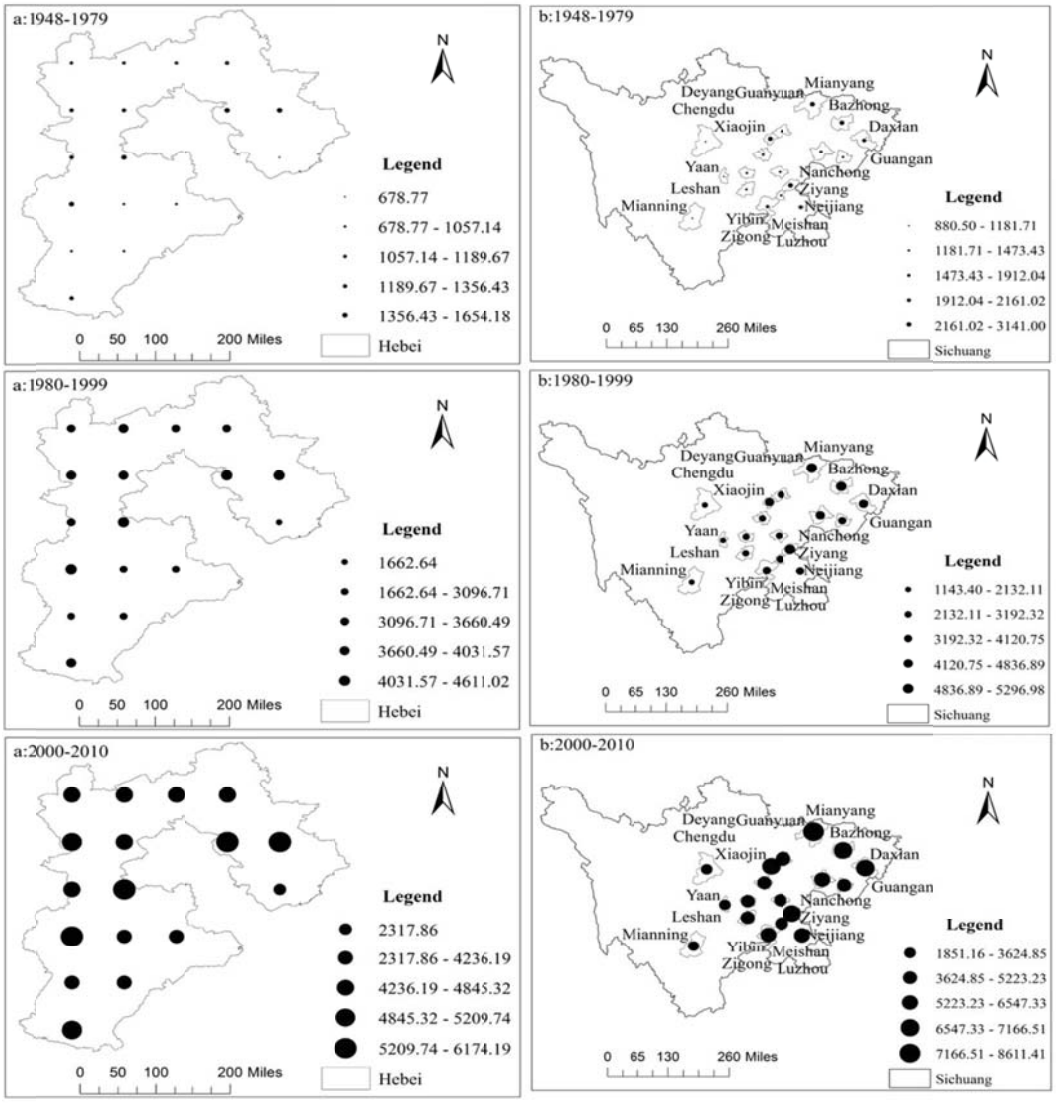

Figure 7. Spatial variations on simulated corn yields in 1948-1979, 1980-1999, and 2000-2010 in the province of (a) Hebei and (b) Sichuan

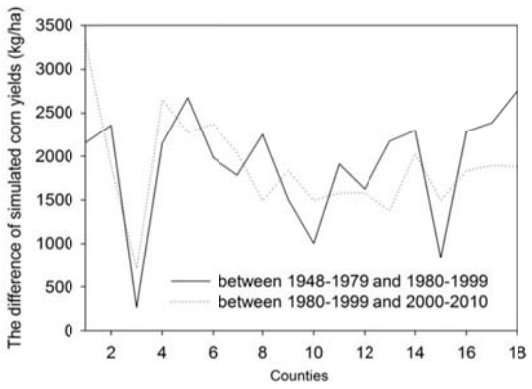

Figure 8. The differences of simulated corn yields between 1980-1999 and 1948-1979, and between 2000-2010 and 1980-1999 in Sichuan. Thereinto, in horizontal ordinate, 1-18 represents Guangyuan, Bazhong, Xiaojin, Mianyang, Daxian, Deyang, Chengdu, Guangan, Meishan, Yaan, Ziyang, Leshan, Zigong, Yibin, Mianning, Luzhou, Neijiang and Nanchong, respectively

3.3 Impact of Regional Warming on Corn Yield

To test the regional warming effect, we removed the daily air temperature warming trend of the forcing meteorological data from 1978 to 2010 in the experiment. Figure 9 shows that, the variations of simulated corn yields with regional warming compared to those without regional warming were different during various phases of 1978-2010. In Hebei province, the annual mean corn yield in the controlled experiment after de-trending was 3794 kg/ha in 1978-2003, 4590 kg/ha in 2004-2007 and 5385 kg/ha in 2008-2010. Compared to the original simulated annual mean corn yields of 3684 kg/ha in 1978-2003, 5156 kg/ha in 2004-2007 and 5097 kg/ha in 2008-2010, mean corn yields increased by 110 kg/ha in 1978-2003 and 288 kg/ha in 2008-2010, respectively, decreased by 565 kg/ha in 2004-2007, with removing the warming trend. The change of mean simulated corn yields in Hebei was not significant ($Z = 0.0095$, $\alpha > 0.05$, resulting from Mann-Kendall test (Fu et al., 2013)).

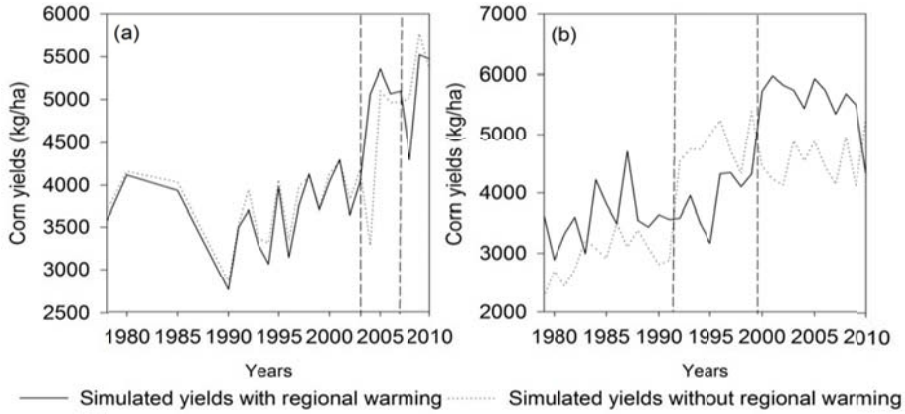

Figure 9. Changes of corn yields with/without regional warming in the province of (a) Hebei and (b) Sichuan

In Sichuan, the simulated mean corn yields before and after de-trending were 3604 kg/ha/yr and 2931 kg/ha/yr in 1979-1991, 3909 kg/ha/yr and 4832 kg/ha/yr in 1992-1999, 5567 kg/ha/yr and 4540 kg/ha/yr in 2000-2010 respectively, which shows that rising air temperature enhanced corn yields in 1979-1991 and 2000-2010, increasing them by 674 kg/ha/yr and 1027 kg/ha/yr on average in these two periods, affected corn growth in 1992-1999, decreasing them by 923 kg/ha/yr. These change were significant ($Z = 1.53$, $\alpha < 0.05$). The reason why rising air temperature has a significant impact on corn yields in Sichuan is that the warming of air temperature extends the summer and increased solar radiation, which allows to plant spring corn, summer corn and silage corn in more regions in a year under the condition of enough precipitation (Figure 6) in 1979-1991 and 2000-2010, so corn yields were increased. In the 1990s, precipitation was the least compared to other periods in Sichuan (Zhou et al., 2011). Therefore, the drought degree intensified in the 1990s in Sichuan with the rising of air temperature and the decrease of precipitation (Figure 6), which led to the reduction of corn yields. However, in Hebei, the warming of air temperature intensified drought and increased evapotranspiration. Sufficient irrigation can compensate for the need of more water in corn growth. Therefore, the rising air temperature did not lead to the significantchange of corn yield in Hebei.

From this, we can see that the sensitivity of simulated corn yields to elevated air temperature was greater in hot and humid environments than in cold and dry environments. In contrast, Liu (2008) reported that the adaptation of crops to the warming of air temperature was low, revealing the inhibition effect on yield in a warm province, and founding that area growth and development of crops were accelerated, but the growth period was shortened, and total dry weight was reduced with rising air temperature. The results in this paper were not controversial to that reported by Liu (Liu, 2008). The increase of corn yields with the rising air temperature is mainly because the enlargement of two bifurcation corn plant area in Sichuan, especially silage corn in large quantities (Duan, 2014). The negative correlation between T_{min} and corn yields (Table 1) also showed that the negative impact of air temperature warming on corn yields, which is in agreement with the report by Liu (Liu, 2008).

The variation of simulated corn yields among different mean growing season air temperature in 1978-2010 in Hebei and in 1979-2010 in Sichuan were calculated and analyzed using the least significant difference (LSD) by SPSS 13.0 software (Table 2). The annual mean growing season air temperature in 1978-2010 in Hebei and in 1979-2010 in Sichuan are taken as control factors, and simulated annual corn yields as the dependent variables. After the control factors were sorted in ascending order, and the dependent variables were correspondingly

sorted with the control factors, the control factors in the different provinces were divided into three levels from small to large, respectively (Table 2). Then, the significance of the difference between the dependent variables among these three levels of the control factors was analyzed using the LSD method. The results showed that, in Hebei, when the mean growing season air temperature increased from 18.69 °C to 19.41 °C, and then to 20.21 °C, there was no significant difference between simulated corn yields, which indicates that there was not a critical threshold value of air temperature influencing corn yields when the growing season air temperature varied in the range of 18.69 °C-20.21 °C. In Sichuan, when the air temperature rose from 22.29 °C to 22.68 °C, simulated corn yields varied non-significantly, but at 23.33 °C, simulated corn yields changed significantly, which shows that 23.33 °C may constitute a critical threshold value influencing simulated corn yields. The change in the growing season air temperature was positively associated with simulated corn yields with the correlation coefficient of 0.456 (sig. = 0.009 < 0.05) (Table 3). Therefore, when the mean growing season air temperature was over 23.33 °C, simulated corn yields increased with the rising of air temperature in Sichuan province. Wolfram and Michael (2009) also found that annual mean air temperature affects corn yields. They indicated that crop yield would increase with elevated air temperature up to 29 °C. Moreover, above this threshold value of air temperature, further warming would have a detrimental effect on yield. The results of this study are not contradictory to those of Wolfram and Michael (2009), and provide a minimum value of air temperature influencing and increasing corn yields. The annual mean air temperature in the growing season of corn (April to September) in Sichuan and Hebei are 22.83 °C and 19.34 °C, respectively. Therefore, when the mean growing season air temperature reaches 23.33 °C-29 °C in Sichuan, corn yields increase with the rising air temperature. It has been found that the 1990s are the warmest 10 years in 1948-2010 because the number of days over 29 °C of actual air temperature in the growing season was more than 190 days in each year of seven years of 1990s. This long period and continuous high temperature are also probably the factors leading to the reduction of corn yields in the 1990s.

Table 2. The least significant difference (LSD) analysis on simulated corn yields among the different growing season air temperature levels in Hebei and Sichuan provinces

Provinces	Air temperature (I) (°C)	Air temperature (II) (°C)	Mean difference (I-J)	Std. Error (°C)	Significance
Hebei	18.69	19.41	998.71*	464.27	0.043
		20.21	120.71	439.25	0.786
	19.41	18.69	-998.71*	464.27	0.043
		20.21	-878.00**	308.16	0.010
	20.21	18.69	-120.71	439.15	0.786
		19.41	878.00**	308.16	0.010
Sichuan	22.29	22.68	-1046.71**	323.42	0.003
		23.33	-1442.88**	526.99	0.010
	22.68	22.29	1046.71**	323.42	0.003
		23.33	-396.16	550.43	0.477
	23.33	22.29	1442.88**	526.99	0.010
		22.68	396.16	550.43	0.477

Note. *. LSD is significant at the 0.05 level (2-tailed); **. LSD is significant at the 0.01 level (2-tailed).

Table 3. Pearson correlation coefficients between the mean growing season air temperature and simulated corn yields in Hebei and Sichuan provinces

Provinces	Correlation coefficients	Significance (2-tailed)
Hebei	0.294	0.163
Sichuan	0.456**	0.009

3.4 Sensitivity Analysis

Anandhi (2016) also analyzed the impact of management decisions, such as plant water use, fertilizer application and hybrids on corn yields, and found inadequate N fertility results in lower yields, whereas over-fertilization results in higher emissions of nitrous oxide. Here, we examine the changes in yields that result from increasing or decreasing inorganic and organic N applications.

3.4.1 Sensitivity to Inorganic N Fertilization Levels

The original fertilization levels (Table 4) were increased or decreased by 50% and 75% without changing other factors. In Hebei province, when the fertilization level was increased by 50% and 75%, mean annual yields from 1979 to 2010 increased by 2136 kg/ha (52%) and 2448 kg/ha (59%), respectively; when the fertilizer level was decreased by 50% and 75%, mean annual yields decreased by 2130 kg/ha (52%) and 3095 kg/ha (75%), respectively (Figure 10a). In Sichuan province, the mean annual yields from 1978 to 2010 were higher by 1467 kg/ha (34%) and 2346 kg/ha (55%) after a 50% and 75% increase in fertilization level, respectively; yields were lower by 2354 kg/ha (55%) and 3369 kg/ha (78%), respectively, when the fertilization level decreased by 50% and 75% (Figure 10b). Fertilization level increases in a cold and dry environment (in Hebei) were more beneficial than in a humid and hot environment (in Sichuan) in terms of percentage change. However, when the fertilization levels were decreased, crop yield in Sichuan suffered more than in Hebei. These results indicated that the sensitivity of the fertilization level influencing corn yield simulation was great in these two provinces. This is probably associated with the original soil fertility condition. It has been reported that the soil in Sichuan was more fertile than that in Hebei because of the humid and hot environment in Sichuan (Zhang, 2014), and because of the higher N fertilization level in Sichuan than that in Hebei (Table 4). In addition, corn yields in Hebei increased slightly with the mean increase amount of 312 kg/ha/yr when the fertilization levels increased from 50% to 75%, which was less than the mean increase amount of 879 kg/ha/yr in Sichuan (Figure 10). This demonstrates that crop yield increases are not proportional to N fertilizer increases, and that fertilizer addition above +50% produces diminishing returns (Zhang, 2014; Luo, 2009).

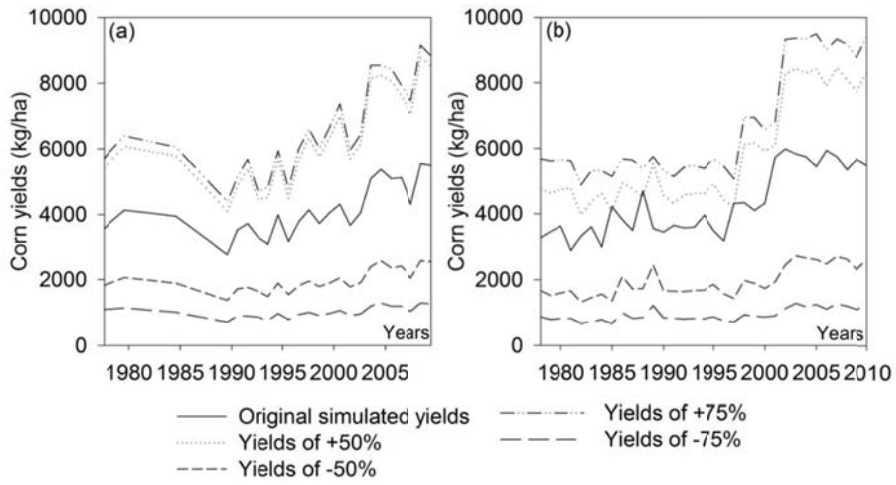

Figure 10. Changes on original simulated corn yields and yields of the control experiment when adding and extracting 50% and 75% of fertilization in the province of (a) Hebei and (b) Sichuan

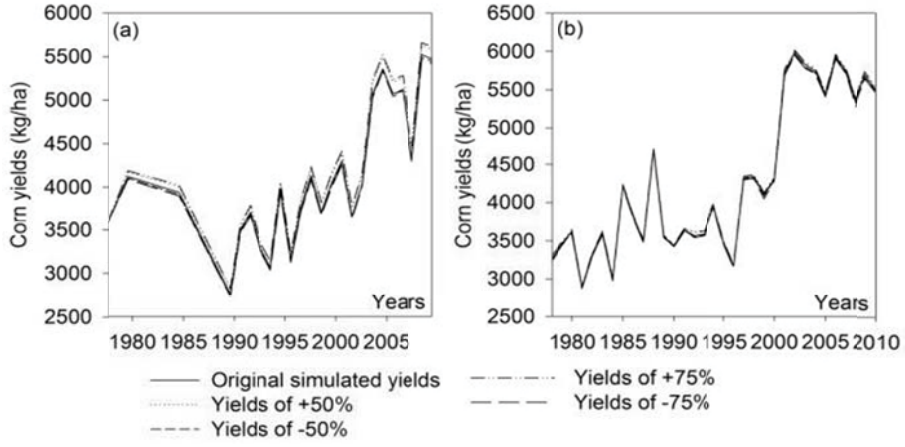

Figure 11. Changes on original simulated corn yields and yields of the control experiment when adding and extracting 50% and 75% of OMAD in the province of (a) Hebei and (b) Sichuan

Table 4. N fertilization levels and OMAD in the control run

Provinces	Phrases set in schedule file	N fertilization level I (kg N/ha/yr)	N fertilization level II (kg N/ha/yr)	Organic C (kg C/ha/yr)
Sichuan	1701-1900	60	60.5	23.1
	1901-1960	60	60.5	23.1
	1961-1970	93	94	57.7
	1971-1980	112	117	69.0
	1981-1996	117	118	81.0
	1997-2000	142	146	92.0
	2001-2010	142	146	92.0
Hebei	1701-1900	18.6	18.6	23.1
	1901-1960	18.6	18.6	23.1
	1961-1976	60.5	63	46.1
	1977-1996	66	66.9	57.7
	1997-2002	66.9	67	57.7
	2003-2010	66.9	67	57.7

3.4.2 Sensitivity to OMAD

After corn is harvested each year, soil fertility needs to be replenished to benefit corn growth in the following year. The amount of organic N that was applied in the control runs was 20 kg/ha/yr. The C:N ratio of the organic matter applied was 15. We conducted sensitivity studies by adding or decreasing 50% and 75% of the original OMAD level. There was no difference in the mean control and experimental simulations yields in 1979-2010 in Sichuan (Figure 11b); whereas, there was a slight increase in 1978-2010 mean yields (59.05 kg/ha/yr) in Hebei (Figure 11a). This is also probably related to the original soil fertilization condition. The higher inorganic N soil fertilization amounts in Sichuan meet the needs of corn growth. There was no need for additional organic N fertilizer in the field. In addition, the reason that OMAD increases are not important to either province is probably different. In Hebei, when corn is harvested in autumn, OMAD cannot efficiently improve soil fertility for drier soil; in Sichuan, OMAD can improve soil fertility for humid soil due to ample precipitation and irrigation.

Generally, fertilization level and OMAD have a larger impact in Hebei (cold and dry environment), where there is less active biophysical activity due to lower soil moisture and warmth. Increased soil fertilization can improve soil biophysical activities and meet the needs of corn growth (Tan et al., 2002).

4. Conclusions

The DayCent model is used to simulate corn yields under different environmental conditions in China. The model reproduced the inter-annual variation of crop yield with a R^2 of 0.71 and 0.85 in Hebei and Sichuan, China, respectively, demonstrating that the DayCent model is capable of accurately simulating corn yield in these two environments.

The annual mean T_{max} and T_{min} increased at a rate of 0.22 °C/10 yr and 0.23 °C/10 yr in Sichuan, respectively, and 0.34 °C/10 yr and 0.55 °C/10 yr in Hebei, respectively. The annual mean downward shortwave radiation (Dswrf) increased at a rate of 1.92 (langleys/day)/10 yr in Sichuan, and decreased at a rate of -0.82 (langleys/day)/10 yr in Hebei. The annual mean RH decreased at a rate of -0.06 %/10 yr in Sichuan, and increased at a rate of -0.45 %/10 yr in Hebei. The annual mean wind decreased at a rate of -0.11 (miles/hour)/10 yr in Sichuan, and increased at a rate of 0.08 (miles/hour)/10 yr in Hebei. The annual mean precipitation decreased at a rate of -0.01 cm/10 yr in both Sichuan and Hebei.

In this paper, the impacts of climatic factors and management amendment levels on corn yields were explored. The results showed that RH and Prcp negatively affected corn yields in Hebei (a cold and dry environment). That impacts of the rising of T_{max} and T_{min} on corn yields were not significant is because corn growth in Hebei mainly depends on irrigation, and is not constrained by the drought resulting from the rising air temperature. Only T_{min} negatively affected corn yields in Sichuan.

Simulated corn yields decreased significantly after de-trending the warm air temperature in Sichuan. There was no critical threshold value of air temperature influencing corn yield simulation in Hebei; whereas, 23.33 °C was the critical threshold value influencing simulated corn yields in Sichuan.

The sensitivity study suggests that crop yield is more sensitive to fertilization level in a cold and dry environment as compared to that in a humid and hot environment. Fertilizer application can remedy the shortage of soil fertility, and accelerate crop growth in a cold and dry environment.

In Sichuan province (a humid and hot environment), current air temperature is limiting to corn production. 23.33 °C-9 °C constitutes the ideal scale of air temperature influencing increase of corn yields.

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Microbial Activity of a Plinthosol With Application of Thiamethoxam Insecticide and Biochar

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Abstract

Although thiamethoxam is an insecticide widely used in agriculture, its high mobility and persistence in the soil can result in contamination of groundwater and alteration in biogeochemical cycles. The objective of this study was to verify the effect of biochar, NPK fertilizer and thiamethoxam insecticide on soil microbial properties. The experiment was conducted in a randomized block design composed of the doses combination of mineral fertilizer NPK (0 and 300 kg ha^{-1} of the formulated 05-25-15), and biochar (0, 8, 16 and 32 t ha^{-1}) in the absence and presence of thiamethoxam. Deformed soil samples were collected in all plots in the 0 to 0.10 m layer to determine the activity of the enzymes: acid and alkaline phosphatase, beta glucosidase and urease, beyond the microbial biomass carbon (MBC), basal respiration rate (C-CO_2) and metabolic quotient (qCO_2). To compare soil microbiology before and after the application of thiamethoxam, multivariate statistical techniques were used. The application of biochar resulted in increased enzymatic activity of urease, acid phosphatase, increase of qCO_2 and basal respiration and reduction of MBC. In contrast, the application of the thiamethoxam insecticide suppressed the enzymatic activity of urease, acid phosphatase, resulting, however, in the elevation of alkaline phosphatase and basal respiration of the soil. Biochar application at doses greater than or equal to 16 t ha^{-1} resulted in elevation of qCO_2 and reduction of MBC, regardless of the absence or presence of NPK chemical fertilization. Biochar effect on soil microbiological attributes is less significant than the effect of thiamethoxam application.

Keywords: soil microbial biomass, enzymatic activity, soil quality

1. Introduction

Thiamethoxam insecticide is used worldwide in agriculture for the control of a wide variety of insect pests (Hilton, Jarvisa, & Ricketts, 2015). In Brazil, 7% of the insecticides used are from the neonicotinoid cluster, with thiamethoxam being one of the main commercialized molecules (IBAMA, 2017). The high demand for the use of this molecule in agricultural systems requires greater attention regarding its effects on the soil microbiota. This concern elapses from the physico-chemical characteristics of thiamethoxam (low sorption interaction, high solubility), which gives it, above all, high soil persistence (Hladik, Kolpin, & Kuivila, 2014). These aspects result in two main concerns with its application: i) high leaching potential and consequently subsurface water contamination; ii) deleterious effects on the soil microorganism and the ecological chain, resulting in toxic effects on several organisms, including humans.

Studies confirm the deleterious effect of thiamethoxam on the soil microbiota, such as toxicity to bacteria involved in the nitrogen cycle (Filimon et al., 2015), reduction in the activity of the urease, phosphatase and β-glucosidase enzymes (Jyot, Mandal, & Singh, 2015) and reduction of microbial biomass carbon (MBC) and basal soil respiration (Portillo, Scorza Junior Salton, Mendes, & Merchant, 2015).

Soil microorganisms have a role of great ecological and agricultural importance, working actively in the processes of genesis, nutrient cycling, decomposition of organic residues, synthesis of organic matter and degradation of organic contaminants (Kirchman, 2018; Mendes, Souza, & Reis Júnior, 2015). Therefore, it is necessary to study techniques to reduce the impact of agrochemicals on the soil microbiota. Due to its characteristics, mainly to the reactivity, the organic matter (OM) is the main component of the soil involved in the remediation of the contaminant potential of agrochemicals (Portilho et al., 2015; Petter et al., 2017). This fact, the OM also happens to be the main compartment of the soil to be improved from the point of view of handling techniques. However, in the tropics the maintenance of OM levels is hampered by high temperatures and precipitation, which requires alternative studies to maintain and/or increase soil carbon stocks. Given the high porosity, high molecular stability, the use of biochar is an alternative to improve carbon stocks in the soil, and can act as a source of nutrients and habitat for microorganisms (Li et al., 2019) and thereby minimize the harmful effects of thiamethoxam on the environment. In addition, although the biochar presents high molecular stability, after its application to the soil, processes of oxidation of the aromatic structures forming new electric charges and reactive functional clusters in the soil occur (Petter et al, 2017). This higher reactivity may represent an improvement in the retention of molecules as it occurs in organic matter (Schmidt et al., 2015).

The effect of biochar in the soil has been the subject of several studies that have shown beneficial effects on soil, such as increase fertility, water retention (Zhu et al., 2017), agrochemicals (Ali, Khan, Li, Zheng, & Yao, 2019; Hladik et al., 2014), increased microbial biomass (Lehmann et al., 2011), significant changes in the composition of the microbial community in clayey soils (Silva et al., 2018; Li, Liang, & Shangguan, 2017), increased enzymatic activities such as urease and β-glucosidase (Huang et al., 2017; Wang et al., 2017), increased nodulation of the root by nitrogen-fixing bacteria, nutrient cycling and carbon sequestration (Scheifele et al., 2017).

However, there is still a lack of studies using biochar in order to reduce the residual effect of pesticides in soil and its effect on long-term biological functions (Palansooriya et al., 2019). Aiming to fill this gap in the research, we propose in our studies to evaluate the use of biochar in the soil as a mitigating technique of the potential effect of the insecticide on the microbiological properties of the soil.

2. **Material and Methods**

2.1 Study Area

The experiment was conducted at Farm Estrela do Sul in Nova Xavantina, Mato Grosso, in the Central West region of Brazil (14°34′50″ S and 52°24′01″ W), with an average altitude of 310 m, and the region located in the 'Cerrado' biome. The climate of the region is hot and humid tropical type (Aw), according to the classification of Köppen-Geiger. The soil is classified according to the Brazilian system of soil classification (Santos et al., 2018) as a Dystrophic Haplic Plinthosol, sandy loam texture, with 763 g kg^{-1} of sand, 67 g kg^{-1} of silt and 170 g kg^{-1} of clay.

2.2 Characterization and Experimental Design of the Study Area

Before the implementation of the experiment the area was native forest until 1985, after it was used for grazing with *Urochloa brizantha* until 2008. The experimental design was randomized blocks in a 2 × 4 factorial scheme with three replications. The treatments consisted of the combination of two doses of NPK fertilizer 05-25-25 (0 kg ha^{-1} and 300 kg ha^{-1}) and four doses of charcoal (biochar) as a source of pyrogenic carbon (0 t ha^{-1}; 8 t ha^{-1}, 16 t ha^{-1} and 32 t ha^{-1}). Each plot was composed of nine soybean/maize lines with a length of 10 m, totaling 40.50 m^2, and the useful area for evaluations of 25.20 m^2.

Before being incorporated into the soil, the eucalyptus biochar was milled and passed through a 2 mm sieve. Its chemical composition is shown in Table 1. This material was applied to the soil only once in December 2008, being incorporated at a depth of 0.10 m by means of a rotary spade. After the incorporation, the experiment was conducted under no-tillage system.

Table 1. Elemental composition (total values) of the biochar used in the experiment

Element	Unit	Concentration
Total Nitrogen (N)		3.3
Phosphorus (P_2O_5 Citric acid)		0.14
Phosphorus (P_2O_5 total)		-
K_2O	g kg^{-1}	1.9
CaO		1.5
MgO		0.9
Sulfur (S)		-
Copper (Cu)		1.0
Zinc (Zn)		36.0
Molybdenum (Mo)	mg kg^{-1}	-
Cobalt (Co)		-
Boron (B)		-
Total carbon (C)		774.0
Humidity	g kg^{-1}	50.0
Total mineral material		-
C:N Ratio		234.5
Specific surface area	m^2 g^{-1}	41.2
Pore volume	cc g^{-1}	0.018
Pore diameter	μm	38.4
Density	g cm^{-3}	0.3
Pyrolysis temperature	°C	400-500

Source: Petter et al. (2012), and Carvalho et al. (2013).

Later, in the two subsequent harvests (2008/2009 and 2009/2010 harvests) after biochar application, rice (*Oryza sativa*) was cultivated in a conventional culture system, and after all the agricultural crops until the time of samples collection for this experiment soybean (*Glycine max*) was cultivated in no-tillage system on millet straw (*Pennisetum glaucum*) that was formed in all crops 45 days before soybean planting in the experimental area. Fertilization was repeated every year, using the same formulation and in the same amounts. To characterize soil fertility in the 2015/2016 harvest, four deformed soil samples were collected from each plot in 0 to 0.10 m layer. Thus, simple samples were mixed composing one single sample per plot (Table 2).

Table 2. Analysis for fertility purposes of a Plinthosol subjected to four doses of charcoal (biochar) as a source of pyrogenic carbon (0 t ha^{-1}; 8 t ha^{-1}, 16 t ha^{-1} and 32 t ha^{-1}) and two doses of NPK fertilizer 05-25-25 (0 kg ha^{-1} and 300 kg ha^{-1}) in the municipality of Nova Xavantina (MT) in the 2015/2016 harvest

Biochar	pH	P	K	Ca	Mg	H+Al	Al	SB	CEC	V%	OM
t ha^{-1}		---- mg dm^{-3} ----		---------- $cmol_c$ dm^{-3} ----------							g dm^{-3}
No fertilization											
0	3.82	18.4	63.0	0.37	0.18	4.35	0.87	0.73	5.08	14.2	11.5
8	3.80	10.2	61.7	0.25	0.12	4.40	1.06	0.53	4.93	10.6	10.5
16	3.85	14.78	57.5	0.37	0.16	4.40	0.83	0.65	5.05	13.3	10.6
32	3.80	17.73	64.8	0.42	0.20	4.93	1.03	0.78	5.30	13.7	10.7
300 kg ha^{-1} of NPK											
0	3.78	24.40	68.5	0.29	0.12	4.35	1.01	0.58	4.93	12.6	11.5
8	3.89	35.35	67.5	0.65	0.37	4.03	0.73	1.13	5.15	19.1	13.5
16	3.88	53.05	65.0	0.49	0.16	4.45	0.80	0.80	5.25	15.4	11.8
32	3.85	35.38	80.0	0.56	0.23	4.58	1.02	1.00	5.58	17.9	10.9

Note. pH at $CaCl_2$; P and K determined by Mehlich-1; Ca, Mg and Al exchangeable extracted by KCl; H + Al extracted by calcium acetate; SB: sum of bases ;CEC: cation exchange capacity at pH 7; V%: soil base saturation; OM: soil organic matter determined by sodium dichromate.

2.3 Soil microbiological Analyzes

After eight years of biochar incorporation, 2018, deformed soil samples were collected with the aid of a Dutch auger in 0 to 0.10 m layer to determine their microbiological attributes. The collection was carried out when the soybean crop was in full bloom. During the collection the samples were packed in polystyrene boxes containing ice to maintain the temperature until dispatch to the laboratory.

After the first sampling, the thiamethoxam insecticide (record dose 105 g ha^{-1} of active) was applied with pressurized CO_2 pump throughout the experimental area (soybean crop). After 48 hours of application, a new soil sample was taken to determine its microbiological attributes, as described in the first collection. Soil moisture was in the same condition as the first collection.

The activities of four soil enzymes were determined: β-glucosidase, acid phosphatase, alkaline phosphatase according to the methods described by Tabatabai (1994), and urease by the method of Kandeler and Gerber (1988). These methods are based on the colorimetric determination of p-nitrophenol (yellow color) formed after the addition of colorless substrates specific to each enzyme evaluated.

For each soil sample, three analytical replicates were performed in the laboratory. The soil enzymatic activity was expressed in μg p-nitrophenol released per gram of dry soil per hour. For the determination of β-glucosidase, phosphatases (acid and alkaline), and urease, the respective substrates were used p-nitrophenol-β-D-Glucopyranoside 0.05 M (PNG 0.05 M), nitrophenol phosphate 0.05 M (PNP 0.05 M) and urea solution Absorbance readings ranged from 400 nm to β-glucosidase, 490 nm to acid phosphatases, 400 nm to alkaline phosphatase, and 690 nm to urease.

The soil basal respiration rate ($C\text{-}CO_2$) was determined by the method described in Anderson and Domsch (1993), and microbial biomass carbon (MBC) by the fumigation-extraction method described by Vance et al. (1987) and Brookes et al. (1985). With data from the biological analyzes, the metabolic quotient (qCO_2) was determined. The qCO_2 is the amount of $C\text{-}CO_2$ produced by unit of soil microbial biomass per unit time (mg $C\text{-}CO_2$ mg^{-1} MBC $hour^{-1}$) (Anderson & Domsch, 1993).

2.4 Statistical Analysis

Residual normality and variances homogeneity among treatments were confirmed by the Shapiro Wilk and Levene tests, respectively. To compare soil microbiological attributes before and after the application of thiamethoxam insecticide, the data were standardized to have mean 0 and variance 1, followed by performing the following multivariate statistical methods: hierarchical cluster analysis, k-means and main components.

A hierarchical cluster analysis was performed only for the insecticide factors and biochar doses, calculating the Euclidean distance between the "accesses" or plots, for the set of 7 variables, and using the Ward algorithm to obtain similar accesses clusters. The result of the analysis was presented in graphical form (dendrogram), which assisted in the identification of the clusters.

The identification of accesses in the clusters was also performed by the k-means analysis (Hair et al., 2009), which belongs to the class of methods of non-hierarchical and unsupervised clusters. In the clusters analysis by k-means, a multivariate analysis of the variables between the established clusters was performed.

Two principal component analyzes were performed: i) with biochar and insecticide factors, and ii) with biochar, insecticide and fertilization factors.

3. Results

3.1 Hierarchical Analysis

In order to define the number of clusters by dendrogram it is considered "jumps" or expressive variations in the distance of connection between the accesses. Among the Euclidean distances from 6 to 9, there was an expressive separation of clusters allowing the definition of 4 clusters (Figure 1). The accesses grouped in clusters 1 and 2 represent the soil without the application of thiamethoxam insecticide, however, it is verified that the biochar doses of 16 and 32 t ha^{-1} modified the microbiological attributes of the soil when compared with the doses of 0 and 8 t ha^{-1}. After applying thiamethoxam insecticide in the soil, it was verified that only 32 t ha^{-1} dose of biochar was able to alter the soil microbiological attributes, isolating the accesses in cluster 4.

From the analysis of hierarchical cluster, it was evident that the application of the thiamethoxam insecticide associated to biochar incorporation altered the microbiological attributes of the soil. However, this analysis alone does not allow the visualization of these changes between the established clusters.

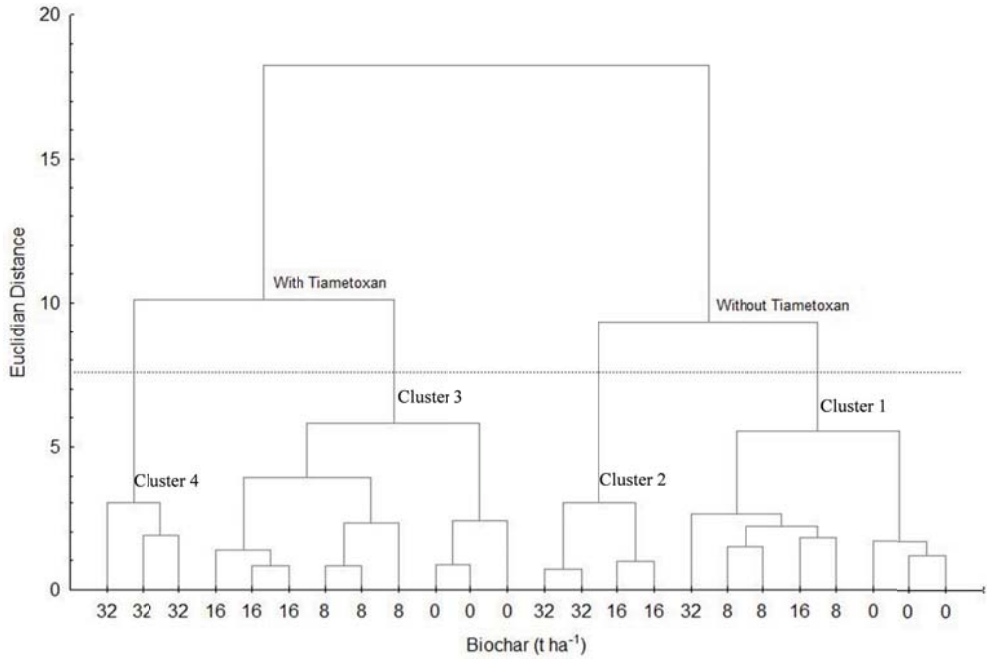

Figure 1. Dendrogram resulting from the hierarchical clusters analysis showing the formation of clusters according to acidic and alkaline phosphatase, β-glucosidase, urease, microbial biomass carbon, C-CO_2: basal respiration and qCO_2: metabolic quotient of a Plinthosol subjected to biochar doses

3.2 K-means Analysis

To characterize the clusters formed in the dendogram, the non-hierarchical k-means analysis was performed, considering four clusters established in the dendogram. By means of multivariate analysis of variance (MANOVA), it was possible to verify that all attributes of the soil presented differences of means among the four clusters (Table 3), confirming appropriate cutting height of the dendrogram for cluster definition.

Table 3. Analysis of variance for microbiological attributes of a Plinthosol submitted to biochar doses among the clusters formed by the non-hierarchical analysis of k-means clusters

Variables	Sum of squares among clusters	Degrees of freedom	Sum of squares among clusters	Degrees of freedom	Fc	Prob
Acid P	19.92	3	3.07	20	43.13	<0.001
Alkaline P	15.27	3	7.72	20	13.18	<0.001
β-Gluco	12.19	3	10.80	20	7.52	<0.01
Urease	16.55	3	6.44	20	17.13	<0.001
MBC	15.53	3	7.46	20	13.86	<0.001
C-CO_2	15.49	3	7.50	20	13.75	<0.001
qCO_2	13.89	3	9.10	20	10.17	<0.001

Note. Acid P: acid phosphatase; Alkaline P: alkaline phosphatase; β-Gluco: Beta glucosidase; MBC: microbial biomass carbon; C-CO_2: basal respiration; qCO_2: metabolic quotient; Fc: value of F calculated; Prob: probability of obtaining a value of $F \geq Fc$.

Clusters 1 and 2 represent the microbiological attributes of the soil without the application of thiamethoxan (Figure 1). Within cluster 1 it is verified that the incorporation of 8 t ha^{-1} of biochar was not sufficient to modify the microbiological attributes of the soil. Likewise, in cluster 2, the doses of 16 and 32 t ha^{-1} also did not present differences for the attributes of the studied soil. The difference between clusters 1 and 2 can be verified by the centroids of the K-means analysis and the standard errors of the standardized means of each attribute (Figure 2). Therefore, it can be inferred that in the absence of insecticide in the soil, doses equal to or greater than 16 t ha^{-1} resulted in a significant increase of β-glucosidase, C-CO_2 and qCO_2, and also reduced MBC.

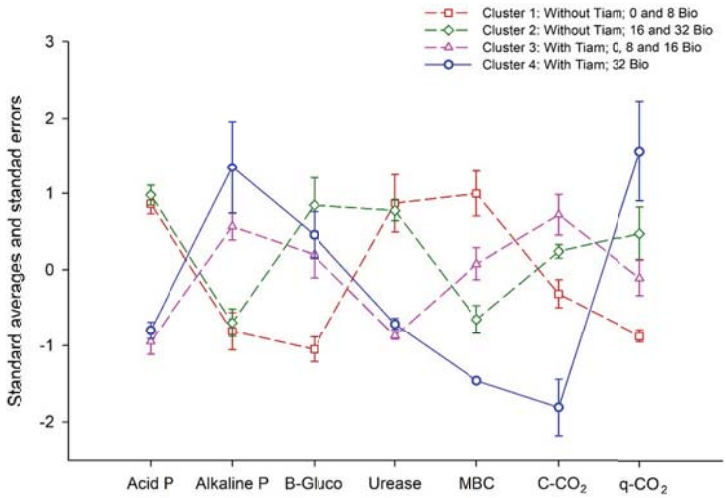

Figure 2. Standardized means of microbiological attributes of a Plinthosol submitted to biochar doses and clusters by non-hierarchical k-means analysis. Acid P: acid phosphatase; Alkaline P: alkaline phosphatase; β-Gluco: Beta glucosidase; MBC: microbial biomass carbon; C-CO_2: basal respiration and qCO_2: metabolic quotient

For the soil condition with insecticide application, observed in clusters 3 and 4, differences in soil attributes were verified only at the dose of 32 t ha^{-1}, occurring increase of qCO_2 and reduction in MBC and C-CO_2 values when compared to cluster 3.These results evidenced that the application of biochar did not attenuate the deleterious effects of thiamethoxam on the soil microbiota, do not confirming our initial hypothesis.

By the method of k-means analysis (Figure 2), it is generally verified that the main changes imposed on the soil by the application of the insecticide were the elevation of alkaline phosphatase and reduction of acid phosphatase and urease.

3.4 Principal Component Analysis

3.4.1 In the Absence of NPK Fertilizer

In order to evaluate the importance of variables in the separation of the four clusters formed in the hierarchical and not-hierarchical analysis, it was carried out the principal components analysis (PC's). The components with eigenvalues greater than or equal to 1 were selected, with only three principal components defined. The eigenvalues found were 3.01, 1.61 and 1.00 for the principal components as one (PC1), two (PC2) and three (PC3), respectively. With these three components it was possible to explain 80.25% of all data variability.

These PCs were constructed by combining the eigenvectors, which are values that represent the weight of each attribute in the components (Silva et al., 2015). In order to select the significant variables for analysis of principal components, we considered only eigenvectors with correlation greater than or equal to 0.5 according to the criteria proposed by Coelho (2003). All soil attributes showed correlations above 0.50 in some of the three components. Only the variable C-CO_2 did not present significant correlation in PC1 and PC2, being isolated in PC3.

The biplot plot of PC1 versus PC2 shows that the application of the insecticide actually altered the microbiology of Plinthosol (Figure 3), confirming the statements made according to the dendrogram and the k-means analysis. Accessions without thiamethoxam application on the soil showed a positive correlation with urease, acid phosphatase and MBC. However, within this cluster it is verified that the biochar incorporation to the soil promoted increase of the urease and acid phosphatase, as well as the reduction of the MBC.

In contrast, the cluster formed by the accesses with application of insecticide negative correlation presentation with urease and acid phosphatase positive with β-glucosidase, alkaline phosphatase and qCO_2 (Figure 3). Within this cluster it is verified that biochar incorporation also provided reduction of MBC and increase of qCO_2.

As the PC3 explained only 14.26% of the total data variability, being represented only by the C-CO_2 attribute the biplot graph was not presented. What can be observed is that the separation of the large clusters according to the application of the insecticide was maintained as a function of PC1. However, there was a confounding among the accesses due to the biochar doses within each large cluster. It was possible to only infer that the accesses with the

presence of thiamethoxam associated with 32 t ha^{-1} dose of biochar had lower values of C-CO_2 compared to the other accessions.

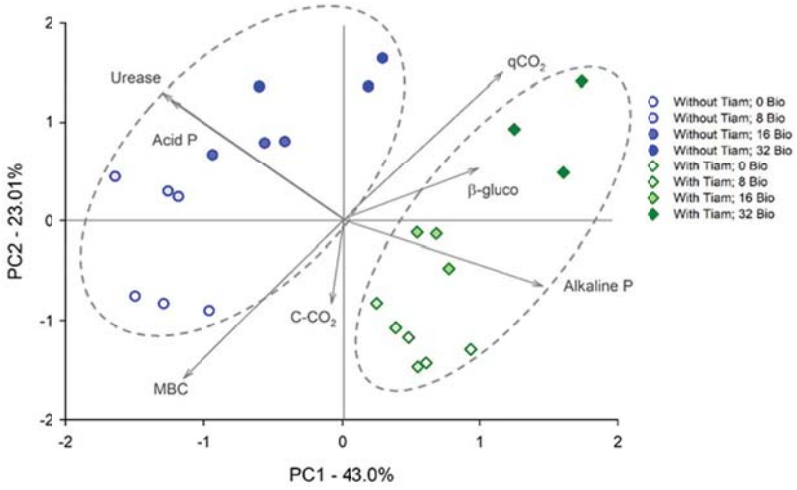

Figure 3. Principal component analysis of a microbiological attributes of a Plinthosol subjected to biochar doses in the absence of fertilization. Acid P: acid phosphatase; Alkaline P: alkaline phosphatase; β-Gluco: Beta glucosidase; MBC: microbial biomass carbon; C-CO_2: basal respiration and qCO_2: metabolic quotient. Without Tiam: without application of the thiamethoxam insecticide. With Tiam: soil submitted to the application of thiamethoxam insecticide. Bio: biochar doses in t ha^{-1}

3.4.2 In the Presence of NPK Fertilizer (300 kg ha^{-1})

With the previous analyzes it was possible to evaluate the consequences of thiamethoxam application on the soil microbiological attributes and its interaction with biochar doses. However, the analyzed plots were not fertilized, leaving doubts as to whether the results found would be similar in fertilized areas. Due to this, another analysis of principal components was performed with the factors such as insecticide, biochar doses and soil fertilization (Figure 4). Only the first two principal components (PCs) were considered. The first principal component (PC1) had eigenvalue of 3.05, and the second (PC2) eigenvalue of 1.53. These two PCs explained more than 65.4% all of variability of the soil microbiological attributes. The greatest variability was retained in the first principal component (PC1), summarizing in one axis of the biplot graph 43.5% of all variability found in the experiment (Figure 4).

The microbiological attributes that presented significance for the separation of the clusters in PC1 were acid and alkaline phosphatase, urease and basal respiration, with eigenvectors or correlations ≥ 0.50, which according to Silva et al. (2015) are highly significant variables. For PC2 only MBC, qCO_2 and β-glucosidase had correlations above 0.50, these variables being responsible for the separation of clusters on the y axis of the biplot graph (Figure 4).

The biplot graph shows the formation of 4 clusters, one in each quadrant (Figure 4). Clusters 1 and 2 are composed of accessions or plots of the experiment in which the soil received application of thiamethoxam insecticide. By analyzing only PC1, it can be verified that the accessions of these clusters showed a high correlation with alkaline phosphatase and basal respiration. Clusters 3 and 4, however, presented a high correlation with acid phosphatase and urease.

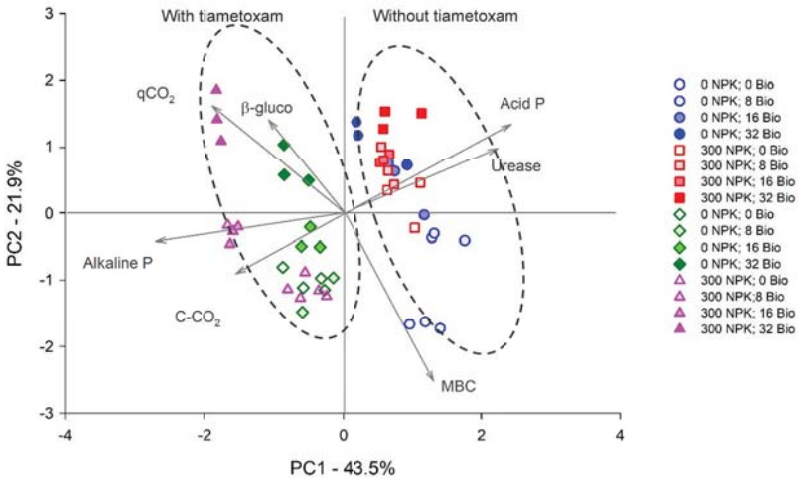

Figure 4. Principal component analysis of microbiological attributes of a Plinthosol subjected to biochar doses and fertilizer (NPK). Acid P: acid phosphatase; Alkaline P: alkaline phosphatase; β-Gluco: Beta glucosidase; MBC: microbial biomass carbon; C-CO_2: basal respiration and qCO_2: metabolic quotient. NPK: 0 or 300 kg ha-^{1} of the formulated 05-25-25, Bio: biochar doses in t ha^{-1}

With this, we can affirm that the soil without the presence of thiamethoxam insecticide presented high values for acid phosphatase and urease, being these soil attributes suppressed with the application of thiamethoxam, resulting in elevation of alkaline phosphatase and basal respiration.

Analyzing PC2, that is, only the separation of clusters in the y-axis, is verified that clusters 1 and 3 showed high correlation with qCO_2 and β-glucosidase. Clusters 2 and 4, however, had a high correlation with MBC. This separation was not performed due to the application of the thiamethoxam insecticide, but due to biochar doses to the soil with thiamethoxam application (clusters 1 and 2) and biochar and NPK doses to the soil without insecticide application (clusters 3 and 4). Cluster 1 was formed by accesses that received the highest biochar dose (32 t ha^{-1}) independent of fertilization, cluster 2 formed predominantly by accessions that received lower biochar doses (0, 8 and 16 t ha^{-1}). Cluster 3 consists of accessions, most of which received the highest biochar doses (16 and 32 t ha^{-1}) without fertilizers and all samples with application of 300 kg ha^{-1} of NPK, and cluster 4 formed predominantly by accessions that received the lowest biochar doses (0 and 8 t ha^{-1}) without fertilizer.

Due to this, it can be stated that independently of the insecticide and NPK application, biochar application at doses greater than or equal to 16 t ha^{-1} resulted in elevation of qCO_2 and reduction of MBC of Plinthosol. The same occurred with application of 300 kg ha^{-1} of NPK independent of biochar dose resulted in elevation of qCO_2 and reduction of MBC of Plinthosol.

The changes imposed by biochar and fertilization application (PC2) on soil microbiological attributes are less significant than the effect of thiamethoxam application (PC1).

4. Discussion

4.1 Biochar in Soil Microbial Properties

Even after eight years of biochar incorporation it was observed an increase of urease and acid phosphatase enzymes of Plinthosol (Figures 2 and 3). Huang et al. (2017) observed increased activity of specific enzymes related to the use of N in the soil in the presence of biochar. Li, Song, Singh, and Wang (2019) also investigated the increase in microbial activity and improvements in soil properties with the release of nutrients from biochar.

Possible explanations for our results would be due to: i) the biochar serving as a substrate of N (N-biochar) released slowly to the soil, resulting in an increase of the total nitrogen as shown in the studies of Petter et al. (2016). The results presented by the authors correspond to biochar application effect after five years of its incorporation into the same Plinthosol of this experiment and six years in an Oxisol. With this, we can affirm that the results verified by Petter et al. (2016) still persist in the soil even after eight years of biochar application. Although not completely elucidated, biochar may promote greater interaction with native organic matter of the soil, generating a positive priming effect, which would result in greater availability of nutrients (*e.g.*, nitrogen and phosphorus) in the soil, especially in sandy soils and of low fertility, thus justifying in part the effect on acid phosphatase and urease. This fact was verified by Wang et al. (2016), in which a 20% increase in OM

mineralization was observed with the application of biochar in sandy soils; the biochar serves as micro-habitat for the soil microbiota through its pores that protect the colonies of fungi and bacteria from natural predators, thereby the enzymes remain for a longer period in the soil (Petter et al., 2018; Scheifele et al., 2017; Pietikäinen, Kiikkilä, & Fritze, 2003). Furthermore, biochar porosity increases the water availability, as noted in the study of Petter et al. (2016), and Carvalho et al. (2013) in the same soil of our study. In periods of scarcity, this water retention in the biochar pores can promote greater survival of microorganisms (Junna, Bingchen, & Gang, 2014), especially during drought periods.

The decrease of the microbial biomass carbon (MBC) and increase of qCO_2 demonstrates that the soil microbiota was under stress. Some studies report that MBC is closely related to the C/N ratio and soil organic carbon (SOC) (Li et al., 2017), decreasing significantly after biochar application biochar (Dempster, Gleenson, Solaiman, Jones, & Murphyet, 2012; Santos, Madari, & Tsai, 2013). Our results indicate that biochar application in the soil reduces MBC proportionally to the applied doses (Table 3). There are several possible reasons that would explain these results: i) the high molecular stability of polycondensed aromatic structures of the biochar carbon formed by the slow pyrolysis at high temperature, resulting in recalcitrant C and more resistant to degradation by microorganisms (Pietikäinen et al., 2019; Chintala et al., 2015; Farrel et al., 2013). Although, the biochar applied in this experiment contained C-labile, after eight years of its application, it was practically no longer observed. This effect was verified in a three-year study after biochar application and incorporation in this same soil and experiment, where it was characterized that there still was C-labile (oxidizable) from biochar available (Petter et al., 2016). The origin of this C-labile would be related to the condensable compounds formed in the biochar pyrolysis process; ii) with the permanence of the biochar to the soil, the oxidation of the biochar carbon-labile associated to a possible positive priming effect in the native OM may have contributed to the reduction of C-labile and increase of the C-recalcitrant in the soil, resulting in an organic matrix with high C: N ratio. This effect would result in greater difficulty of gain for MBC or even MBC loss, as verified in the present study; iii) no less important, the long residence time of biochar in the soil can induce changes in the mineralization rate of the SOC as suggested by Li et al. (2018), especially carbon from recent soil inputs. This may be related to the modification of the microorganisms' abundance related to the C and N cycle of the soil, observed by Xu et al (2014).

4.2Thiamethoxam in Soil Microbial Properties

The reduction of urease and acid phosphatase enzymatic activity may be directly related to the toxicity of the insecticide on the soil microbiota, corroborating the results of Filimon et al. (2015), which verified the decrease of urease and acid phosphatase in the presence of thiamethoxam and its toxic effect on the bacteria involved in the nitrogen cycle. It is well known in the literature (Moreira & Siqueira, 2006) the perception of the sensitivity of soil nitrifying bacteria to agrochemicals as herbicides, fungicides and insecticides.

On the other hand, the increase of enzymatic activity of β-glucosidase and alkaline phosphatase with the application of thiamethoxam may be related to the soil microbiota modification selecting through selective pressure microorganisms capable of producing these enzymes, as well as providing energy in their degradation. A study by Myresiotis, Vryza, and Papadopoulou-Mourkidou (2012) showed that the increasing of bacterial growth resulted in greater degradation of thiamethoxam. The β-glucosidase enzyme is essential for carbon degradation to generate energy for the microorganisms through the catalysis of cellobiose hydrolysis into two glucose molecules (Adetunji et al., 2017). Thus, the selective microbiota possibly acted on the degradation of thiamethoxam resulting in momentarily high levels of β-glucosidase.

Thiamethoxam degradation in soil primarily involves bacterial activity of *Pseudomonas* sp genre, resulting in metabolites of the 'magic-nitro' cluster (=N-NO_2) and subsequently transformed into metabolites such as nitroguanidine, desnitro/guanidine (THX-II) and urea (THX-III) (Pandey et al., 2009). According to these authors, 'magic-nitro' clusters (=N-NO_2) can be converted to bacterial enzymes in a nonspecific way, which would explain in part the increase in the activity of β-glucosidase and acid phosphatase even in momentary events after the application of thiamethoxam.

It is noticeable while on one hand biochar provides increased enzymatic activity of urease and acid phosphatase, on the other hand, thiamethoxam provides precisely the reduction of the activity of such enzymes, showing that biochar little attenuates the deleterious effect of this molecule on soil microbial activity. This fact is further confirmed by the reduction of MBC, increase of qCO_2 and C-CO_2 with the application of both. These results reinforce the need for long-term studies after application of biochar on microbial activity in agricultural areas submitted to the intensive use of agrochemicals, since they have high sorption interaction with biochar, especially polar pesticides. Thus, this greater sorptive interaction could provide greater availability of access of

microorganisms to these molecules and provide long-term significant change in the community and microbial activity, whose results may alter the biogeochemical cycles of nutrients in the soil and the growth and development patterns of the plants.

4.3. NPK fertilizer in Soil Microbial Properties

The increase of qCO_2 and reduction of the MBC of Plinthosol in the presence of chemical fertilization, independent of biochar dose seems to be related to the adaptation of microorganisms to the soil environment with Biochar + NPK. These results confirm the observations of recent studies in which high doses of biochar (> 16 t ha^{-1}) provided lower biodiversity when compared to control soil (Santos, 2013), reduction of enzyme activity and microbial abundance, besides alter the microbial community structure (Huang et al., 2017). The reports justify the hypothesis above, since it was expected that, with the application of chemical fertilization, there would be higher MBC due to the greater contribution of vegetal residues.

Thus, it seems that the biochar interferes in the soil organic matter dynamics and this on the microbial activity in the presence of chemical fertilization under three main points: i) lower biodiversity and alteration of microbial community structure; ii) priming effect on organic carbon derived from plant residues as previously discussed, a fact that would reduce the effect of the higher contribution of labile carbon on plant residues on MBC; iii) presence of inhibitor substances (ethylene, phenolic compounds) of soil microbial processes in the presence of high biochar doses (Deenik, McClellana, Ueharaa, Antal, & Campbell, 2010; Spokas et al., 2010).

5. Conclusions

Microbial properties of Plinthosol showed different responses after eight years of biochar incorporation. The increase of biochar doses resulted in an increase in the production of urease and acid phosphatase enzyme, increase of qCO_2 and basal respiration and reduction of MBC. The application of biochar in larger doses than or equal to 16 t ha^{-1} resulted in elevation of qCO^2 and reduction of MBC.

The application of thiamethoxam insecticide suppressed the enzymatic activity of urease and acid phosphatase, resulting in elevation of alkaline phosphatase and reduction of basal respiration of the soil.

The application of thiamethoxam insecticide led to more significant modifications on the soil microbiota than biochar.

The application of biochar in the soil did not attenuate the negative effects of thiamethoxam on the soil microbiota.

The results of the present study suggest that the application of biochar in the soil may result after long term in significant transformations in the soil microbiota, either through the selection of microorganisms or the alteration of microbial and enzymatic activity.

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Physical Quality Indicators of an Oxisol Under Grass in the Agreste Region of Paraiba, Brazil

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Abstract

The aim of this study was to evaluate the influence of different grass cultivars on physical attributes of an Oxisol in the Agreste region of Paraíba. The experiment was set up in 2005 in experimental areas of the Center of Agricultural Sciences of the Federal University of Paraíba, Areia-PB. The experimental design adopted was that of randomized complete blocks (RCB) with 5 treatments and 4 replications, in experimental plots of 50 m^2. The treatments were the following: I. *Brachiaria decumbens* Stapf., II. *Brachiaria brizantha*, III. *Brachiaria humidicola* (Rendle) Schweickvr., IV. *Brachiaria brizantha* cv. MG5-Vitória and V. *Brachiaria ruziziensis*. In October 2018 samples of soil with disturbed and undisturbed structure were collected at the center of each experimental plot in the depth of 0.0-0.10 m for the determination of the following variables: soil porosity (total, macro and micro), bulk density, compaction degree, saturated hydraulic conductivity, field capacity, permanent wilting point, available water content, soil aeration capacity, clay dispersed in water, flocculation degree and aggregate stability index. The analysis of variance was performed and the means were compared by Tukey's tests, principal component analysis and Pearson's correlation analysis ($p < 0.05$). It is concluded that after the 13-year period, *Brachiaria brizantha* promoted improvements to the field capacity of the Oxisol. The other attributes were not physically altered. Main component analysis showed that the correlation values were more significant for the *Brachiaria brizantha* component. Pearson's correlation was significant between field capacity and soil aeration capacity.

Keywords: soil physics attributes, *Brachiaria*, aggregates stability index, bulk density, hydraulic conductivity

1. Introduction

The land usage and management system, when performed incorrectly can alter the physical attributes of the soil, promote degradation, reduce quality and cause damage to its sustainability. In general, the factors of greatest impact on the physical and structural quality of the soil are the excessive traffic of agricultural machines and input of agricultural implements, animal trampling (Gasparetto et al., 2009), predatory removal of vegetation cover (Sales et al., 2018) and surface burning of residues (Redin et al., 2011).

The physical degradation of the soil reflects directly on its structural quality, varying through different degrees of intensity. When the soil structure changes negatively, there may be a reduction in crop productivity due to changes in water retention and availability, oxygen diffusion, soil resistance to root penetration (Guimarães et al., 2014) and aggregates stability (Bonini & Alves, 2011).

Different attributes have been used to evaluate soil physical quality, such as: bulk density, macroporosity, microporosity, total porosity, aggregates stability, soil penetration resistance (Lima et al., 2014) and saturated hydraulic conductivity, for being a parameter of great importance in the water movement through the soil (Soto & Kiang, 2018).

Generally, in production systems where vegetation cover is suppressed, changes in soil physical attributes occur, reducing the sustainability of agricultural production, especially in environments with climatic irregularities. In this context, it is noted an urgent need to adopt management systems that maximize food production, without compromising the physical properties of the soil. The maintenance of the vegetation cover has been used in a quite satisfactory way in conservationist production systems, considering the physical improvements that it promotes in the soil, especially in situations of low water availability (Costa et al., 2007; Oliveira et al., 2018).

The maintenance of permanent vegetation cover with grasses favors numerous improvements to the environment, especially the reduction in erosion rate caused by surface runoff (Silva et al., 2018), improvements in water productivity and availability indexes (Braga et al., 2017) and formation of aggregates with higher stability index (Santos et al., 2012). All of this derives from the action of the root system, which, depending on the species cultivated, may show a higher or lower intensity of improvement in the physical attributes of the soil.

Thus, knowing the importance of the vegetation cover for the improvement of the physical attributes of the soil and the lack of information about which species of the *Brachiaria* genus are more efficient in this improvement, this study aimed to evaluate the influence of different cultivars of grasses of such genus on physical attributes of anOxisol in the Agreste region of Paraíba.

2. Method

2.1 Characterization of the Experimental Area

The experiment was set up in 2005 in an experimental area belonging to the Center of Agricultural Sciences of the Federal University of Paraíba, in Areia-PB. The city is located within the micro-region of Brejo and mesoregion of the Agreste of Paraíba (6°58′12″ S; 35°41′15″ W; and altitude of 620 m). In 2017 the micro-region of the Brejoparaibano was inserted in the climatic domain of the Brazilian Semiarid region (Dry lands), due to the irregularities in the local climate, caused by poor rainfall distribution and the increase in the annual average temperature (SUDENE, 2017).

According to Köppen classification, the predominant climate in the municipality is As'-tropical, hot and humid, with rains during autumn and winter and mean annual precipitation of 1400 mm (Almeida et al., 2014), with 62.0% occuring between April and July. The annual mean temperature is 24.5 ºC (75.2 °F) and the relative humidity of the air ranges in average of RH = 80.0% (Carmo et al., 2012). The soil of the experimental area is classified as Dystrophic Yellow Oxisol (Santos et al., 2018), with sandy-clay-loam texture. The physical and chemical characterization of the soil can be found in Tables 1 and 2, respectively.

Table 1. Physical soil characterization of the experimental area for the layer of (0.0-0.10 m)

Sand					Silt	Clay	Textural classification*	S/C	PD
VC	C	M	F	VF					
---------- g kg^{-1} ----------									g cm^{-3}
43	199	196	112	24	69	357	Sandy clay loam	0.194	2.58

Note. VC = Very coarse; C = Coarse; M = Medium; F = Fine; VF = Very Fine; SC = Silt/Clay relation; PD = Particle Density; * = According soil taxonomy.

Table 2. Soil chemical characterization of the experimental area for the layer of (0.0-0.10 m)

pH	Ca^{2+}	Mg^{2+}	Al^{3+}	$H+Al^{3+}$	SB	CEC	Na^{+}	SOM	P	K^{+}
$H_2O_{(1:2.5)}$	----------	----------	----------	$cmol_c dm^{-3}$	----------	----------	----------	g kg^{-1}	---- mg dm^{-3} ----	
5.3	1.89	1.43	0.30	8.33	3.46	11.79	0.06	49.52	1.82	28.37

Note. pH = Hydrogen potential; Ca^{2+} = Calcium; Mg^{2+} = Magnesium; Al^{3+} = Exchangeable aluminum; Na^{+} = Sodium; SB = Sum of bases; CEC = Cation exchange capacity; SOM = Soil organic matter; P = Phosphorus; K^{+} = Potassium.

The grasses were implanted in 2005 in experimental plots with dimensions of 10 × 5 m, adding up to 50 m^2 of useful area (Figure 1). Plotswere 1 meter apart and blocks were 2 meters apart.

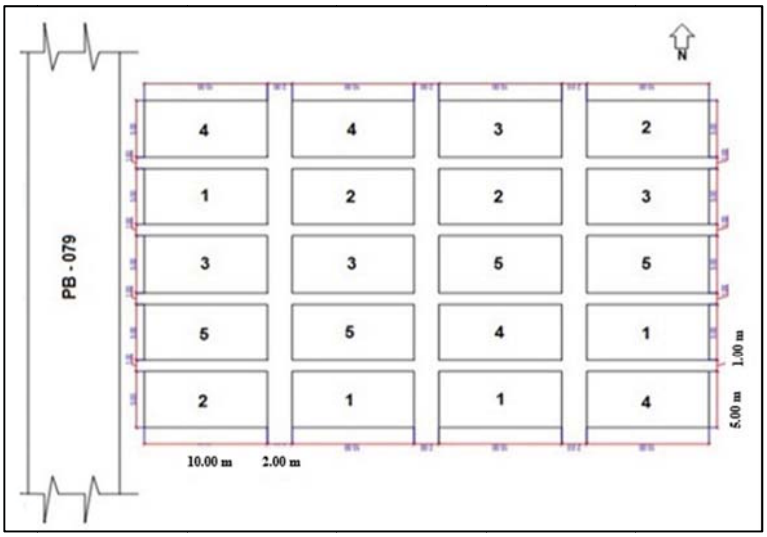

Figure 1. Detailing of experimental plots with evaluated treatments

Note. 1 = *Brachiariadecumbens* Stapf., 2 = *Brachiaria brizantha*, 3 = *Brachiaria humidicola* (Rendle) Schweickvr., 4 = *Brachiaria brizantha MG5* and 5 = *Brachiaria ruziziensis*.

In the initial year of the experiment, 553 kg ha^{-1} of NPK mixture (60-80-45) was fertilized in the experimental plots with the following nutrient sources: ammonium sulfate, triple superphosphate and potassium chloride. The fertilizer was sowed in the beginning of the rain season, after cutting and standardizing the grasses, a practice that was repeated until 2010 (Almeida et al., 2014). Later, the experiment was conducted annually, but without fertilization.

2.2 Experimental Design

The experimental design adopted was that of randomized complete blocks (RCB) with 5 treatments and 4 replications (5 × 4). The assessed treatments were the following: T1-*Brachiaria decumbens* Stapf., T2-*Brachiaria brizantha*, T3-*Brachiaria humidicola* (Rendle) Schweickvr., T4-*Brachiaria brizantha* cv. MG5-Vitória and T5-*Brachiaria ruziziensis*.

2.3 Physical Attributes Analyzed

In 2018, soil samples with disturbed and undisturbed structure were collected with the aid of the Uhland-type sampler in volumetric rings of 98.17 cm^{-3} in the layer of 0.0-0.10 m. The samples with preserved structure were collected at the center of each experimental plot. They were then taken to the Laboratory of Soil Physical Analysis of the Center of Agricultural Sciences of the Federal University of Paraíba to determine the following attributes: Total porosity (TP), microporosity and macroporosity (Mi & Ma), soil aeration capacity (SAC), bulk density (BD), compaction degree (CD), field capacity (θFD), permanent wilting point (θPWP), available water content (θAWC), saturated hydraulic conductivity (Kθ),weighted meansdiameter of wet and dry aggregates (WMDda ad WMDwa), aggregate stability index (ASI), clay dispersed in water (CDW) and flocculation degree (FD), as described in Teixeira et al. (2017).

Total porosity was calculated through the humidity corresponding to the saturation volume, as described in Equation 1.

$$TP\ (m^3\ m^{-3}) = Mssat - Mds/Vt \quad (1)$$

Where, Mssat is the mass of saturated soil (kg); Mds is the mass of dry soil at 105 °C (kg) and Vt is the total volume of the soil sample in the cylinder (cm^{-3}).

Microporosity (Mi-$m^3\ m^{-3}$), was calculated by applying the matrix potential of 6 kPa in the tension table in saturated soil samples for the minimum period of 48 hours. Macroporosity (Ma—$m^3\ m^{-3}$), was obtained through the difference between TP and Mi, according to Equations 2 and 3:

$$Mi\ (m^3\ m^{-3}) = Ms6kPa - Mds/Vt \quad (2)$$

$$Ma\ (m^3\ m^{-3}) = TP - Mi \quad (3)$$

Where, Mi is the microporosity of the soil; Ms6kPa is the humidity of the soil stabilized in the tension table; Mds is the mass of dry soil 105 °C; VT is the total volume of the cylinder; TP is the total porosity and Ma is the macroporosity of the soil.

The Soil Aeration Capacity variable (SAC), was determined as described in Equation 4:

$$SAC\ (m^3\ m^{-3}) = PT - \theta FC/TP \quad (4)$$

Where, TP is the total porosity of the soil ($m^3\ m^{-3}$); θFC is the volumetric content of water in the field capacity ($m^3\ m^{-3}$) determined by the Richards extractor with an applied tension of 10 kPa.

Bulk density (BD) was determined through the ratio between dry soil mass/volume of soil sample in the cylinder Blake and Hartge (1986). To obtain the mass of dry soil the sample was placed in a heating chamber with temperature of 105 °C, for a 48 hours period until reaching a stable weight.

Compaction degree (CD) was determined according to the methodology proposed by Suzuki et al. (2007), in order to define the percentage of soil compaction in relation to its maximum (Equation 5). 1.85 g cm^{-3} was used as the maximum restrictive value of bulkdensity for medium texture Oxisol (Beutler et al., 2005).

$$CD = BD/1.85 \times 100 \quad (5)$$

Where, CD is the compaction degree (%) and BD is the bulk density of the analyzed layer of the soil (0.0-0.10 m).

The matrix potentials: 10 kPa and 1500 kPa were used to determine the moisture in the field capacity (θ_{FC}) and the permanent wilting point (θPWP), as described in Teixeira (2017). Through the ratio between θFC-θPWP, the available water content range was obtained-θAWC ($m^3\ m^{-3}$). The saturated hydraulic conductivity (Kθ-cm h^{-1}), was determined in undisturbed soil samples, with the aid of the constant charge permeameter and calculated with the following Equation 6:

$$K\theta = (Q \times L)/(A \times H \times T) \quad (6)$$

Where, Kθ is the saturated hydraulic conductivity; Q is the water volume percolated and colected in a measuring cylinder (mL^{-1}); L is the height of the soil block in (cm); A is the area of the cylinder in (cm^2); H is the height of the soil block + water sheet (cm) and T is the time in hours of collection of percolated water volume.

The determination of the weighted meansdiameter of soil wet aggregates (WMDwa) and weighted meansdiameter of soil dry aggregates (WMDwa) followed the methodology proposed by Kemper and Chepil (1965), with changes proposed by Carpenedo and Mielniczuk (1990) and Silva and Mielniczuk (1997), where the principle is to evaluate the resistance that the aggregates offer when submitted to oscillations in sieves in water. The aggregate stability index (ASI) was estimated by the ratio between the WMDwa/WMDda.

The clay dispersed in water was obtained by the means of soil granulometric analysis, according to the method of Bouyoucos densimeter method (Teixeira et al., 2017) however, without using chemical dispersant. For the total clay the same previous procedure was utilized, but using sodium hydroxide (NaOH—1N) as dispersant agent. The flocculation degree was obtained as described in Equation 7:

$$D_{FLO} = (Clay - Clay_{H_2O})/Clay \times 1000 \quad (7)$$

Where, D_{FLO} is the flocculation degree (g kg^{-1}), Clay is the clay content dispersed in sodium hydroxide—NaOH (g kg^{-1}), and $Clay_{H2O}$ is the clay content dispersed in water (g kg^{-1}). The silt clay relation (S/C), was determined by the ratio of silt content (g kg^{-1}) and the total clay (g kg^{-1}) in the soil sample.

2.4 Statistical Analysis of Data

The analysis of variance (ANAVA) was performed and the means were compared by Tukey's test ($p < 0.05$), using statistical software *R* (R Devedolpemt, 2013). The Pearson correlation analysis (r) and the principal components analysis (PCA) were performed in order to evaluate the spatial dependence between the analyzed variables ($p < 0.05$).

3. Results

There was no significant statistical variation for total porosity (TP) ($p < 0.05$) level between the assessed treatments (Table 3), however the values varied from 0.44 $m^3\ m^{-3}$ in the *Brachiaria ruziziensis* treatment to 0.49 $m^3\ m^{-3}$ in the *Brachiaria decumbens* Stapf. treatment. Other variables such as macroporosity, microporosity, soil aeration capacity, bulk density and degree of compaction were not modified throughout different *Brachiaria cultivars*.

Table 3. Mean values of soil porosities, soil aeration capacity, bulk density and compaction degree (CD) of an Oxisol under grasses in the Agreste region of Paraíba (0.0-0.10 m)

Treatments	TP	Ma	Mi	SAC	BD	CD
	---------- $m^3\ m^{-3}$ ----------				$g\ cm^{-3}$	%
Brachiaria decumbens	0.49 a	0.16 a	0.33 a	0.29 a	1.18 a	63.8a
Brachiaria brizantha	0.46 a	0.13 a	0.33 a	0.21 a	1.20 a	65.0 a
Brachiaria humidicola	0.46 a	0.14 a	0.32 a	0.25 a	1.19 a	64.2 a
B. brizantha cv. MG5	0.48 a	0.16 a	0.32 a	0.27 a	1.18 a	64.0 a
Brachiaria ruziziensis	0.44 a	0.13 a	0.31 a	0.23 a	1.20 a	64.6 a
CV (%)	5.0	11.9	4.0	11.5	4.4	4.4

Note. TP = Total porosity; Ma = Macroporosity; Mi = Microporosity; SAC = Soil aeration capacity; BD = Bulk density; CD = Compaction degree; CV = Coefficient of variation. Mean values followed by the same letter in the column do not differ by Tukey test ($p < 0.05$).

As for macroporosity (Ma), the values varied between 0.13 and 0.16 $m^3\ m^{-3}$, with a higher average value in *Brachiaria decumbens* Stapf. and *B. brizantha* cv. MG5 treatments. Microporosity (Mi) varied from 0.31 to 0.33 $m^3\ m^{-3}$, with a lower value in the *Brachiaria ruziziensis* treatment (Table 3).

It can be seen in Table 3 that the highest soil aeration capacity (SAC) value was found in the *Brachiaria decumbens* Stapf., treatment, but the SAC was not influenced by the different cultivars of *Brachiari*a. The bulk density (BD) ranged from 1.18 to 1.20 g cm^{-3}, however with no significant statistical variation between the assessed treatments (Table 3). The compaction degree of the soil (CD) did not vary among the evaluated treatments ($p < 0.05$), as verified in Table 3.

Table 4 shows the mean values of field capacity (θFC), permanent wilting point (θPWP), available water content (θAWC) and saturated hydraulic conductivity (Kθ). It was verified that a significant statistical variation for the θFC ($p < 0.05$). The values ranged from 0.205 to 0.243 $m^3\ m^{-3}$, with a better result for the *Brachiaria brizantha* treatment (0.243 $m^3\ m^{-3}$).

Table 4. Field capacity, permanent wilting point, available water content and saturated hydraulic conductivity of an Oxisol under grasses in the Agreste region of Paraíba (0.0-0.10 m)

Treatments	θFC	θPWP	θAWC	Kθ
	---------- $m^3\ m^{-3}$ ----------			$cm\ h^{-1}$
Brachiaria decumbens	0.205 b	0.144 a	0.060 a	38.3 a
Brachiaria brizantha	0.243 a	0.162 a	0.081 a	29.4 a
Brachiaria humidicola	0.211 ab	0.144 a	0.067 a	40.7 a
B. brizantha cv. MG5	0.206 ab	0.142 a	0.064 a	34.2 a
Brachiaria ruziziensis	0.207 ab	0.149 a	0.058 a	37.1 a
CV (%)	5.5	7.7	12.4	43.6

Note. θFC = Field capacity; θPWP = Permanent wilting point; θAWC = Available water content; Kθ = Saturated hydraulic conductivity; CV = Coefficient of variation. Mean values followed by the same letter in the column do not differ by Tukey test ($p < 0.05$).

Regarding saturated hydraulic conductivity (Kθ), it can be observed in Table 4, that there was no significant statistical variation between the treatments ($p < 0.05$), however the Kθ in the *Brachiaria humidicola* treatment was 40.7 cm h^{-1}, higher than the others evaluated treatments, due to two main factors, the low degree of soil compaction and an increase in the total pore volume.

Table 5 shows the average values of mean weighted diameter of wet and dry aggregates (WMDwa and WMDda), aggregate stability index (ASI), clay dispersed in water (CDW) and flocculation degree (FD). It was verified that there was no significant statistical variation among the treatments evaluated ($p < 0.05$).

Table 5. Mean weighted diameter, aggregates stability index, clay dispersed in water and flocculation degree of an Oxisol under grasses in the Agreste region of Paraíba (0.0-0.10 m)

Treatments	WMDwa	WMDda	ASI	CDW	FD
	---------- mm ----------			---------- g kg^{-1} ----------	
Brachiaria decumbens	2.44 a	3.12 a	0.782 a	19.25 a	942 a
Brachiaria brizantha	3.13 a	3.52 a	0.889 a	3.25 a	992 a
Brachiaria humidicola	2.75 a	3.21 a	0.856 a	22.75 a	934 a
B. brizantha cv. MG5	2.60 a	3.70 a	0.702 a	19.00 a	944 a
Brachiaria ruziziensis	2.74 a	3.36 a	0.815 a	6.50 a	982 a
CV (%)	13.3	13.9	9.6	65.3	2.8

Note. WMDwa = Weighted meansdiameter of wet aggregates; WMDda = Weighted meansdiameter of dry aggregates; ASI = Aggregates stability index; CDW = Clay dispersed in water; FD = Flocculation degree; CV = Coefficient of variation. Mean values followed by the same letter in the column do not differ by Tukey test ($p < 0.05$).

For the clay dispersed in water (CDW), it was verified that there was a reduction after nine years from the start of the experiment (Table 5), reflecting in high values for the flocculation degree of the soil, as for example 992 g kg^{-1} in the *Brachiaria brizantha* treatment. This action shows that the use of permanent grasses as soil cover, favors the formation of more stable aggregates in the soil, verified through the mean values of FD and CDW (Table 5).

Table 6 reveals the correlation values of the principal components for physical attributes of the Oxisol evaluated in the experiment.

Table 6. Correlation values of the principal componentsanalysis (PCA) for physical attributes of an Oxisol under grasses in the Agreste region of Paraíba (0.0-0.10 m)

Components of Variance	Principal Components			
	1	2	3	4
Autovalues (%)	13.740	4.012	3.237	2.011
Proportions (%)	50.738	17.444	14.073	8.745
Accumulated (%)	59.73	77.18	91.25	100.00
Variables	---------- Correlation with principal components ----------			
TP	-0.180	-0.108	0.367	0.182
Ma	-0.144	-0.260	0.354	0.131
Mi	-0.012	0.065	0.518	0.232
BD	0.264	0.027	-0.059	-0.102
Kθ	-0.191	-0.010	-0.357	0.204
CD	0.264	0.027	-0.059	-0.102
WMDda	0.081	0.277	0.275	-0.419
WMDwa	0.262	0.100	0.011	0.085
ASI	0.219	-0.061	-0.221	0.285
θFC	0.243	0.005	0.149	0.175
θPWP	0.257	-0.111	0.108	0.047
θAWC	0.208	0.114	0.246	0.277
SAC	-0.257	-0.065	0.146	0.025
Sand	-0.259	0.125	-0.033	-0.045
Silt	0.204	0.196	-0.135	0.325
Clay	0.235	-0.219	0.101	-0.066
VCS	-0.180	0.346	-0.121	0.104
CS	-0.236	0.129	0.096	-0.260
MS	-0.193	-0.321	0.130	0.090
FS	0.036	-0.469	-0.076	0.200
VFS	0.100	0.427	0.000	0.253
CDW	-0.231	0.159	0.004	0.282
FD	0.235	-0.149	-0.018	-0.270

Note. TP = Total porosity, Ma = Macroporosity, Mi = Microporosity, BD = Bulk density, Kθ = Saturated hydraulic conductivity, CD = Compaction degree, WMDwa = Weighted meansdiameter of wet aggregates; WMDda = Weighted meansdiameter of dry aggregates, ASI = Aggregates stability index, θFC= Field capacity, θPWP = Permanent wilting point, θAWC = Available water content, SAC = Soil aeration capacity, VCS = Very coarse sand, CS = Coarse sand, MS = Medium sand, FS = Fine sand, VFS = Very fine sand, CDW = Clay dispersed in water and FD = Flocculation degree.

It can be observed that 77.1% of the coefficient of variation was explained by quadrants 1 and 2 for most assessed attributes. The *Brachiaria brizantha* and *Brachiaria ruziziensis* components were the most influential on the correlation values of the analyzed variables (Figure 2).

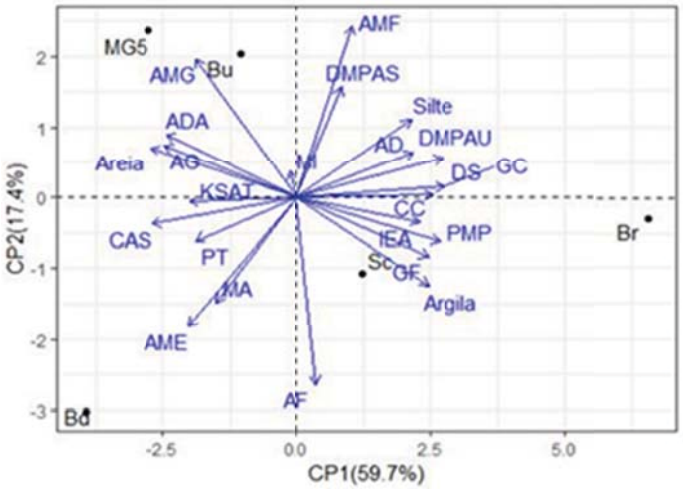

Figure 2. Principal components analysis for physical attributes of an Oxisol under grasses in the Agreste region of Paraíba (0.0-0.10 m)

Note. CP = Principal component, Bd = *Brachiaria decumbens*, Bu = *Brachiaria humidicola*, Br = *Brachiaria brizantha*, Sc = *Brachiaria ruziziensis*, MG5 = *Brachiaria brizantha* MG5 cv. Vitória. PT = Total porosity, Ma = Macroporosity, Mi = Microporosity, DS = Bulk density, K_{SAT} = Saturated hydraulic conductivity, GC = Compaction degree, DMPAs = Mean weighted diameter of dry aggregates, DMPAu = Mean weighted diameter of wet aggregates, IEA = Aggregates stability index, CC = Field capacity, PMP = Permanent wilting point, AD = Available water content, CAS = Soil aeration capacity, AMG = Very coarse sand, AG = Coarse sand, AME = Medium sand, AF = Fine sand, AMF = Very fine sand, ADA = Clay dispersed in water and GF = Flocculation degree.

The main variables influenced by the *Brachiaria brizantha* MG5 cv. Vitória and *Brachiaria humidicola* components were the clay dispersed in water and the coarse fractions of Sand (Coarse and very coarse sand). For the *Brachiaria ruziziensis* component, the most important attributes were θFC, θPWP, ASI and FD. This result shows that *B. ruziziensis*, through the action of its aggressive root system, favors improvements in soil physical attributes used as indicators of soil quality, especially those related to structure such as FD and ASI.

Brachiaria brizantha was the most influential component on the variability of the soil attributes verified in the principal component's analysis (Figure 2). The BD, CD, Wet and WMDwa and WMDdawere the most influenced attributes. These results demonstrate the efficacy of this species in soil management and conservation. The attribute that was less influenced by the different grasses treatments was microporosity, since it is sensitive to variations in the texture of the soil and not to management practices.

Table 7 shows the correlation values for the physical attributes of Oxisol under different grasses cultivars. The criteria for interpretation of the Pearson correlation coefficient (r) used in this study followed the one proposed by (Oliveira et al., 2018), being: $0.7 \leq r \leq 1.0$ for strong correlation; $0.4 \leq r < 0.6$ for moderate correlation; $0.1 \leq r < 0.3$ for weak correlation and $0.0 \leq r < 0.1$ for null correlation.

Table 7. Pearson correlation (r) for physical attributes of an Oxisol under grasses in the Agreste region of Paraíba (0.0-0.10 m)

	TP	MA	MI	BD	CD	Kθ	WMDda	WMDwa	ASI	θFC	θ_{PWP}	θ_{AWC}	SAC	CDW	FD
TP	1	0.66**	0.65**	-	-	-	-	-	-	-	-	-	0.85***	-	-
MA		1	-	-0.55*	-0.55*	-	-	-	-	-	-	-	0.65**	-	-
MI			1	-	-	-	-	-	-	-	-	-	-	-	-
BD				1	1***	-	-	-	-	0.45*	0.55*	-	-0.52*	-	-
CD					1	-	-	-	-	0.45*	0.55*	-	-0.52*	-	-
Kθ						1	-	-	0.48*	-	-	-	-	-	-
WMDda							1	0.70***	-	-	-	-	-	-	-
WMDwa								1	0.48*	0.49*	-	0.59**	-	-	-
ASI									1	-	-	-	0.47*	0.46*	-
θ_{FC}										1	0.83***	0.72***	-0.71***	-	-
θ_{PWP}											1	-	-0.57*	-	-
θ_{AWC}												1	-0.57**	-	-
SAC													1	-	-
CDW														1	1***
FD															1

Note. * = Meaningful at ($p < 0.05$); ** = Meaningful at ($p < 0.01$); *** = Meaningful at ($p < 0.001$); (-) = Not meaningful; WMDwa = Weighted meansdiameter of wet aggregates; WMDda = Weighted meansdiameter of dry aggregates; ASI = Aggregates stability index; CDW = Clay dispersed in water; FD = Flocculation degree; θ_{FC} = Field capacity; θ_{PWP} = Permanent wilting point; θ_{AWC} = Available water content; K_{SAT} = Saturated hydraulic conductivity; PT = Total porosity; Ma = Macroporosity; Mi = Microporosity; SAC = Soil aeration capacity; BD = Bulk density; CD = Compaction degree.

The most significant values of positive correlation were verified between CDW and FD (r = 1.0), being considered strong, since they are inherently linked attributes. The most significant negative correlation was found between SAC and θFC (r = -0.71). Another strong correlation was found between θFCand θPWP (r = 0.83). The θAWC attribute presented r = 0.72 with θFC. However, it can be observed that θAWC did not present a significant correlation value with θPWP, showing that it is an attribute which is not dependent on high matrix potentials. The correlation between PT and SAC was r = 0.85, which is considered strong. The SAC showed moderate correlation with Ma (r = 0.85).

One last strong correlation was verified between WMDwa and WMDda with r = 0.70, this means that the increase in the mean weighted diameter of dry aggregates leads to increase in the mean weighted diameter of wet aggregates and the aggregates stability index. It is also important to highlight the moderate correlation values found between θPWP, CD and BD with r = 0.55.SAC showed strong negative correlation with θFC r = -0.71.

There was a moderate negative correlation between the physical attributes of Ma, BD and CD r = -0.55. Other values of moderate negative correlation were verified between BD, CD and SAC (r = -0.52). There was a strong and perfectly positive correlation (r = 1.0) between CD and BD.

4. Discussion

The permanence of total porosity (TP) values above 0.44 m^3 m^{-3} in all assessed treatments is related to the keeping of vegetation cover and to the development of the grass root system, which grows favoring the formation of porous spaces in the soil. Other factors such as reduction of soil mobilization and constant deposition of organic matter on the surface favor the increase in the pore volume of the soil (Melo et al., 2016; Sales et al., 2018), due to the action of microorganisms, which bind the particles together and contribute to the formation of pores between the aggregates of the soil.

Another perceived factor concerns the critical macroporosity values of the soil, as it was observed that all values were above the critical limit, which according to Reichert et al. (2007) is 0.10 m^3 m^{-3}. Unlike macroporosity that fits into the structural porosity category, the microporosity is classified as a component of soil textural porosity and is therefore little influenced by soil management practices (Ramos et al., 2014).

The soil aeration capacity (SAC) was not influenced by the different cultivars of *Brachiari*a. However, the values ranged from 0.21 to 0.29 m^3 m^{-3}, being above the critical limit used as standard for most soils and crops, which according to Tormena et al. (2002) is 0.10 m^3 m^{-3} (10%). When SAC is greater than 0.34 m^3 m^{-3}, some

factors start to affect the development of crops, mainly due to problems related to water retention in the soil (Silva et al., 2018).

The lowest bulk density (BD) value was verified in the *Brachiaria decumbens* Stapf. and *B. brizantha* cv. MG5 treatments = 1.18 g cm^{-3}, well below the critical range, which is 1.30 to 1.40 g cm^{-3} for soils of clayey texture (Reinert et al. 2008). When BD values area above the critical limit, some abnormalities will affect the development of the plants, due to the reduction in aeration capacity and the low power of roots penetration in compacted layers of the soil (Reinert et al., 2008). The BD values found in this study were much lower than those verified by Tormena et al. (2002), while evaluating the influence of BD and TP on the development of *Brachiaria brizantha* on Yellow Oxisol, with values between 1.43 and 1.57 g cm^{-3}.

The compaction degree of the soil (CD) did not vary among the evaluated treatments ($p < 0.05$). However, the values obtained remained between 63.8 and 65.0%, a trend verified as a function of the BD increase, since the CD is closely related to BD. While working with CD evaluation in Oxisol and Argisol, Suzuki et al. (2007) found that the restrictive CD for most crops is 75.0%, well above the maximum value verified in this study, which was 65.0% in the *Brachiaria brizantha* treatment.

It is observed that there is a direct relationship between the volume of water in the field capacity and the degree of soil compaction in the *Brachiaria brizantha* treatment. The 65.0% elevation in the CD favored not only the water volume in the θFC, but also the water volumes in the θPWP and the θAWC. Several factors are involved in the increase of θFC in the *Brachiaria brizantha* treatment when compared to the other treatments, among them are the greater presence of vegetal cover in surface, greater volume of micropores and reduction in SAC.

However, the accumulation of organic matter on the surface by the input of dry biomass from the grass *Brachiaria decumbens* Stapf. may have favored the increase in the water volume stored under θFC (Silva et al., 2019). Therefore, the maintenance of vegetal residues on the surface reduces the loss of organic carbon through the formation of aggregates and, among other benefits it leads to the preservation of soil water content (Carmo et al., 2012).

Aggregate stability index (ASI) values shown in table 5 were higher than those verified by Almeida et al. (2014), in a work done on the same experimental area, where it was evaluated the effect of fertilization on *caespitosa* and *decumbens* Poaceae over soil aggregation. They found ASI values of 0.790 in 2010 for the *Brachiaria brizantha* treatment. Nine years later the ASI found for the same treatment was 0.889, an increase of 11.0%. Brandão and Silva (2012) working with formation and stabilization of aggregates by the *Brachiaria* root system, observed that the use of grasses increased the WMDwa and the ASI of the soil. They concluded that this process ocurred due to the release of exudates and the greater density of grasses' roots.

The continuous supply of organic matter by grasses or root excretions, whose products are composed of organic molecules in several stages of decomposition, act as agents for formation and stabilization of aggregates, providing improvements in soil structure (Bonini & Alves, 2011).

The aggregation arising from the union of particles (clay-ion-organic matter, sand and silt) in environments under conservationist management reduces the impact of rain drops on the clay dispersion (Sales et al., 2010). It is then verified the great importance in managing the soil, permanently keeping the vegetation cover on the surface.

On the coefficient of variation (CV) of the statistical analysis, it is noted that the majority of the treatments presented low CV values, which should be equal to or less than 10.0%. Taking into account the classification proposed by Oliveira et al. (2018), the attributes that presented the lowest CV were PT, Mi, BD and CD (Table 3), θFCe θPWP (Table 4), ASI and FD (Table 5), all with values ranging from 4 to 9.6%, with the lowest CV being 2.2% for the FD attribute. These values indicate that they are attributes that can be used as indicators of soil quality, due to the high reliability presented (Cavallini et al., 2010).

In relation to Pearson's correlation, the higher the content of clay dispersed in water, the lower the flocculation degree of the soil. As the CDW values were low for the conditions to which the soil was evaluated, it favored an increase in the FD of the soil.

The strong positive correlation found between θFC and θPWP it indicates that both attributes have a close relationship with the soil matrix, directly influencing the water available to the plants. The SAC showed moderate correlation with Macroporosity, therefore, the increase of macroporosity contributes with the increase of the aeration capacity of the soil. As the pore classes of the soil are interconnected, the modification of one can positively or negatively affect the variation of the other. Specially the structural pores, because they are more sensitive to soil management practices (Klein et al., 2008).

The correlation verified between θPWP, CD and BD, means that the higher the compaction degree of the soil, the greater the water potential in the θPWP, due to the reduction of larger pore spaces such as Ma. Rodrigues et al. (2015) verified a negative correlation between θPWP and BD, with an average value of r = -1.0 for a medium texture Red Oxisol. SAC showed strong negative correlation with θ_{FC}, this shows that the larger the volume of water retained as θFC, the smaller are the spaces occupied by air.

There was a moderate negative correlation between the physical attributes of Ma, BD and CD, therefore, these attributes are very sensitive to changes in soil management. Because, the larger the BD, the smaller the volume of Ma in the soil. Sampietro et al. (2015) verified the reduction of Ma of the soil with the increase of BD. This increase in BD can lead to changes in the physical and hydraulic attributes of the soil, especially the retention and availability of water to the plants. Montanari et al. (2013) verified that the increase in BD promoted a decrease in the production of bean pods cultivated on Oxisol in the state of Mato Grosso do Sul. In their interpretation this fact is due to the increase in the compaction degree and reduction of soil porosity. Because unlike grasses, legumes have a less aggressive root system, reducing to a certain extent the volume of soil explored and performing lower absorption of water and nutrients (Melo et al., 2016).

The correlation verified between BD, CD and SAC, show it SAC is an attribute directly related to the increase of density and, that it can be used as an indicator of soil quality, for indirectly predicting the compaction degree of the evaluated soil layer.

5. Conclusion

After the 13 years period, it was concluded that *Brachiaria brizantha*, promoted improvements to the field capacity in the Oxisol. The other attributes were not significantly modified after the same time lapse. Principal component analysis showed that the correlation values were more significant for the *Brachiaria brizantha* component. Pearson's correlation was more significant between field capacity and soil aeration capacity.

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Post-Harvest Conservation of Baby Corn Under Controlled Atmosphere and Refrigeration

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Abstract

The baby corn has been gaining ground in the market and arousing interest of producers. However, there is a barrier in its production chain due to the lack of scientific knowledge in the harvest and post-harvest strategies. Therefore, the objective of this study was to evaluate the changes in the physicochemical characteristics of the baby corn stored at different temperatures and under controlled atmosphere. The studies were performed at UNIMONTES with the 'AG 1051' baby corn spikletes. Two tests were performed, one considering the spikelets in the straw and the other with the husked spikelets. The tests were carried out under CRD, in a 2 × 2 × 6 factorial scheme, that is two storage temperatures (16 and 25 °C), two controlled atmosphere conditions (with and without PVC plastic wrap) and six evaluation periods (0, 3, 6, 9, 12 and 15 days after harvest) with four replications. The quality characteristics of the spikelets were analyzed in the post-harvest. the post-harvest quality preservation of baby corn in the straw and the husked ones was affected by temperature, controlled atmosphere and evaluation period. The best storage condition to maintain the main quality characteristics of the spikelets at post-harvest was observed at the temperature of 16 ºC with controlled atmosphere use. For the spikelets preserved with the presence of straw, the maximum storage time for maintenance of post-harvest characteristics was four days, and for spikelets stored without straw, the maximum storage time was two days and 12 hours, both at refrigerated temperature (16 °C) and under controlled atmosphere.

Keywords: storage, quality, *Zea mays*

1. Introduction

Baby corn is the corn spikelet (*Zea mays* L.), harvested in the early stage of development, prior to fertilization (Rattanachai & Kanlayanarat, 2015). Considered a refined product, with crispy texture, slightly sweet flavor and delicate appearance, can be found *in natura* or in the form of canned acidified food (Lima, Melo, Oliveira, Tolentino, & Branco, 2015). Due to its diversified uses, this vegetable has gained ground in the market and has aroused producer's interest (Asaduzzaman et al., 2014). However, their production is limited due to the lack of scientific knowledge in the phytotechnical adjustments and the harvest and post-harvest strategies (Rodrigues, Silva, & Mori, 2004).

The baby corn cycle is considered short, ranging around 45 to 70 days (Brandão, 2015). Its harvest extends for several days after its beginning, which generates different harvested volumes. Thus, in order to obtain the required volume for transport to the agroindustries, it is occasionally necessary to store the spikelets harvested previously.

However, this vegetable presents a short post-harvest life, due to its active metabolism, and the shelf life of the product varies inversely with the respiration rate, causing reduction in water content, weight loss, changes in aroma, flavor and darkening of spikelets, making them undesirable regarding quality and appearance of the product (Bakry, El-Shorbagy, El-Desuki, El-Behairy, & Ibrahim, 2015; Saltveit, 2016).

Aiming to develop the best strategy of storage of these horticultural crops, studies are necessary to guarantee the best yield and less waste of them. Some treatments, associated to refrigeration and controlled atmosphere storage, have shown good results in delaying senescence and prolonging life during storage (Vani, Rajasekhar, & Reddy, 2013).

Thus, the present study had as objective to evaluate the changes in physicochemical characteristics of the baby corn, under conditions of storage at different temperatures and under the use of the controlled atmosphere.

2. Materials and Methods

The study was conducted at the State University of Montes Claros (Unimontes), Campus Janaúba-MG, in 2016. The analyzes were carried out at the Technology Laboratory for the Processing of Vegetable Origin Products of Unimontes. Baby corn spikelets of AG 1051 cultivar were used, marketed by the company Agroceres. The baby corn cultivation was carried out in the experimental area of Plant Science located in the same University, at the coordinates 15°47′18″ South latitude and 43°18′18″ West longitude, with an altitude of 515 meters.

The research consisted of two concomitant experiments, in the experiment I was carried out the physicochemical quality assessment of the conserved baby corn spikelets in the straw, and the experiment II was carried out in a similar way, but with husked spikelets storage.

The treatments were distributed under a completely randomized design (CRD), in a 2 × 2 × 6 factorial scheme, and four replications for both experiments. The treatments consisted of two storage temperatures, 16 °C (refrigerated) and 25 °C (room temperature), in two conditions, being, controlled atmosphere with PVC plastic wrap, presenting 20 μm thickness and self-adhesive and without cover, evaluated in six different periods of post-harvest comprising 0, 3, 6, 9, 12 and 15 days after harvest.

Harvesting of spikelets was performed in the morning. The samples were placed in plastic boxes and sent to the laboratory, where they were washed under running tap water and sanitized with a sodium hypochlorite solution containing 100 mg L^{-1} for 15 minutes and then placed on paper towels to dry at room temperature. For experiment I were selected spikelets inside the straw, standardizing their size and diameter, to compose the treatments. In experiment II, the spikelets were husked and those that met the requirements of 8 to 12 cm in length (commercial standard) were selected, then washed and sanitized once more with a sodium hypochlorite solution of 20 mg L^{-1} for 15 minutes, after this procedure, the samples were placed on paper towel until the total drying at room temperature.

In both experiments, the following physicochemical characteristics, related to the quality of baby corn post-harvest, were evaluated for each time period considered: Fresh Matter Loss (FML); Titratable Acidity (TA); Hydrogen-ion potential (pH); Soluble Solids (SS); and the ratio of soluble solids and titratable acidity (SS/TA) of spikelets.

The FML was measured in electronic balance accurate to 0.001 g, and the results expressed as a percentage. A TA was determined by neutralization titration, which consists of diluting 10 mL of spikelet juice, triturated with the aid of a Mallory Robot® UP 150w mixer in 90 mL of distilled water, using 0.1 % phenolphthalein solution as indicator and titration with 0.1 N NaOH solution. The obtained results were expressed as percent of malic acid. To determine the pH was used a mPA210 pH meter from Ms Tecnopon®, calibrated using pH 4 and 7 buffer solutions. The SS were determined by refractometry using HI 96801 digital refractometer by HANNA® mark and the results expressed in °Brix. The SS/TA ratio was determined by the quotient between soluble solids and titratable acidity (Instituto Adolfo Lutz, 1985).

The variables were submitted to the normality test of Lilliefors and the Bartlett test to verify the normality of the data and homogeneity of the variances, respectively. After confirming these assumptions, the variables were submitted to an analysis of variance ($p < 0.05$) to verify the existence of interactions between the factors studied in both experiments. In the affirmative of the interactions, the qualitative factors (temperature and packaging) were compared by the F test ($p < 0.05$) while the quantitative factor (storage times) were studied by regression. The regression models were adjusted according to their significance ($p < 0.05$) and to the explanatory power of the biological effect in it. Statistical analyzes were performed with the aid of statistical software GENES (Cruz, 2016).

3. Results and Discussion

The experimental coefficient of variation (CV%) allows comparisons between variables of different natures and provides an idea of the accuracy of the data. At first it is considered that the lower the CV%, the more homogeneous the data and the greater the experimental precision. In field experiments when the CV% is lower than 10% is considered low, between 20 to 30% medium and when over 30% considered high (Pimentel-Gomes, 2000). In general, it is observed in this study that the experimental precision, evaluated by CV%, was considered low for the variable pH, indicating high precision and classified as average for FML, TA, SS e SS/TA, which is a good precision in both experimental tests (I and II) (Tables 1 and 3), judging correct the inferences raised in the experiment.

3.1 Experiment I

It was observed lower fresh matter loss when the spikelets were kept at a lower temperature (16 °C) and stored with PVC platic wrap package (Table 1), verifying that at 15 days after harvest, there is greater conservation of spikelets when stored in this condition (Table 2).

According to Soares, Guimarães, Silva, Durigan, and Silva (2018), packaging reduces the loss of corn mass during the days of storage, providing a better product appearance. Losses in the order of 3% to 6% are sufficient to cause

Table 1. Physicochemical parameters of baby corn conserved with straw (Experiment I), considering different temperatures, storage forms and post-harvest days. Janaúba-MG, Unimontes, 2019

Temperature (ºC)	Package (PVC plastic wrap)	Periods (days)						CV (%)
		0	3	6	9	12	15	
Fresh matter loss								
16	w [1]	0 aA	3.7 bB	9.6 bB	13.9 bB	19.0 bB	24.6 bB	13.27
	w/o [2]	0 aA	16.6 bA	33.1 bA	39.8 bA	45.7 bA	90.7 aA	
25	w	0 aA	11.4 aB	22.2 aB	31.5 aB	40.6 aB	49.4 aB	
	w/o	0 aA	29.4 aA	43.0 aA	50.5 aA	56.8 aA	62.7 bA	
Titratable acidity								
16	w	0.19 aA	0.19 aA	0.16 bB	0.15 bB	0.26 bA	0.19 bB	17.62
	w/o	0.21 aA	0.21 bA	0.27 aA	0.23 bA	0.30 bA	0.34 bA	
25	w	0.19 aA	0.19 aB	0.31 aA	0.32 aA	0.32 aB	0.36 aB	
	w/o	0.19 aA	0.28 aA	0.25 aA	0.31 aA	0.41 aA	0.46 aA	
Hydrogen-ion potential								
16	w	5.67 aA	5.72 aA	5.84 aA	6.24 aA	5.81 aA	6.06 bA	4.22
	w/o	5.67 aA	5.68 aA	5.55 aA	6.01 aA	5.81 aA	5.75 aA	
25	w	5.67 aA	5.71 aA	5.58 aA	5.97 aA	5.85 aA	6.43 aA	
	w/o	5.67 aA	5.58 aA	5.49 aA	5.89 aA	6.13 aA	6.06 aB	
Soluble solids								
16	w	8.32 aB	7.93 aA	8.90 aB	6.48 aB	7.23 aB	6.80 aB	17.72
	w/o	10.82 aA	10.30 aA	11.48 aA	14.57 aA	11.93 aA	12.87 aA	
25	w	8.69 aA	8.28 aA	10.63 aA	8.65 aA	7.75 aB	6.23 aA	
	w/o	8.73 aA	10.13 aA	11.83 aA	10.76 bA	10.55 aA	8.55 bA	
Ratio of soluble solids and titratable acidity								
16	w	3.04 aA	2.89 aA	3.89 aA	3.02 aA	1.95 aB	2.45 aA	19.33
	w/o	3.52 aA	3.35 aA	2.93 aB	3.72 aA	2.68 aA	2.54 aA	
25	w	3.04 aA	2.88 aA	2.34 bB	1.85 bA	1.75 aA	1.16 bA	
	w/o	3.06 aA	2.45 bA	3.24 aA	2.36 bA	1.82 bA	1.27 bA	

Note. * Means followed by different lowercase letters, presented in the column, differ from each other by the F test ($p < 0.05$) as a function of the temperature factor interaction within the packaging factors and storage times.

Means followed by different upper case letters, presented in the column, differ from each other by the F test ($p < 0.05$) as a function of the interaction of the packaging factor within the temperature factors and storage times.

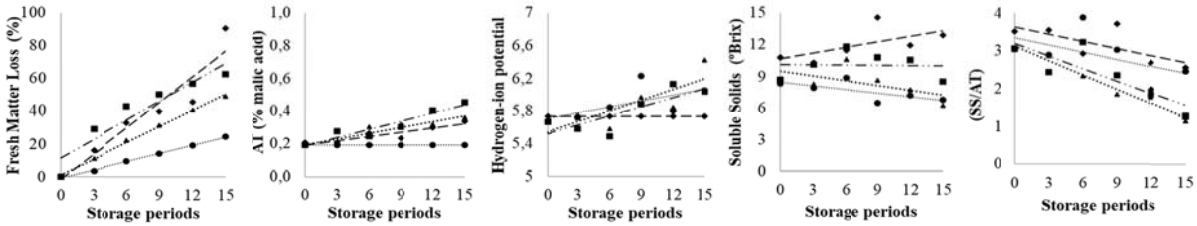
[1] With; [2] Without.

a significant decline in quality (Mamede et al., 2009). Considering these assertions, it is possible to affirm that the treatments without the PVC plastic wrap protection do not meet this criterion from four days of storage, even submitted to the cooling (16 ºC).

Inside baby corn there is a high moisture content, causing an intense metabolic activity, as stated by Santos and Oliveira (2012). This can lead to large post-harvest losses, such as high dehydration and physicochemical changes, restricting the period of commercialization. In sweetcorn the use of refrigeration and controlled atmosphere are shown to be promising technologies, with positive reports evidenced by Mamede, Fonseca, Soares, Pereira Filho, and Godoy (2015) and Soares et al. (2018), also in the present study, for baby corn, these technologies reduced dehydration and maintained the post-harvest characteristics of the products.

From the third day, the packaged spikelets preserved at the lowest temperature (16 °C), obtained the lowest values of titratable acidity (Table 1). The spikelets acidity increased along the days of storage, with a linear and positive increase over the temperatures and packages, except for those under temperature of 16 ºC with packaging, that no differences were observed regarding storage days (Table 2).

Table 2. Equations for the variables considered in the experiment I of baby corn conserved with straw in different times of post-harvest. Janaúba-MG, Unimontes 2019



Variables (With straw)	Temperature (ºC)	Package (PVC plastic wrap)	Equation		R^2	P_{value}	Days	Y_{max}
Fresh Matter Loss	16	w[1]	1•	$\hat{y} = -0.5640 + 1.6499^{**}x$	0.99	0.0001	15	24.18
		w/o[2]	2♦	$\hat{y} = -1.4783 + 5.2153^{**}x$	0.90	0.0001	15	73.75
	25	w	3▲	$\hat{y} = 1.3004 + 3.2731^{**}x$	0.99	0.0001	15	50.40
		w/o	4■	$\hat{y} = 11.58 + 3.8434^{**}x$	0.98	0.0001	15	69.23
Titratable Acidity	16	w	1•	$\hat{y} = 0.19$	-	>0.05	-	0.19
		w/o	2♦	$\hat{y} = 0.1921 + 0.008714^{**}x$	0.83	0.0001	15	0.32
	25	w	3▲	$\hat{y} = 0.1932 + 0.01174^{**}x$	0.86	0.0001	15	0.37
		w/o	4■	$\hat{y} = 0.1880 + 0.01671^{**}x$	0.90	0.0001	15	0.44
Hydrogen-ion potential	16	w	1•	$\hat{y} = 5.7038 + 0.0245^{*}x$	0.41	0.0140	15	6.07
		w/o	2♦	$\hat{y} = 5.74$	-	> 0.05	-	5.74
	25	w	3▲	$\hat{y} = 5.5395 + 0.0436^{**}x$	0.83	0.0001	15	6.19
		w/o	4■	$\hat{y} = 5.5179 + 0.0380^{**}x$	0.66	0.0001	15	6.19
Soluble Solids	16	w	1•	$\hat{y} = 8.4749 + 0.1156^{*}x$	0.48	0.0001	15	10.21
		w/o	2♦	$\hat{y} = 10.6894 + 0.1739^{*}x$	0.40	0.012	15	13.30
	25	w	3▲	$\hat{y} = 9.5031 + 0.1512^{*}x$	0.75	0.0270	15	11.77
		w/o	4■	$\hat{y} = 10.09$	-	> 0.05	-	10.09
SS/TA	16	w	1•	$\hat{y} = 3.3498 - 0.06352^{**}x$	0.30	0.0030	0	3.35
		w/o	2♦	$\hat{y} = 3.5558 - 0.05783^{**}x$	0.47	0.0060	0	3.56
	25	w	3▲	$\hat{y} = 3.1195 - 0.1265^{**}x$	0.97	0.0001	0	3.12
		w/o	4■	$\hat{y} = 3.2008 - 0.1113^{**}x$	0.71	0.0001	0	3.20

Note. [1] With; [2] Without.

Low values TA, due to storage of products under controlled atmosphere and at low temperatures, are attributed a reduction of organic acids amounts acids due to deceleration in the respiratory process and metabolic activity of the spikelets (M. I. F. Chitarra & A. B. Chitarra, 2005). Besides the degradation process, the decrease of the moisture contents favors the concentration of acidity, explaining the fact that the spikelets stored at higher temperatures (25 ºC) and unpackged presented the highest values of acidity (Table 1). However, even with a higher acidity (which is responsible for the flavor of the spikelets), it is not only attributed to organic acids, but also to a beginning of acid fermentation by microorganisms during storage (Sena et al., 2016). Considerable factor since these were in direct contact with the environment.

At the 15th day of storage, the lowest pH values were observed when the spikelets were stored at 16 °C and stored without the package (Table 1). The hydrogenionic potential of spikelets increased in function of days of storage. The highest pH increase occurred at 25 °C with the use of package, and the lowest increment and pH observed at 15 days of storage occurred at the temperature of 16 °C with the use of the package (Table 2).

The pH of the food is an important variable, since it allows the determination of possible changes caused by the action of microorganisms, as well as indicates its parameters of acidity and flavor (M. I. F. Chitarra & A. B.

Chitarra, 2005). The pH results obtained in this study agree with Lima et al. (2015) that evaluated the quality of six varieties of organic baby corn, 'Pipoca', 'Super doce', 'Doce cristal', 'Eldorado', 'Cate-tinho' and 'Branco', confirming that the baby corn is an acid food, regardless of the studied varieties, without altering in great magnitude the level of acidity.

During the storage process of the spikelets, it was possible to notice that the conditions with higher temperature and without package favored the increase of the organic acids and the pH; contributed with a possible microbiological activity in the spikelets, although this action has not been evaluated (Table 1). At the lowest temperature (16 °C) with presence of package it is observed the maintenance of the spikelets acidity levels found on the harvest day.

A concentration in the SS contents of the baby corn spikelets is observed after the post-harvest days. The spikelets submitted to the temperature of 16 °C without package obtained the highest increase and final soluble solids content (Table 2). The same behavior was observed in the fresh matter loss, pointing out a possible ratio between these variables. If this occurs, the lower the moisture content, the greater the concentration of soluble solids in the spikelets, indicating in grams, the solids that are dissolved in the juice or in the pulp, may increase with maturation by means of synthetic processes or by the degradation of the polysaccharide (M. I. F. Chitarra & A. B. Chitarra, 2005). Perfeito et al. (2017), observed for sweet corn, soluble solids values ranging from 15.0 to 17.0 °Brix, while Lima et al. (2015) working with baby corn, detected values ranging from 3.0 to 6.0 °Brix, and the results observed in the present study are lower than those found with sweetcorn and higher than the results obtained to baby corn by the authors (Table 1). In the evaluation of the SS/TA ratio, the samples stored at 25 °C without package were lower, evidencing a greater imbalance between the contents of sugars and acids of this vegetable (Table 1).

A decrease in SS/TA can be observed as days of storage increase, where the highest reduction is associated with the temperature of 25 °C, with greater accentuation with the use of package to store the spikelets (Table 2). The reduction of this ratio observed during storage days is indicative of quality losses. This change is affected by the physical and metabolic degradation of the spikelets and constitutes an important parameter to define the best alternative for storage (Menezes et al., 2017).

3.2 Experiment II

The spikelets stored at 25 °C had the highest fresh matter losses, and the treatment without package had significant losses at both temperatures (Table 3). In all treatments there was an increase of FML. However, lower losses were observed in conserved baby corn at the temperature of 16 °C with the use of the package (Table 4). In accordance with Mamede et al. (2009) in which the fresh matter losses for spikelets may not exceed 6%, the condition that provided the longest storage time was under controlled atmosphere and refrigeration of 16 °C, providing a maximum time of 2.5 days.

The lower loss of matter caused by the use of the controlled atmosphere can be explained by the low concentration of O_2, along with the increase of CO_2 concentration in the vegetables conserved in package, causing the reduction of the respiratory rate and consequently decreasing the matter loss (Soliva-Fortuny & Martín-Beloso, 2003). Araújo, Campos, and Gomes (2014) studying sweetcorn, accomplished that the use of the modified atmosphere associated with refrigeration helps to maintain the quality and improve the appearance of sweetcorn.

From the 3rd day of storage, the packaged baby corn exhibited lower acidity at the two temperatures studied (Table 3). As for FML, there was an increase in TA over the stored days, and the highest acidity values were observed at 25 °C without the use of the package (Table 4). Spikelets stored without package has an increase in their respiratory activity, undergoing influences of dehydration, that is, occurs greater synthesis and accumulation of organic acids and, consequently, increases the titratable acidity (Oliveira et al., 2017).

pH differences between treatments were observed only from the 9th day of evaluation, in which was detected in the packaged spikelets a lower pH at a temperature 16 °C when compared to the samples at 25 °C in the same condition (Table 3). Treatment with unpacked spigots at 25 °C obtained the lowest pH at 2.2 days of storage, while in the treatment with unpacked spikelets at 16 °C, the lowest pH value was observed only at 8.9 days after storage (Table 4). According to Miranda, Marques, Passos, and Oliveira (2017), the decrease in pH indicates the increase of spikelets acid fermentation, leading to deterioration of the product and senescence. The lowest pH, in shorter storage time, was observed in the spikelets conserved at 25 °C compared to those maintained at 16 °C. Thus, a lower temperature induces a delay in pH elevation, consequently contributing to the better post-harvest quality and shelf life of this horticultural.

Table 3. Physicochemical parameters of conserved baby corn without straw (Experiment II), considering different temperatures, storage forms and post-harvest days. Janaúba-MG, Unimontes, 2019

Temperature (ºC)	Package (PVC plastic wrap)	Periods (days)						CV (%)
		0	3	6	9	12	15	
Fresh matter loss								
16	w [1]	0 aA	6.1 bB	16.1 bB	23.1 bB	30.8 bB	39.3 bB	10.70
	w/o [2]	0 aA	50.9 aA	36.8 bA	51.3 bA	64.3 bA	72.6 aA	
25	w	0 aA	16.8 aB	31.6 aB	44.3 aB	56.8 aB	67.3 aB	
	w/o	0 aA	29.8 bA	49.2 aA	62.9 aA	71.7 aA	77.0 aA	
Titratable acidity								
16	w	0.22 aA	0.22 aA	0.28 bB	0.28 aB	0.30 bB	0.26 bB	13.30
	w/o	0.27 aA	0.27 aA	0.40 aA	0.41 bA	0.57 aA	0.58 bA	
25	w	0.19 aA	0.25 aB	0.36 aB	0.34 aB	0.55 aA	0.58 aB	
	w/o	0.19 bA	0.33 aA	0.46 aA	0.58 aA	0.56 aA	0.72 aA	
Hydrogen-ion potential								
16	w	5.67 aA	5.61 aA	5.59 aA	5.76 bA	5.62 bA	5.98 bA	3.05
	w/o	5.67 aA	5.62 aA	5.48 aA	5.62 aA	5.68 bA	6.16 aA	
25	w	5.67 aA	5.44 aA	5.52 aA	6.12 aA	5.91 aB	6.22 aA	
	w/o	5.67 aA	5.44 aA	5.54 aA	5.61 aB	6.38 aA	6.04 aA	
Soluble solids								
16	w	9.08 aB	8.65 aB	10.63 aA	8.80 bB	8.83 bB	8.00 aB	10.57
	w/o	11.58 aA	11.03 aA	12.27 bA	14.18 bA	15.78 bA	16.25 bA	
25	w	8.71 aA	9.60 aB	9.70 aB	13.13 aB	12.45 aB	6.16 aB	
	w/o	8.68 bA	12.03 aA	15.5 aA	21.05 aA	23.64 aA	25.10 aA	
Ratio of soluble solids and titratable acidity								
16	w	2.81 aA	2.68 aA	2.56 aA	2.50 aA	2.00 aA	2.07 aA	16.60
	w/o	2.94 aA	2.79 aA	2.12 aA	2.38 aA	1.86 bA	1.90 aA	
25	w	3.03 aA	2.75 aA	1.87 bA	2.65 aA	1.56 aB	0.97 bB	
	w/o	3.05 aA	2.41 aA	2.28 aA	2.45 aA	2.62 aA	2.34 aA	

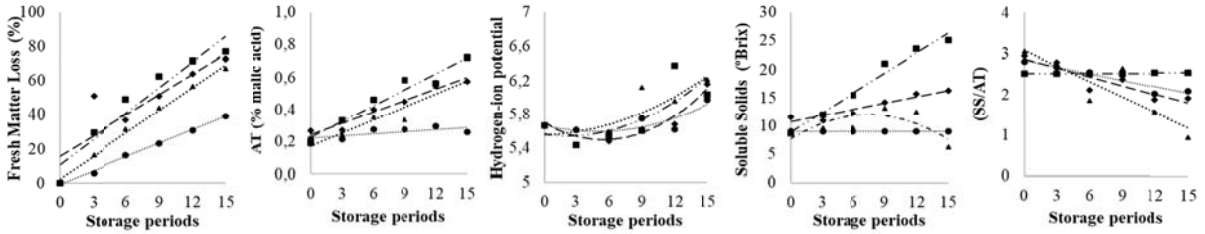
Note. * Means followed by different lowercase letters, presented in the column, differ from each other by the F test ($p < 0.05$) as a function of the temperature factor interaction within the packaging factors and storage times.

Means followed by different upper case letters, presented in the column, differ from each other by the F test ($p < 0.05$) as a function of the interaction of the packaging factor within the temperature factors and storage times.

[1] With; [2] Without.

From the 6th day of storage, the soluble solids contents were higher for the treatments kept at 25 °C, and the lowest contents were observed in the packaged spikelets, under a temperature of 16 °C (Table 3). It is observed the higher solids contents in the husked spikelets without plastic wrap cover, which have positive linear increase along the storage period, being this increase in the contents more noticeable in the temperature of 25 °C (Table 4). The interaction between the variables Soluble Solids with Fresh Matter Loss, largely explains the obtained results. Spikelets, along storage days, tend to lose moisture and to concentrate SS, such ratio is higher in unpacked spikelets, preserved at higher temperatures (25 °C) and without natural cover (straws). The soluble solids content determines the concentration of the organic acids and other sugars dissolved in the solution within the food, presenting an important indicator of their quality and flavor. In addition, it largely determines consumer acceptance of the product (Silva, Martins, Nascimento, Moraes, & Santos, 2018).

Table 4. Equations for the variables considered in the experiment II of baby corn conserved without straw in different post-harvest seasons. Janaúba-MG, Unimontes, 2019



Variables (without straw)	Temperature (ºC)	Package (PVC plastic wrap)		Equation	R^2	P_{value}	Days	Y_{max}
Fresh Matter Loss	16	w[1]	1●	$\hat{y} = -0.5911 + 2.6421^{**}x$	0.99	0.0001	15	39.04
		w/o[2]	2♦	$\hat{y} = 16.1357 + 3.9796^{**}x$	0.76	0.0001	15	75.83
	25	w	3▲	$\hat{y} = 2.6469 + 4.4672^{**}x$	0.99	0.0001	15	69.65
		w/o	4■	$\hat{y} = 10.9935 + 4.9913^{**}x$	0.92	0.0001	15	85.86
Titratable Acidity	16	w	1●	$\hat{y} = 0.2226 + 0.004429^{*}x$	0.55	0.0310	15	0.29
		w/o	2♦	$\hat{y} = 0.2401 + 0.02326^{**}x$	0.92	0.0001	15	0.59
	25	w	3▲	$\hat{y} = 0.1739 + 0.02698^{**}x$	0.92	0.0001	15	0.58
		w/o	4■	$\hat{y} = 0.2287 + 0.03295^{**}x$	0.95	0.0001	15	0.72
Hydrogen-ion potential	16	w	1●	$\hat{y} = 5.6754 - 0.03174^{*}x + 0.003214^{*}x^2$	0.69	0.047	4.9	5.60
		w/o	2♦	$\hat{y} = 5.7102 - 0.07566^{**}x + 0.006815^{**}x^2$	0.92	0.0001	8.9	5.58
	25	w	3▲	$\hat{y} = 5.6381 - 0.04717^{**}x + 0.005533^{**}x^2$	0.63	0.0001	4.3	5.54
		w/o	4■	$\hat{y} = 5.5660 - 0.01867^{*}x + 0.004251^{**}x^2$	0.59	0.0009	2.2	5.55
Soluble Solids	16	w	1●	$\hat{y} = 8.9925$	-	>0.05	-	8.99
		w/o	2♦	$\hat{y} = 10.6919 + 0.3762^{**}x$	0.91	0.0001	15	16.33
	25	w	3▲	$\hat{y} = 7.6902 + 1.1531^{**}x - 0.07733^{**}x^2$	0.56	0.0001	7.5	11.99
		w/o	4■	$\hat{y} = 8.9161 + 1.1667^{**}x$	0.98	0.0001	15	33.92
SS/TA	16	w	1●	$\hat{y} = 2.8112 - 0.05554^{**}x$	0.89	0.0010	0	2.81
		w/o	2♦	$\hat{y} = 2.8808 - 0.0735^{**}x$	0.82	0.0001	0	2.88
	25	w	3▲	$\hat{y} = 3.08476 - 0.1260^{**}x$	0.77	0.0001	0	3.08
		w/o	4■	$\hat{y} = 2.52$	-	>0.05	-	2.52

Note. [1] With; [2] Without.

The ratio between soluble solids and titratable acidity in the stored spikelets without straw decreased as a function of the increase in storage days, except for samples at 25 °C without the presence of plastic wrap packaging, which did not fit the statistical model (Table 4).

The packaging and lower temperatures provided better values of SS/TA because these storage conditions promote a reduction of moisture loss. Thus, the maintenance of post-harvest quality of the spikelets is preserved and the ratio between soluble solids and acidity are more stable, leading to a higher flavor of the baby corn. The decrease in SS/TA over the storage days can be attributed to changes in soluble solids contents and acidity of the spikelets, because even increasing the values observed in these isolated variables, their imbalance leads to a decrease in the flavor, being an important characteristic, linking this variable with the smooth and pleasant taste of them (Ferreira et al., 2010).

4. Conclusions

The best storage condition for the spikelets with straw and husked ones, aiming at maintaining the main characteristics of quality in the post-harvest, are obtained at the temperature of 16 ºC and with the use of the controlled atmosphere, through the packaging with the PVC plastic wrap.

For spikelets preserved by the presence of straw, the maximum storage time for maintenance of characteristics in the post-harvest was four days, and for spikelets stored without straw, the maximum storage time was two days and 12 hours, both at refrigerated temperature (16 °C) and under controlled atmosphere.

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Effect of Farming Systems on the Spatial Variability of Soil Physical Properties and Soybean Yield

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Abstract

In no-tillage (NT) and minimum tillage (MT) areas, spatial variability of soil physical properties may affect crop yield. The aim of this study was to assess the spatial distribution of soil physical properties, as well as the yield components and grain yield of soybean (GY), based on the mapping of areas under soil conservation farming systems. We assessed yield components, GY and the physical properties of an Oxisol, under NT and MT using the t-student test, and geostatistics to assess spatial variability. The largest population of NT plants showed no spatial dependence and did not influence GY, but the components related to plant height and soil properties differed between systems. From a spatial standpoint, the kriging maps demonstrated that mass of one thousand grains (MOG), total porosity (TP) and soil bulk density (BD) influenced GY under NT, whereas TP1 exerted the most influence under high soil moisture conditions and MT. The maps make it possible to assess the spatial distribution of soil physical properties and the influence on GY, making them an important tool for more accurate production planning in soil conservation systems.

Keywords: *Glycine max* L., minimum tillage, no-tillage, soil physical properties

1. Introduction

The Brazil advances to become the largest producer of soybeans in the world, this should happen with greater investment in production technology (Pereira et al., 2018a). This is partly due to the use of conservation farming systems, such as NT (no-tillage) and MT (minimum tillage), which promote improved soil quality, enabling cropping systems to cover vast areas (Cortez et al., 2017).

Soil conservation systems, which leave crop residue on the soil surface, include MT (using scarifiers) and NT (Dam et al., 2005). NT is based on the application of a set of technologies to avoid soil disturbance, the permanent maintenance of soil surface with crop residue and crop rotation (Moraes et al., 2017). Its worldwide use is increasing because it results in faster and more efficient operations, in addition to improving soil physical conditions for better crop development and growth (Tavares Filho et al., 2012; Pereira et al., 2018a). According to Kassam et al. (2018), NT encompassed 180 million hectares in 2015/16, 32 million of which were in Brazil, making the country's agriculture one of the most sustainable in the world (Freitas & Landers, 2014).

However, compacted and subcompacted layers emerge as a result of fewer soil operations (Franchini et al., 2011), making it a limiting factor in achieving maximum crop yield (Araújo et al., 2004). With the higher BD, penetration resistance (PR) and microporosity (MI) increase, causing a decline in TP, macroporosity (MA) and hydraulic conductivity (Rosa Filho et al., 2009). Scarification has been adopted to reverse this process, based on the principles of NT (Bertolini & Gamero, 2010; Nunes et al., 2014).

The effects of soil turning vary, with crops responding differently in terms of grain yield (Moraes et al. 2017; Pereira et al., 2018a). To better understand and analyze the spatial behavior of soil and plant properties, geostatistics is used to detect the variability and spatial distribution of the properties, by constructing maps

considering the position of the sample, generated according to interpolated data (Aubert et al., 2012; Alves et al., 2014; Betzek et al., 2017; Cortez et al., 2018). Their use is important in selecting production processes to increase agricultural profits with minimal environmental impact by adapting soil management to variable field conditions (Bottega et al., 2017). In this respect, our aim was to assess the spatial distribution of yield components, GY and soil physical properties by mapping of soybean crop areas, under NT and MT.

2. Method

2.1 Experimental Area

The study was conducted in 2015/2016 in an experimental area belonging to São Paulo State University (UNESP), at the School of Natural Sciences and Engineering of Ilha Solteira, São Paulo state, located in the city of Selvíria-MS (Brazil), at coordinates 20°20′ S and 51°24′ W, and average altitude of 344 m. The climate in the region is Aw, according to Köppen's classification, characterized as tropical wet-dry, with a rainy season in the summer and dry season in the winter, average annual rainfall of 1,300 mm and temperature of 23.7 °C. Figure 1 presents the monthly temperature, humidity and rainfall during the experiment.

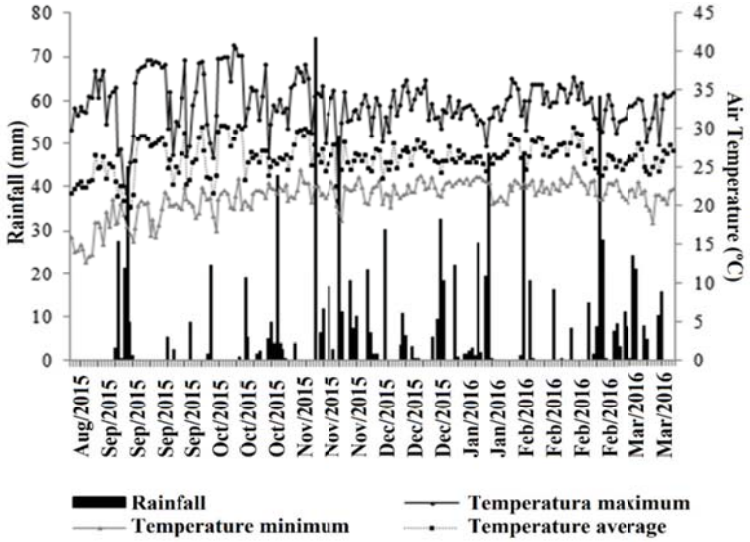

Figure 1. Rainfall (mm), minimum, maximum and mean temperature (°C) during the 2015/2016 growing season

The soil in the area is classified as Latossolo Vermelho distrófico, Brazilian classification (Santos et al., 2013), corresponding clayey dystrophic Oxisol with granulometry averaging 440 g kg^{-1}, 165 g kg^{-1} and 395 g kg^{-1} for clay, silt and sand, respectively. Before the experiment, the soil exhibited the following chemical characteristics: phosphorous (P_{resin}): 31 mg dm^{-3} and 20 mg dm^{-3}; organic matter (OM): 27 g dm^{-3} and 19 g dm^{-3}, pH ($CaCl_2$): 5.1 and 4.7, potassium (K): 5 $mmol_c$ dm^{-3} and 2.4 $mmol_c$ dm^{-3}, calcium (Ca): 27 $mmol_c$ dm^{-3} and 10 $mmol_c$ dm^{-3}, magnesium (Mg): 20 mmolc dm^{-3} and 10 $mmol_c$ dm^{-3}, aluminum (Al): 0 mmolc dm^{-3} and 4 mmolc dm^{-3}, H+Al: 38 mmolc dm^{-3} and 42 mmolc dm^{-3}, SB: 52.8 mmolc dm^{-3} and 28.4 mmolc dm^{-3}, CTC: 90 mmolc dm^{-3} and 70.4 mmolc dm^{-3}, V: 58% and 40%. Chemical analysis and fertilization were carried out according to Raij (2011) and Cantarella et al. (1997). Thus, with a view to increasing base saturation, 2.08 t ha^{-1} of lime were applied to the area, with CaO: 28.0% and MgO: 20.0%.

2.2 History of the Area

Before conducting the experiment, the 6,400 m^2 area, which undergoes center pivot irrigation, was used for annual crops until 1987. In 1998 and 2003, the soil was prepared using conventional tillage (plowing). In the other years, the crops were planted under NT until 2010. In 2011/2012, the area was used to plant corn for silage, together with Urochloa brizantha and Megathyrsus maximum. From 2012/2013 onward, corn in summer and bean in winter were the last successor crops in the area before the experiment. In September 2015, millet (*Pennisetum glaucum* L.), cultivar BRS 1501, was planted and dessicated with glyphosate 40 days after planting [1,440 g ha^{-1} of the active ingredient (i.a.)].

2.3 Experimental Design

The area was divided in December 2015, and 3,200 m^2 were allocated to MT and the rest to NT. The MT consisted of mechanical soil scarification, up to 0.37 m, followed by leveling and crushing. Next a sample grid consisting of 51 sampling points was divided into three 160 m transections, in order to cover each 3,200 m^2 area.

2.4 Crop Planting

Soybean was planted using the M 7110 IPRO cultivar, with indeterminate growth. Between-row spacing was 0.45 m, with a density of 15.9 plants per meter in the row. In line with crop recommendations, 250 kg ha^{-1} of 04-20-20 (N-P-K) formulation was applied. The fungicide carboxin (50 g ha^{-1} do i.a.) + thiram (50 g ha^{-1} do i.a.) was used to treat 100 kg of seed, followed by the liquid inoculant *Bradyrhizobium* sp. to supply 600,000 cells per seed.

2.5 Analyses of Yield Components and Grain Yield of Soybean

Soil collections, assessments and harvests occurred in March 2016, in the phenological stage R8 soybean. The final population of soybean plants (POP, plants ha^{-1}) was counted along eight meters of the crop line around each sampling point. The plants were manually harvested for mechanical threshing GY (kg ha^{-1}) was calculated, corrected to 13.0% (wet basis), and MOG was measured on a scale accurate to±0.01 g, at 13.0% (wet basis). During harvesting, 10 plants were separated at each sampling point to determine the following: height of insertion of the first legume (HL, m), measuring the distance from the ground to the first legume, number of legumes per plant (LP), by counting the legumes in plants containing seeds and dividing by the number of plants sampled, plant height (HP, m), measuring the distance from the ground to the apex of the main stem, number of grains per plant (GP), by counting the seeds and dividing by the number of plants sampled, number of grains per legume (GL), by counting the number of seeds and dividing by the number of legumes in the sampled plants.

2.6 Analyses of Soil Properties

Sample were collected in layers 0.00-0.10 m and 0.10-0.20 m around each sampling point to determine soil physical properties. Undisturbed soil samples in volumetric cylinders were used to calculate MA (m^3 m^{-3}), MI (m^3 m^{-3}), TP (m^3 m^{-3}), and BD (kg dm^{-3}) values, applying the tension table method described by Teixeira et al. (2017). An impact penetrometer was used to determine PR (MPa), according to Stolf et al. (2014). Disturbed samples were collected to determine gravimetric moisture (GM, kg kg^{-1}) and volumetric moisture (VM, m^3 m^{-3}), in line with the methodology described by Donagema et al. (2011), and volumetric moisture using Hydrosense system (Vh, m^3 m^{-3}).

2.7 Statistical Analysis

The average values of yield components, soybean yield and soil physical properties were submitted to the Shapiro-Wilk test of normality, followed by analysis of variance applying the F test ($p \leq 0.05$). When a significant difference was observed, the average plant and soil properties were compared using the t-student test (≤ 0.05) and R software (R Core Team, 2014).

2.8 Geostatistical Analysis

Statistical analysis was used to obtain the mean and frequency distribution, using SAS® software (SAS Institute, 2002). Spatial dependence was analyzed for each property by calculating a semivariogram, the nugget effect (C_o), range (A_o), sill ($C_o + C$), coefficient of determination (r^2) and residual sum of squares (RSS). Kriging maps were obtained by interpolation in order to analyze the spatial dependence between the properties, using Gamma Design version 7.0 (GS^+, 2004).

3. Results and Discussion

The effects of MT and NT influenced POP, HP and HL, with no effect on GY, MOG, LP, GP and GL (Table 1). POP was lower in MT, and soil turning with scarifier blades likely affected seed deposition and cover in the soil, exposing them to heat, thereby hindering plant emergence because planting occurred after scarification. This did not occur in NT, since planting maintains crop residue on the soil surface, providing favorable plant emergence conditions. Cortez et al. (2017) also found lower POP in scarified soil compared to NT. The authors attribute this behavior to less seed-soil contact when exposed to higher temperatures, which affected water absorption.

Table 1. Effect of minimum and no-tillage on yield components and soybean yield (Mean±SD)

Properties[a]		Minimum tillage	No-tillage	Pr < w[b]	
				Minimum tillage	No-tillage
GY (kg ha^{-1})	NS	4.25±507.29	4.43±456.18	0.112^{NO}	0.484^{NO}
MOG (g)	NS	16.24±0.92	16.19±0.84	0.007^{IN}	0.020^{TN}
LP	NS	49.53±6.95	48.87±7.36	0.055^{NO}	0.042^{TN}
GP	NS	114.30±16.05	112.00±19.83	0.089^{NO}	0.736^{NO}
GL	NS	2.31±0.15	2.28±0.16	0.474^{NO}	0.496^{NO}
HP (m)	**	1.00±0.05 a	0.95±0.07 b	0.020^{TN}	0.015^{TN}
HL (m)	*	0.15±0.01 b	0.16±0.01 a	0.437^{NO}	0.347^{NO}
POP (pl ha^{-1})	**	253.15±18.19 b	281.80±20.76 a	0.798^{NO}	0.051^{NO}

Note. [a]GY, grain yield of soybean; MOG, mass of one thousand grains; LP, number of legumes per plant; GP, number of grains per plant; GL, number of grains per legume; HP, plant height; HL, height of insertion of the first legume; POP, final population of soybean plants. [b]Frequency distribution: NO, normal; TN, tending to normal; IN, indeterminate. Different lower-case letters in each column indicate differences between farming systems: NS not significant; * P < 0.05% and ** P < 0.01 (with the t-student test).

Table 1 shows that HL under NT was higher than that of MT, and the greatest HP was obtained in MT. Soil scarification may have favored root distribution, allowing greater soil exploitation and increasing plant height. However, since water availability was the same between systems, the best plant development did not lead to a rise in GY. The HL and HP are important characteristics in crop development, since plants with HL lower than 0.15 m exhibit greater crop losses, while taller plants display a higher lodging incidence, which may limit the performance of harvesters.

Despite the similar GY between NT and MT (Table 1), yields were higher than those reported by Rosa Filho et al. (2009), who assessed NT in an Oxisol, and similar to those observed by Girardello et al. (2014), who also found no GY difference in Oxisol under NT and a scarified area. The use of scarifiers in planting systems does not always increase soybean yield (Cortez et al., 2017). Soybean is considered a rustic crop, with a limited response to soil interventions under favorable climate conditions (Pivetta et al., 2011). The results confirm that changes in crop yield may not be only a function of soil, which means that the best physical soil condition may not necessarily result in higher yields (Nunes et al., 2014).

Mechanical soil scarification prompted a reduction BD and an increase in GM, VM, MA, MI, TP and PR, in both layers (Tables 2 and 3). BD increased in NT, with a change in TP, a predominance of MI and decline in MA, caused by less soil turning. With no soil turning, the pressures caused by farm machinery traffic and the natural settlement of particles raise soil compaction, thereby reducing pore size (Godoy et al., 2015). Pereira et al. (2018b) assessed native forest in the Cerrado and in found BD values in natural Oxisol close to those recorded under MT, indicating compaction in the NT area and the effects of decompaction in MT. Additionally, higher GM and VM values may be related to the increase in MI under MT, retaining more water in the soil, despite the NT exhibiting more plant residue on the soil surface. The MA values in NT were lower than 0.10 m^3 m^{-3} (Tables 2 and 3), which is considered the limit for the development of most species (Collares et al., 2008). However, they did not restrict crop development, given that yield was similar between systems. These results are corroborated by Silveira et al. (2008), who found that NT obtains higher BD values, and lower MA and TP values, due to the absence of soil turning. Mazurana et al. (2011) observed that scarification reduced BD and PR, in addition to increasing water infiltration.

Table 2. Effect of minimum and no-tillage on soil physical properties, in the 0.00 to 0.10 m layer (Mean±SD)

Properties[a]		Minimum tillage	No-tillage	Pr<w[b]	
				Minimum tillage	No-tillage
MA1 (m^3 m^{-3})	**	0.126±0.04 a	0.054±0.02 b	0.941^{NO}	0.008^{IN}
MI1 (m^3 m^{-3})	*	0.369±0.03 a	0.354±0.02 b	0.644^{NO}	0.513^{NO}
TP1 (m^3 m^{-3})	**	0.496±0.04 a	0.408±0.03 b	0.801^{NO}	0.397^{NO}
BD1 (kg dm^{-3})	**	1.317±0.11 b	1.542±0.09 a	0.917^{NO}	0.056^{NO}
GM1 (kg kg^{-1})	**	0.264±0,02 a	0.218±0.02 b	0.268^{NO}	0.683^{NO}
VM1 (m^3 m^{-3})	**	0.347±0.04 a	0.336±0.03 b	0.410^{NO}	0.148^{NO}
Vh1 (m^3 m^{-3})	*	0.285±0.05 b	0.310±0.04 a	0.503^{NO}	0.000^{IN}
PR1 (MPa)	**	0.381±0.20 b	0.588±0.25 a	0.155^{NO}	0.050^{NO}

Note. [a]MA, macroporosity; MI, microporosity; TP, total porosity; BD, bulk density; GM, gravimetric moisture; VM, volumetric moisture; Vh, volumetric moisture using Hydrosense system; PR, penetration resistance. b Frequency distribution: NO, normal; TN, tending to normal; IN, indeterminate. Different lower-case letters in each column indicate differences between farming systems: NS not significant; * P < 0.05% and ** P < 0.01 (with the t-student test).

Table 3. Effect of minimum and no-tillage on soil physical properties, in the 0.10 to 0.20 m layer (Mean±SD)

Properties[a]		Minimum tillage	No-tillage	Pr<w[b]	
				Minimum tillage	No-tillage
MA2 (m^3 m^{-3})	**	0.096±0.03 a	0.054±0.01 b	0.564^{NO}	0.345^{NO}
MI2 (m^3 m^{-3})	**	0.377±0.02 a	0.346±0.02 b	0.413^{NO}	0.897^{NO}
TP2 (m^3 m^{-3})	**	0.474±0.02 a	0.401±0.02 b	0.991^{NO}	0.055^{NO}
BD2 (kg dm^{-3})	**	1.378±0.09 b	1.554±0.05 a	0.269^{NO}	0.900^{NO}
GM2 (kg kg^{-1})	**	0.286±0.02 a	0.230±0.21 b	0.109^{NO}	0.065^{NO}
VM2 (m^3 m^{-3})	**	0.395±0.03 a	0.357±0.30 b	0.085^{NO}	0.006^{IN}
Vh2 (m^3 m^{-3})	NS	0.380±0.03	0.374±0.02	0.423^{NO}	0.001^{IN}
PR2 (MPa)	**	1.018±0.37 b	1.603±0.32 a	0.002^{IN}	0.092^{NO}

Note. [a]MA, macroporosity; MI, microporosity; TP, total porosity; BD, bulk density; UG, gravimetric moisture; VM, volumetric moisture; Vh, volumetric moisture using Hydrosense system; PR, penetration resistance. [b]Frequency distribution: NO, normal; TN, tending to normal; IN, indeterminate. Different lower-case letters in each column indicate differences between farming systems: NS not significant; * P < 0.05% and ** P < 0.01 (with the t-student test).

Scarification was efficient in diminishing PR, which is lower than under NT (Tables 2 and 3). One of the main objectives of mechanical scarification is to decrease PR in soil (Girardello et al., 2014). Scarification helped break up compacted layers, increased MI and favored GM and VM, reducing cohesion between soil particles and PR values. Since the latter is greater in dry periods (Collares et al., 2008). Similar results were obtained by Souza et al. (2010), who also observed a drop in PR to 0.15 m, in an Oxisol, due to soil turning under conventional tillage. The PR that limits root growth is 2 MPa and no limitation to plant development was observed in either treatment. Higher PR values were reported by Dalchiavon et al. (2011) in Oxisol under NT. However, Almeida et al. (2008) studied Oxisol under NT and conventional tillage and found no difference for BD and GM up to 0.20 m. These results indicate that the effect of scarification could be temporary, and that physical properties may not improve after a certain time (Nicoloso et al., 2008).

Except for HL in MT (Table 4) and GP, HL and POP in NT (Table 5), which exhibited a pure nugget effect, the remaining plant and soil properties (Tables 6 and 7) showed spatial dependence. These were fitted in the spherical and exponential model, indicating that spatial distributions were not random. Similar behavior was observed by Dalchiavon et al. (2011), who found a spherical model for LP, GP and seed weight per plant, in Oxisol planted with soybean under NT. The spherical model has been applied primarily to describe the variability of soil properties (Cambardella et al., 1994; Oliveira et al., 2013; Ribeiro et al., 2016). Cortez et al. (2017) reported greater exponential model fit for PR, in both NT and scarification. Ribeiro et al. (2016) observed spatial dependence, with spherical models for BD and moisture in Oxisol, under NT and conventional tillage. The nugget effect showed a value of zero for all the components and properties assessed. Nugget effect values close to zero indicate more accurate estimates using kriging (Cortez et al., 2018). This occurs because the lower

the nugget effect in relation to the variogram baseline, the higher the continuity of the phenomenon and variance of the estimate, and the more reliable the estimate (Bottega et al., 2013; Cortez et al., 2018).

Table 4. Simple semivariogram parameters of the yield components and soybean yield under minimum tillage

Properties[a]	Model[b]	Nugget Effect (C_0)	Sill ($C_0 + C$)	Range (m)	r^2	RSS[c]
GY (kg ha^{-1})	sph (79)	5.750×10^4	2.467×10^5	31.3	0.863	1.430×10^9
MOG (g)	sph (82)	4.220×10^{-1}	1.13	56.5	0.966	1.000×10^{-2}
LP	sph (66)	1.961×10	4.020×10	43.7	0.980	3.83
GP	exp (67)	2.360×10	1.783×10^2	24.9	0.709	7.340×10^2
GL	exp (66)	2.620×10^{-3}	1.544×10^{-2}	23.4	0.845	2.031×10^{-6}
HP (m)	sph (67)	8.210×10^{-4}	2.572×10^{-3}	43.9	0.959	5.558×10^{-8}
HL (m)	nef.	3.690×10^{-4}	3.690×10^{-4}	-	-	-
POP	exp (67)	4.010×10^7	2.880×10^8	30.3	0.901	8.970×10^{14}

Note. [a]GY, grain yield of soybean; MOG, mass of one thousand grains; LP, number of legumes per plant; GP, number of grains per plant; GL, number of grains per legume; HP, plant height; HL, height of insertion of the first legume; POP, final population of soybean plants. [b]Models; exp, exponential; sph, spherical; nef, pure nugget effect and in parentheses the number of pairs in the first lag. [c]RSS, Residual sum of squares.

Table 5. Simple semivariogram parameters of the yield components and soybean yield under no-tillage

Properties[a]	Model[b]	Nugget Effect (C_0)	Sill ($C_0 + C$)	Range (m)	r^2	RSS[c]
GY (kg ha^{-1})	sph (70)	8.300×10^3	1.851×10^5	21.1	0.815	7.530×10^8
MOG (g)	sph (64)	3.310×10^{-1}	6.720×10^{-1}	31.1	0.632	$1.750 \times 10\text{-}^2$
LP	sph (75)	2.190×10	5.596×10	32	0.839	6.070×10
GP	sph (72)	1.641×10^2	3.359×10^2	36.4	0.957	4.410×10^2
GL	nef.	2.898×10^{-2}	2.898×10^{-2}	-	-	-
HP (m)	sph (69)	2.210×10^{-3}	5.440×10^{-3}	94.6	0.929	7.570×10^{-7}
HL (m)	nef.	2.630×10^{-4}	2.630×10^{-4}	-	-	-
POP	nef.	4.187×10^8	4.187×10^8	-	-	-

Note. [a]GY, grain yield of soybean; MOG, mass of one thousand grains; LP, number of legumes per plant; GP, number of grains per plant; GL, number of grains per legume; HP, plant height; HL, height of insertion of the first legume; POP, final population of soybean plants. [b]Models; exp, exponential; sph, spherical; nef, pure nugget effect and in parentheses the number of pairs in the first lag. [c]RSS, Residual sum of squares.

Table 6. Simple semivariogram parameters of soil properties under minimum tillage

Properties[a]	Model[b]	Nugget Effect (C_0)	Sill ($C_0 + C$)	Range (m)	r^2	RSS[c]
TP1 (m^3 m^{-3})	sph (76)	8.150×10^{-4}	1.640×10^{-3}	77.40	0.814	1.086×10^{-7}
BD2 (kg dm^{-3})	sph (75)	4.930×10^{-3}	1.046×10^{-2}	59.40	0.870	3.226×10^{-6}
PR1 (MPa)	exp (62)	1.024×10^{-2}	3.308×10^{-2}	49.80	0.804	3.496×10^{-5}
PR2 (MPa)	exp (78)	1.780×10^{-2}	1.1416×10^{-1}	35.40	0.878	3.509×10^{-4}

Note. [a]TP, total porosity; BD, bulk density; PR, penetration resistance. 1 and 2 are soil layers 0.00-0.10 m and 0.10-0.20 m, respectively. [b]Models; exp, exponential; sph, spherical; nef, pure nugget effect and in parentheses the number of pairs in the first lag. [c]RSS, Residual sum of squares.

Table 7. Simple semivariogram parameters of soil properties under no-tillage

Properties[a]	Model[b]	Nugget Effect (C_0)	Sill ($C_0 + C$)	Range (m)	r^2	RSS[c]
TP1 (m^3 m^{-3})	sph (68)	4.300×10^{-4}	8.610×10^{-4}	68.2	0.853	2.339×10^{-8}
BD1 (kg dm^{-3})	exp (82)	1.200×10^{-3}	9.700×10^{-3}	23.1	0.502	3.726×10^{-6}
VM1 (m^3 m^{-3})	sph (68)	4.100×10^{-4}	8.400×10^{-4}	56.2	0.885	1.411×10^{-8}
Vh1 (m^3 m^{-3})	sph (72)	7.750×10^{-4}	1.560×10^{-3}	34.4	0.889	2.246×10^{-8}

Note. [a]TP, total porosity; BD, bulk density; VM, volumetric moisture; Vh, volumetric moisture using Hydrosense system. 1 and 2 are soil layers 0.00-0.10 m and 0.10-0.20 m, respectively. [b]Models; exp, exponential; sph, spherical; nef, pure nugget effect and in parentheses the number of pairs in the first lag. [c]RSS, Residual sum of squares.

The highest range obtained under MT were MOG, HP, LP, GY, TP1 and BD2 (Tables 4 and 6), and HP, GP, TP1, VM1 and Vh1 under NT (Tables 5 and 7), indicating that they can be used to create more accurate kriging maps since they exhibit less spatial data variability in the area. Range is the distance over which sampling points are spatially correlated (Ribeiro et al., 2016). In relation to thematic maps, the higher the range, the more accurate the kriging estimate (Alves et al., 2014). In this respect, according to Dalchiavon et al. (2011), the range values used in geostatistical packages that feed the computer packages applied in precision agriculture should consider the smallest ranges. As such, they should not be less than 23.4 m and 21.1 m, respectively for MT and NT, since these are the distances within which the values of a certain property are equal.

The behavior of plant and soil properties was assessed using kriging maps (Figures 2 and 3), classified into five amplitude classes. In Figure 2 under MT, the highest GY are observed from the eastern to western part of the map. Spatially, the highest MOG and GL and lowest HP values were obtained in the eastern part of the map, indicating that these components influenced GY. Thus, MOG exhibited greater influence on GY, given that a 2 g variation in MOG increased GY by more than 500 kg ha^{-1}. Moreover, the region with the highest GY (eastern region) coincides with the highest TP1 and lowest BD2 values, because MT provides better decompaction conditions, prompting better plant development. Thus, from the spatial standpoint of the study area, MOG, GL, TP1 and BD2 showed the same trend and are good indicators of GY under MT.

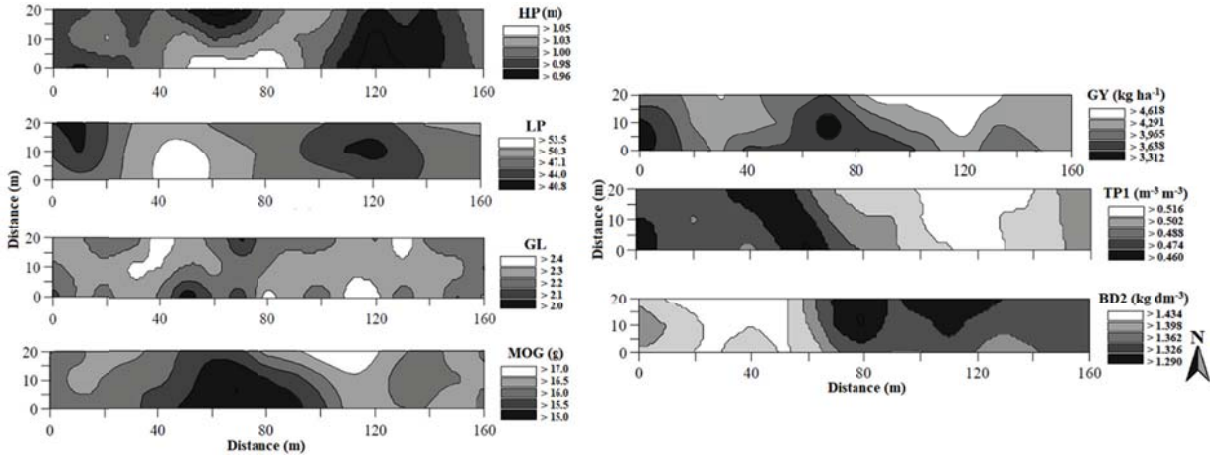

Figure 2. Kriging maps of physical properties and yield components, grain yield of soybean and soil physical properties in Oxisol under minimum tillage

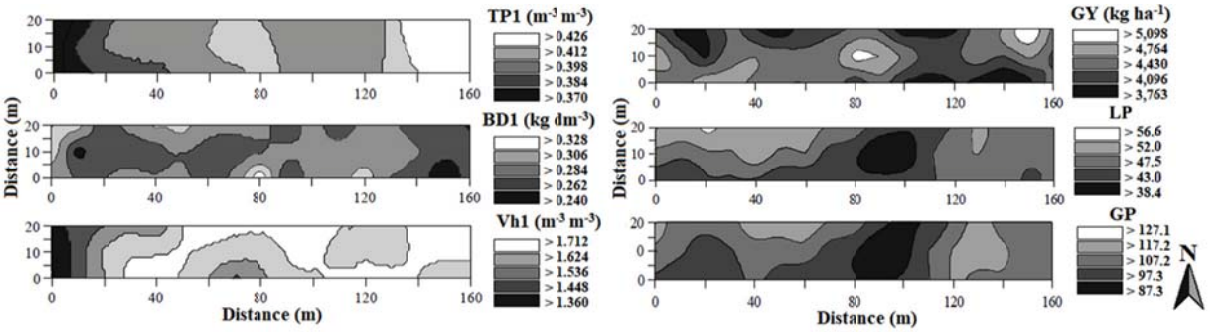

Figure 3. Kriging maps of physical properties and yield components, grain yield of soybean and soil physical properties in Oxisol under no-tillage

The higher GY in NT (Figure 3) is located in the central region moving toward the eastern region of the map, while LP and GP obtained the lowest values in these regions. This suggests that the yield components of soybean followed different patterns between the systems assessed. As such, the higher GY in NT was not a function of the yield components. Spatially, the kriging map (Figure 3) shows that TP1 was similar to GY, demonstrating the former's greater influence, given that Vh1 was homogeneous in the entire area, confirming that the higher GY in the NT is a result of TP1. Thus, in the NT area, TP1, VM1 and Vh1 are possible indicators of GY,

suggesting the importance of plant residues on the soil surface, since direct relationships were observed between soil moisture and GY, in order to create more accurate kriging maps, HP, GP and TP1 can be used under high soil soil moisture conditions.

3. Conclusion

The largest population of NT plants showed no spatial dependence and did not influence GY, but the components related to plant height were different between systems.

The MA, MI, TP, BD, GM, VM and PR, in the layers assessed were higher with scarification.

Spatially, the maps show that in MT, GY was influenced by MOG and GL, and greater in areas with higher TP1 and BD2 values, demonstrating that using some of these properties may produce more accurate maps.

The kriging maps illustrate the distribution of soil physical properties and their influence on soybean yield, making them an important tool for more accurate seed production planning in these areas.

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Migdolus fryanus Damage Causes Decrease in the Starch Content in *Manihot esculenta*

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Abstract

The beetle *M. fryanus* causes serious damage to cassava in Brazil. However, due to the subterranean behavior of the larvae and the recent appearance of this pest in cassava, little is known about the main behavioral characteristics and damage caused to this crop. The main aim of this study is to demonstrate the variation of starch production in the roots of *M. esculenta* as a result of the intensity of damage caused by *M. fryanus*. The study was carried out in a commercial cassava farm in Paraná, Brazil. The proposed scale for damage was: (1) No apparent root damage; (2) roots with scrapings; (3) roots with scraping across the cortex; (4) damaged roots and galleries; (5) roots with galleries and presentation of rot. The parameters evaluated were the damage caused by *M. fryanus* in roots and starch content, estimated by using a hydrostatic scale, starch extracted by cassava processing, and the starch reduction percentage in damaged roots, assessed by using the hydrostatic scale (SC) and processing methods (SCP). It was observed that there was manifestation of damage in approximately 60% of the roots collected directly from the ground, with levels representing loss of starch produced by the plant. Decreases in the productive parameters, such as starch content, were measured according to the increase of the proposed damage levels in the two cassava cultivation cycles, with a starch reduction rate higher than 20% when the roots suffered the most severe damage.

Keywords: root-feeding beetle, drill roots, cassava, soil-borne pest, long-horned beetle

1. Introduction

Manihot esculenta Crantz (Malpighiales: Euphorbiaceae) is an important source of calories in the Neotropical region, and because it is rich in carbohydrates, this plant is used for human and animal consumption through "*in natura*" consumption or through industrial processing. The main products of this plant are flour and starch (FAO, 2008). It is a perennial plant, but its roots can be marketed from six- to twenty-four months after planting. The high amplitude in the harvest period evidences positive aspects, such as, the possibility of market evaluation before harvest, and negative, such as, greater period of plant exposure to pests and diseases (Sagrilo et al., 2006).

Among the pests damaging the cassava crop, the following stand out: *Erinnyis ello* (L.) (Lepidoptera: Sphingidae), *Bemisia tuberculata* (Bondar) e *Aleurothrixus aepim* (Goeldi) (Hemiptera: Aleyrodidae), *Phenacoccus herreni* Cox & Williams e *Pseudococcus mandio* Williams (Hemiptera: Pseudococcidae), *Vatiga* spp. (Hemiptera: Tingidae), and recently *Migdolus fryanus* Westwood (Coleoptera: Vesperidae) (Bellotti, Smith, & Lapointe, 1999; Bellotti, Campo, & Hyman 2012).

Larvae of *M. fryanus* compromise the root system of *Eucalyptus*, *Pinus*, grapevine, mulberry, cotton, beans, coffee, pasturage, sugarcane, and cassava plants. However, the main studies and quantification of damage was performed only for sugarcane, with observed reduction in production, of up to 25% (Machado, Habib, Leite, & Mendes, 2006; Machado, Habib, Leite, & Carregari, 2006; Wilcken, Orlato, & Ottati, 2005; Ugwu & Ojo, 2015).

M. fryanus is native to South America, with prominent occurrence seen in the sandy soil regions. The larval period of this beetle is extended with subterranean habitat, so studies on the behavior and determination of damage to host cultures are incipient (Bellotti, Campo, & Hyman 2012; Nakayama et al., 2017).

The *M. fryanus* beetle has caused serious damage to the cultivation of cassava in the Northwest of the State of Paraná, a region that is part of the Arenito Caiuá formation, which has basic sandy soils with an advanced degree of physical and chemical degradation and critical levels of organic matter, especially in pastures and areas of sugarcane reforestation (Machado, Habib, Leite, & Mendes, 2006; Machado & Habib, 2006; Pietrowski et al., 2010).

In the cassava, *M. fryanus* larvae can cause failure in the development of the plant by feeding on its stem cuttings. They can cause loss of starch during production due to damage that may be caused early in the cortex sweeps to the opening in the gallery roots, causing soil deposition and allowing entry of the opportunistic pathogens that predispose it to the occurrence of rot (Machado, Habib, Leite, & Carregari, 2006; Bellotti, Campo, & Hyman 2012).

Faced with increased attacks on plants by *M. fryanus* and lack of information about the damage and loss it causes in cassava plants, conducting a research to elucidate its consumption habits and the injuries it causes in this culture is essential (Bellotti, Campo, & Hyman, 2012; Pietrowski et al., 2010).

The aim of this study is to demonstrate the variation in starch production in *M. esculenta* roots due to the intensity of damage caused by *M. fryanus.*

2. Method

2.1 Place of Study

The study was conducted in Cascata Farm in a commercial plantation of cassava *M. esculenta*, variety IAC-90, located in the northwest region of Parana in Perobal, PR, Brazil (23°51′17.57″ South Latitude, 53°20′40.39″ West Longitude, and 425 m average altitude).

Planting was carried out in 2014 and evaluations were conducted during the first and second crop cycles, 12 and 18 months after planting. A conventional planting system was used, with spacing of 1.00 × 0.60 m, the fertilizer used was 1000 kg ha^{-1} of simple superphosphate, covered with 80 kg ha^{-1} of potassium chloride.

Under the conditions of the first crop cycle, for the identification of the insects (adult males) and to evaluate the damage caused by the larvae in cassava roots, pitfall traps were installed containing Migdo® pheromone, in 78 evaluation points, with a spacing of 70 × 30 m between plants, located along the trajectory of the spatial points, delimited by the software Google Earth® and Quantum GIS 2.8.3®.

A Global Positioning System (GPS) receiver (Datum WGS 84) and Universal Transverse Mercator (UTM) projection system, zone 22 S, were used according to the categorization of the sample grids. Furthermore, at each evaluated point, three random plants were selected and removed from the soil to distinguish the damages caused by the feeding of *M. fryanus*. The collected insects, *M. fryanus* adults and larvae, were sent to the Laboratory of Chemical Ecology and Insect Behavior, Department of Entomology and Acarology, “Luiz de Queiroz” School of Agriculture - University of São Paulo.

2.2 Proposed Visual Scale for Scores to Quantify Damage by M. fryanus

Based on field observations, the scale of notes with damage intensity caused by *M. fryanus* on cassava roots and its influence on the production of starch in the culture was elaborated using a visual scale (Pietrowski et al., 2010). The notes were established as follows: Note 1: No apparent damage (scraping) at the root; Note 2: Scraping at the root without overcoming the cortex; Note 3: Scraping at the root overlying the cortex, but without galleries; Note 4: Root with galleries; Note 5: Root with galleries and presence of rot (Figures 1 and 2).

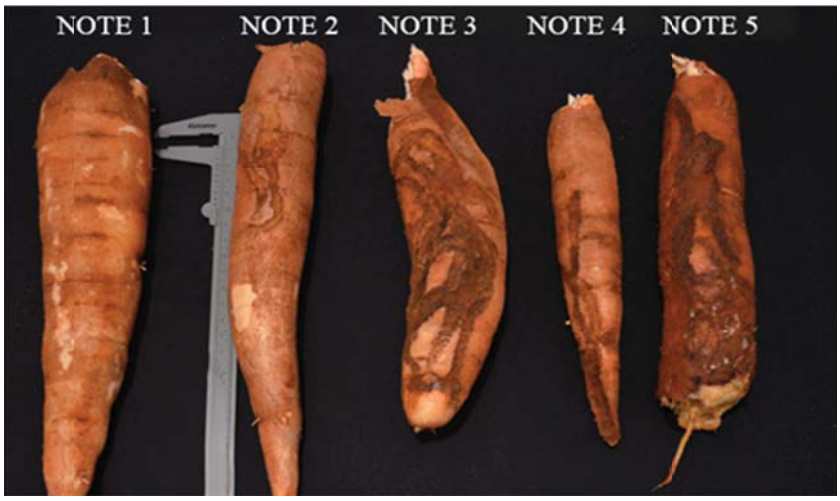

Figura 1. Notes to define scale of *M. fryanus* damage in *M. esculenta*. Umuarama, Paraná, 2015 and 2016

2.3 Starch Production and Influence of M. fryanus Damage

For the evaluation of starch production of cassava roots in the first and second cropping cycles (harvest at 12 and 18 months after planting), samples of roots that were already extracted from the soil and ready to be sent to the industry were collected. The samples were collected in a targeted manner, according to the damage intensity proposed in this study. The productive parameters of the roots were analyzed in the Laboratory of Entomology of the State University of Maringá, Campus of Umuarama, Paraná.

Figura 2. Detail of roots of *M. esculenta* damaged by larvae of *M. fryanus* according to proposed damage scale. Umuarama, Paraná, 2015 e 2016

2.4 Starch Content in the Tuberous Roots Using a Hydrostatic Scale

With the separation of root groups (according to the damage intensity proposed) yield analysis was performed (specific mass of starch) using a hydrostatic scale (SC). To this aim 3-kg root samples were weighed, initially on the upper plate of the scale (mass in the air) and then in a wire basket coupled to the lower part of the same scale, immersed in a water tank (mass in water). This kind of evaluation is standardized by the industry to estimate the starch in cassava roots (Juste Júnior et al., 1983; Maeda & Dip, 2000).

The estimation of the dry matter and starch content found in the roots was determined after drying at 105 ºC. It showed that the dry matter content in the roots increased by an order of 0.0564% for each unit increase of the sample weight in water. Thus, the following equation was obtained by Sagrilo et al. (2006):

$$Y = 15.75 + 0.0564x \quad (1)$$

Where, "Y" is the dry matter content and "x" is the specific starch mass of 3-kg roots in water, determined on a hydrostatic scale. The starch content (SC) in the tuberous roots was calculated by subtracting the constant 4.65 dry matter content (Y) (Maeda & Dip, 2000; Oliveira et al., 2011).

$$SC = Y - 4.65 \quad (2)$$

The variation of the starch percentage content in the tuberous roots using a hydrostatic scale (SC), according to the proposed damage scale, was evaluated using a completely randomized design, with 15 replications. The

scores referent to damage by *M. fryanus* were considered for treatment purposes and evaluated in the first and second crop cycle, totaling to 75 root samples in each harvest cycle.

2.5 Starch Content Obtained With Cassava Root Processing (SCP)

Samples (1 kg) of cassava were processed and separated according to the proposed damage scale. The woody parts at the end, near the stem of the plant, film, and cortex of each sample were removed. Smaller pieces were cut and using an industrial blender with 1000 mL drinking water, a grinding process was performed (Oliveira et al. 2011). The mixture was filtered through cotton cloth and the crushed mass washed to remove the starch until the liquid showed no whitish color. The liquid obtained from the washing (starch milk) was left in pots protected with a plastic bag for 24 hours, to decant the starch. The supernatant was rejected and the rest of the material submitted to the drying process in a controlled environment until reaching a constant mass. The starch was measured using a semi-analytical scale.

The starch content obtained with cassava root processing (SCP) according to the proposed damage scale was evaluated using a completely randomized design, with 15 replications. The scores referent to damage by *M. fryanus* were considered for treatment and evaluated in the first and second crop cycle, totaling 75 root samples in each harvest cycle.

2.6 Data Analysis

Data analysis of damage caused by *M. fryanus* larvae on cassava roots, and the influence of larvae consumption in the estimation of starch content using the hydrostatic scale (SC) and estimation of starch content using the root and residue extraction method (SCP) were performed with normality tests (Shapiro-Wilk) and homogeneity (Levene) and analysis of variance. After verifying the significance, the Tukey test was performed for the quantitative parameters evaluated. The starch reduction percentage was calculated by comparing the starch content in undamaged roots with that in damaged roots. By using the hydrostatic scale (SC) and processing methods (SCP), linear regression analysis for starch content in the root was obtained. Statistical tests were performed with the Agroestat® software (Barbosa & Maldonado Júnior, 2015).

3. Results

Collection of cassava plant roots of the variety IAC 90, directly from the ground was carried out under the conditions of the first cycle (harvest at 12 months after planting). It was possible to confirm the presence of *M. fryanus* and to observe the damage intensities, proposed in this study. In this evaluation we also found larvae of *M. fryanus* feeding inside the deeper roots containing galleries, as shown in Figure 3.

Figure 3. Galleries and Larva of *Migdolus fryanus* (Coleoptera: Vesperidae) in roots of *Manihot esculenta* (Euphorbiaceae) variety IAC-90. Umuarama, Paraná, 2016

It was possible to evaluate the occurrence of more than one damaged root in the same plant, with different injuries observed (Figure 4). The total amount of root means observed in each plant (4.32), about 60% had at least one *M. fryanus* damage symptom. In addition, in most of the plants, more than 20% of the roots showed symptoms of damage with a proposed grade above note 4. This represented the highest losses in starch, roots with galleries, and presence of rot (Tables 1 annd 2, Figures 5 and 6).

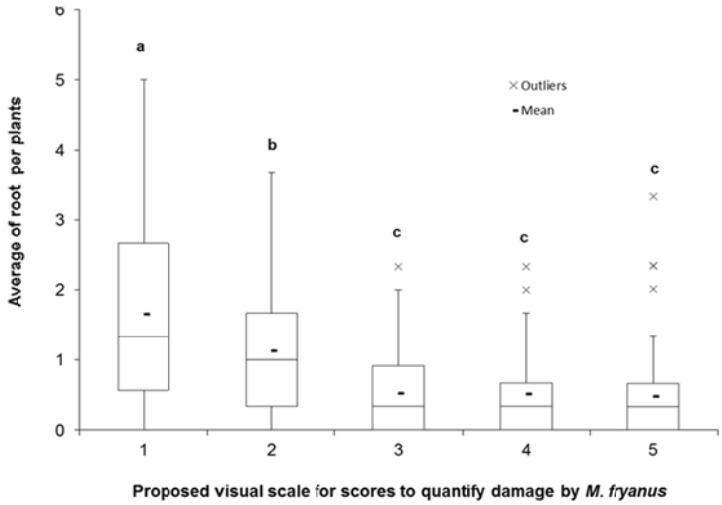

Figure 4. Boxplot showing median, mean, range and interquatile of roots average per plants with their proposed visual scale for scores to quantify damage by *M. esculenta*. Umuarama, Paraná, 2015 and 2016

The occurrence of roots with symptoms of different damage in the same plant indicates that larvae are walking between the roots and consumption in different roots in the same plant may occur, and in some cases it indicates the presence of more than one insect around the attacked plant.

In the evaluation of productive characteristics directed only to the roots with symptoms of damage, it was noted that the roots collected under the conditions of the first cycle (agricultural year 2015) with damages seen on two, three, four and five levels, had a decrease in starch content when determined on a hydrostatic balance (SC), with a percentage of starch reduction (RSC) alternating from 5.37% to 21.72%. The roots collected in the second crop cycle (agricultural year of 2016) had a decrease in the starch content that varied from 6.81% to 23.90%, for damages three, four, and five, when compared with the roots that were not damaged (Table 1).

Table 1. Starch contents obtained with hydrostatic balance (SC %), percentage of starch reduction with hydrostatic balance (RSC %). Umuarama, Paraná, 2015 and 2016

Damage notes	Cultivation cycles (Year)			
	1 (2015)		2 (2016)	
	(SC)	RSC %	(SC)	RSC %
1	30.15 a	-	30.71 a	-
2	28.53 b	5.37	30.02 a	2.25
3	27.59 b	8.49	28.62 b	6.81
4	25.82 c	14.36	26.77 c	12.83
5	23.60 d	21.72	23.37 d	23.90
CV %	4.88		4.80	

Note. The averages followed by the same lowercase letter in the column do not differ from each other by the Tukey test applied at the 5% probability level; CV: coefficient of variation.

The decrease in starch content seen when the evaluation method for extraction processing of cassava (SCP) was used, proved the relation obtained for hydrostatic balance (Tables 1 and 2). The reduction of starch seen by using this method was higher than 20% in the first cycle of cultivation for the roots that had damage scores 4 and 5, when compared with those not attacked. In the second crop cycle, the decrease in starch content was less pronounced, with the highest number of roots classified being with damage scores 5, with a reduction of 28.16% for this factor.

Table 2. Starch contents obtained with extraction method with cassava processing (SCP %), and percentage of starch reduction with cassava processing (RSCP %) in roots with respective damage notes. Umuarama, Paraná, 2015 and 2016

Damage notes	Cultivation cycles (Year)			
	1 (2015)		2 (2016)	
	(SCP)	RSCP %	(SCP)	RSCP %
1	17.86 a	-	18.54 a	-
2	16.48 a	7.73	18.08 a	2.48
3	15.90 a	10.97	17.34 ab	6.47
4	13.82 b	22.62	16.42 b	9.82
5	12.51 b	29.96	13.32 c	28.16
CV %	12.56		9.41	

Note. The averages followed by the same lowercase letter in the column do not differ from each other by the Tukey test applied at the 5% probability level; CV: coefficient of variation.

The reduction in the starch content of the manioc roots estimated by the hydrostatic balance (SC) was confirmed by regression analyses between this factor and the scores attributed to *M. fryanus* larvae, with a linear and significant adjustment, indicating the starch content reduction for the first and second crop cycles, respectively (Figure 5).

For the evaluation of the starch content obtained by the extraction method with cassava processing (SCP%), values of significant determination coefficients of 0.8939 and 0.8499, respectively, were also observed for the first and second crop cycle, with better adjustment for the linear condition (Figure 6).

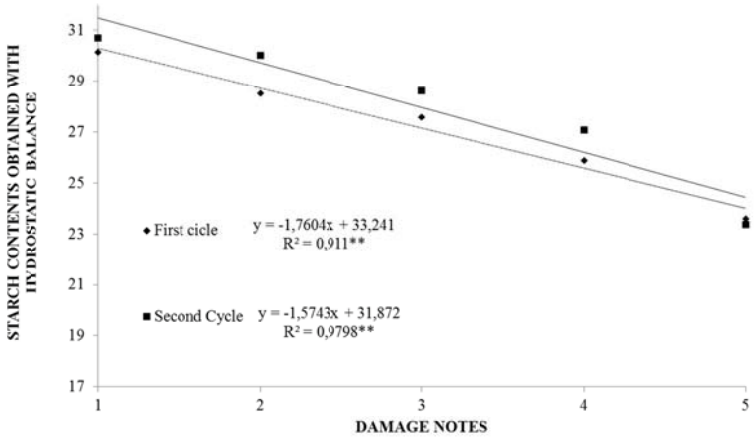

Figure 5. Effect of damage intensity of *M. fryanus* (Coleoptera: Vesperidae) larvae on the starch content of the IAC-90 variety in the first and second cropping cycles cropping cycles, estimated by the hydrostatic balance method, using samples with 3 kg of manioc. Umuarama, Paraná 2016

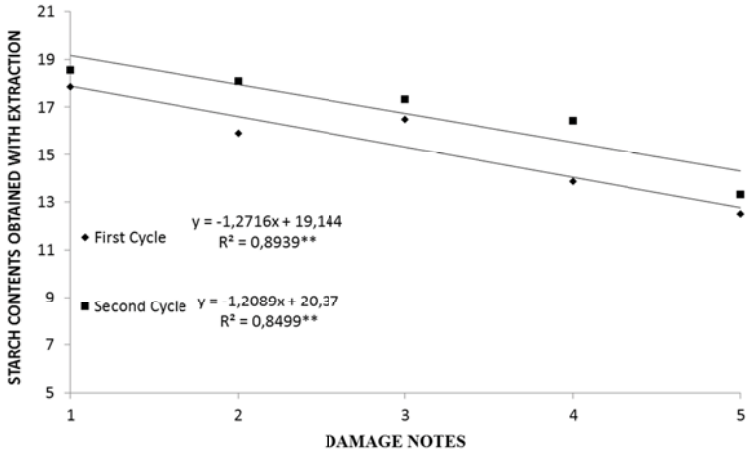

Figure 6. Effect of damage intensity of *M. fryanus* (Coleoptera: Vesperidae) larvae on the starch content of the IAC-90 variety in the first and second cropping cycles, obtained by the root processing method using samples of 1 kg of processed cassava. Umuarama, Paraná 2016

It is possible to affirm that the starch content estimated by the hydrostatic balance method (which determines the specific mass) and the extraction by processing of cassava, could show a decrease in cassava starch production when submitted to the stress caused by *M. fryanus* larvae. In addition, a complete relationship between the increase in the level of damage and the decrease of this evaluated factor has been observed.

4. Discussion

The damages caused by larvae of *M. fryanus* in cassava roots observed in the present study are expressive and provide a decrease in the productive values of this crop. It was possible to observe the beetle feeding on the roots and completely damage them, in all evaluations, in places where the sugar cane crop was previously cultivated. The complex of insects that occur in cassava and that live part of their life in the soil, particularly the Scarabaeidae and Vesperidae families, have increased their importance as agents causing primary damage in the tuberous roots. The record of *M. fryanus* damage in cassava is recent, and the actual damage caused by this pest in the roots and its effect on other parts of the plants until the present time is little known, as noted by Pietrowski et al. (2010) and Bellotti et al. (1999).

It is important to note that the *M. fryanus* species is considered a polyphagous pest, and the polyphagia characteristic observed for this insect can be explained by the presence of proteins from the cysteine and serine peptidase group present in the digestive tract, which leads to a more diverse feeding behavior (Nakayama et al., 2017).

However, the highest occurrence of *M. fryanus* in cassava crops in the northwest of the State of Paraná, is related to the planting sites that previously adopted crops considered preferential to this pest, such as sugarcane and the use of pasturage (Machado & Habib, 2006).

Migdolus fryanus has a habit of living underground, and root damage is due to larval feeding. In the same plant there may be gradations of different root damage, and the more severe damage represented by perforations and the presence of microorganisms may result in a considerable drop in productivity. Similar damages were observed for crops such as sugarcane, pasturage, and pine (Wilcken et al., 2015; Machado et al., 2006b; Machado & Habib, 2006).

The observation of the symptoms of damage in different roots, in the same plant, and with different degrees of severity can be explained by the habit of feeding and walking of the larvae in the soil, which at this stage of the life of the insect occurs both vertically and horizontally. It is dependent on climatic factors characterizing the higher feeding periods in dry seasons of the year, periods coinciding with the presence of more advanced instars near the cassava plants (Machado et al., 2006a; Machado & Habib, 2006).

The behavior of this pest can be explained by the presence in the fatty tissues of the protein larvae of the Otopetrins group, which present a sensorial function in these more advanced insects, which are present near the cassava plants (Nakayama et al., 2017). Thus the management of *M. fryanus* depends on the knowledge of the behavior of all stages of life of the insect, and its relationship with the cultivated plants (Bento et al., 1992; Bento et al., 1993).

The starch content estimated by the hydrostatic balance method and that obtained by the cassava processing method in roots that did not present with *M. fryanus* damage, under the conditions of this study, are within the recommended values for industrial use (Oliveira et al., 2011). However, from the moment of occurrence of symptoms of *M. fryanus* damage in the roots, the decrease in starch content was verified.

Besides water, starch is the most abundant constituent of cassava roots, and its decrease due to damage caused by *M. fryanus*, is because of root rot (Herren, 1994). The greatest drop in the productive parameters, such as starch content, can be caused by the formation of galleries and consequent soil deposition and onset of rot caused by opportunistic microorganisms and *M. fryanus* larvae, which penetrate into the roots and begin their colonization. This causes the most severe damage, such as rotting, and consequent fall in productive factors as noted for cassava and sugarcane (Machado et al., 2006a; Bellotti et al., 2012).

The decrease in starch content evaluated in cassava plants as a function of *M. fryanus* damage was less pronounced in roots that did not suffer severe damage (damage scores 2 and 3), indicating possible recovery of plants in the conditions of the second crop cycle, even with a longer period in contact with the soil conditions, which could lead to greater plant exposure to the larvae of the beetle and the occurrence of opportunistic microorganisms that penetrate the lesions caused by the larvae of *M. fryanus*, according to Camargo (2009).

5. Conclusions

The proposed notes on the scale of damage, allowed for estimating the losses caused by *M. fryanus*, in relation to starch in cassava. Notes four and five were related to the largest falls in starch content. Larger damages by *M. fryanus* larvae were verified under the conditions of the first crop cycle.

The decrease in terms of the amount of starch produced may lead to intensified losses to suppliers and the industry.

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Combined Effects of Biological and Chemical Treatment on Rice Seed Physiological and Sanitary Quality

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Abstract

This study aimed to evaluate the efficiency of integrated biological and chemical control of pathogens in rice seeds and their effects on seed quality. The experiment was conducted in a 2 × 5 factorial completely randomized design. Fungicide-treated (carboxin/thiram) and untreated rice seeds were inoculated with distilled water (control), *Azospirillum brasilense*, *Bacillus subtilis*, *Pseudomonas fluorescens*, or *Trichoderma harzianum*. Seed vigor and viability, shoot and root length, and seedling dry weight were determined. The blotter test was carried out to assess seed health. Fungicide treatment improved seed vigor and viability and reduced the incidence of fungi. Biological treatment did not enhance the physiological quality of seeds but was able to control fungi. *A. brasilense*, *B. subtilis*, *P. fluorescens*, and *T. harzianum* controlled *Phoma sorghina*; *B. subtilis*, *P. fluorescens*, and *T. harzianum* were effective against *Aspergillus flavus*; *P. fluorescens* and *T. harzianum* controlled *Pyricularia oryzae*; and *T. harzianum* was effective against *Gerlachia oryzae*.

Keywords: bioinoculant, seed pathology, vigor

1. Introduction

Rice (*Oryza sativa*) is a staple food of great economic and social importance in Brazil and several other countries. According to data from the United States Department of Agriculture (USDA), world rice production in 2018/2019 will be of 501.57 million tonnes, an increase of approximately 6.2 million tonnes compared to the 2017/2018. The largest rice producing, importing and consuming country is China. India is already the world's largest exporter of the grain. Forecast of world supply and demand for 2018/2019, production of 501.57 million tons, export of 47.37 million tons and inventory versus consumption ratio of 35.00 million tons (Companhia Nacional de Abastecimento, 2019). In 2018/2019, the yield of rice in Brazil is estimated to reach 5,994 kg ha^{-1} (Companhia Nacional de Abastecimento, 2018).

High rice yields depend, among other factors, on seed health. Seed treatment with chemical agents is an important strategy to control phytopathogens and prevent disease transmission to other plants and areas (Corrêa et al., 2008). An alternative to chemical control is the use of biological agents, which include non-pathogenic microorganisms capable of increasing host resistance or limiting pathogen activity (Choudhary & Johri, 2009; Busby, Ridout, & Newcombe, 2016). Biological agents are able to combat not only seed-borne pathogens but also those present in the soil. The main microorganisms used for biological seed treatment are bacteria (such as *Agrobacterium radiobacter*, *Bacillus* spp., and *Pseudomonas* spp.) and fungi (such as *Aspergillus* spp., *Chaetomium* spp., *Gliocladium* spp., and *Trichoderma* spp.) (Lucca Filho & Farias, 2012).

Some rhizobacteria promote plant growth and control diseases. The beneficial effects of root-colonizing microorganisms decrease production costs and minimize the need for pesticides, consequently reducing the

environmental impacts associated with their use (Harthmann, Mógor, Wordell Filho, Luz, & Biasi, 2009). For instance, studies have shown that *Bacillus* spp. decreases the incidence and severity of pathogen attack by inducing resistance through cytochemical changes in plant tissues (Mertz, Henning, & Zimmer, 2009). Seed inoculation with *Bacillus subtilis* enhances plant growth in common bean (Custódio, Araújo, Ribeiro, Souza Filho, & Machado Neto, 2013). Inoculation with *Azospirillum brasilense* increases plant dry matter, nitrogen accumulation, and grain production (Reis Junior, C. C. T. Machado, A. T. Machado, & Sodek, 2008). *Pseudomonas fluorescens* and other species of the genus promote plant growth and pathogen control by inducing hormone production in plants, producing siderophores and antibiotics, and competing for space and nutrients with pathogenic microorganisms (Corrêa, Bettiol, & Sutton, 2010).

Trichoderma is a genus of free-living and symbiotic fungi. They can survive in the soil, rhizosphere, and within plants. *Trichoderma*-based products are used for seed, substrate, and foliar treatment; to prevent damping-off of seedlings; and to reduce the severity of soil-borne diseases, such as those caused by *Pythium*, *Rhizoctonia*, *Sclerotium*, *Sclerotinia*, *Fusarium*, and *Phytophthora* (Pomella & Ribeiro, 2009).

Despite the various benefits of biological agents, there is no conclusive evidence that chemical treatment affects biological agents or that their combined use can promote beneficial effects. Therefore, we aimed to investigate the efficacy of integrated chemical and biological control of seed-borne pathogens in rice and evaluate their effects on the physiological quality of seeds.

2. Methods

The experiment was carried out at the Seed Technology Laboratory of the Department of Agronomy of the State University of Maringá, Umuarama, Paraná, Brazil. A 2 × 5 factorial completely randomized design was carried out with four repetitions. A commercial seed lot of cultivar SCS 112 was used. We evaluated the physiological quality of fungicide-treated and untreated seeds inoculated with distilled water (control), *A. brasilense*, *B. subtilis*, *P. fluorescens*, or *Trichoderma harzianum*. The fungicide combination carboxin/thiram (Vitavax-Thiram 200 SC®) was used at 300 mL 100 kg^{-1} seed. *A. brasilense* (Az Total®), *B. subtilis* (Accelerate BS®), and *P. fluorescens* (Accelerate PF®) were used at 100 mL 100 kg^{-1} seed. *T. harzianum* (Ecotrich WP®) was used at 3×10^{12} colony forming units 100 kg^{-1} seed, diluted in 300 mL of distilled water. For each treatment, seeds were placed in a plastic bag and received the addition of the corresponding amount of fungicide and/or biological agent. The bag was then shaken vigorously to ensure a homogeneous distribution of the agents.

Seed health was evaluated by a standard filter paper method (blotter test) using four repetitions of 100 seeds per treatment (Brazil, 2009a). Seeds were placed, 1 cm apart from each other, on top of three sheets of germination paper, moistened with a volume of distilled water equal to 2.5 times the weight of a dry sheet, and placed in a germination box. Each repetition was composed of four boxes containing 25 seeds each. Boxes were kept at 20±2 °C in a BOD incubator equipped with white fluorescent lamps under a photoperiod of 12:12 h light/darkness for 8 days. After incubation, seeds were examined individually for any sign of fungal fruiting bodies using a stereomicroscope (4-10× magnification). Fungal species were identified by comparing fruiting bodies with reference slides of identified fungal structures. Results are expressed as percentage of incidence of fungi in seeds.

The germination test was carried out with four repetitions of 50 seeds per treatment. Seeds were placed on top of two sheets of germination paper, covered with a third sheet, moistened with a volume of distilled water equal to 2.5 times the weight of a dry sheet, rolled, and placed in a BOD incubator at 25±2 °C under a photoperiod of 12:12 h light/darkness. Seeds were examined on days 5 and 10 of incubation to determine, respectively, seed vigor and viability (Brazil, 2009b).

Shoot length was determined with four repetitions of 10 seeds sown in line on the upper third of a previously moistened paper substrate, as described for the germination test. Substrates were rolled and placed vertically in a BOD incubator at 25 °C under a photoperiod of 12:12 h light/darkness. After 14 days, the shoot length, length of the primary root, and number of seedlings were determined. Subsequently, remaining seed reserve tissue was removed, and seedlings were packed in paper bags and dried in a forced-air oven at 65 °C for 48 h. Seedling dry weight was determined by dividing the weight of the sample by the number of normal seedlings (Nakagawa, 1999).

Data were tested for normality by the Shapiro-Wilk test and found to be normally distributed. Analysis of variance was performed, and means were compared by Tukey's test using Sisvar version 5.3 (Ferreira, 2011).

3. Results

Seed vigor was positively influenced by fungicide treatment (Table 1). Biological treatment did not increase seed vigor in comparison with control, regardless of association with fungicide treatment. *A. brasilense* and *P. fluorescens*, without fungicide treatment, reduced seed vigor in relation to control, *B. subtilis* and *T. harzianum*.

Seed viability was majorly affected by fungicide treatment, increasing this parameter with the use of the chemical product.

Table 1. Vigor and viability of fungicide-treated and untreated rice seeds inoculated with biological agents

Biological agent	Seed vigor (%)	
	Fungicide-treated	Untreated
Control (distilled water)	91.5 Aa	74.0 Bab
Azospirillum brasilense	88.0 Aa	63.0 Bbc
Bacillus subtilis	84.5 Aa	76.0 Aa
Pseudomonas fluorescens	89.0 Aa	57.0 Bc
Trichoderma harzianum	81.5 Aa	74.5 Aab
	Viability (%)	
	Fungicide-treated	Untreated
	91.7 A	82.4 B

Note. Means followed by the same lowercase letter in each column or uppercase letter in each row are not significantly different (Tukey's test, $p < 0.05$).

P. fluorescens and *T. harzianum* reduced shoot and root length in relation to *B. subtilis* treatment, but no treatment changed this parameters in relation to control (Table 2).

Table 2. Shoot and root lengths of rice seedlings treated with biological agents and fungicides

Biological agent	Shoot length (cm)	Root length (cm)
Control (distilled water)	9.28 ab	15.11 ab
Azospirillum brasilense	9.40 ab	15.63 ab
Bacillus subtilis	10.26 a	17.90 a
Pseudomonas fluorescens	8.44 b	14.31 b
Trichoderma harzianum	8.43 b	13.86 b

Note. Means followed by the same letter are not significantly different (Tukey's test, $p < 0.05$).

Seedling dry mass was determined; however, no significant differences were observed between treatments (data not shown).

For the analysis of fungi incidence on seeds (Table 3) note that a single seed can be infected by more than one species of fungus. Application of fungicide was efficient in controlling *Aspergillus flavus*, *Pyricularia oryzae, Gerlachia oryzae* and *Phoma sorghina*. Moreover, fungicide-treated seeds had the highest percentage of seeds without signs of fungal infection. There was no additional effect in fungi control when fungicide and biological treatment were associated.

B. subitilis, *P. flourescens* and *T. harzianum*, without application of fungicide, were able to control *A. flavus* and *P. oryzae* (Table 3). Additionally, *G. oryzae* was best controlled by *T. harzianum*, followed by *A. brasilense*, than by *P. fluorescens* and *B. subitilis* afterall. All inoculants were equally effective against *P. sorghina*.

Biological agents, without application of fungicide, showed different performances in combating fungi in rice seeds (Table 3). *B. subtilis*, *P. fluorescens*, and *T. harzianum* were more effective against *A. flavus* than *A. brasilense*. *T. harzianum* was the most effective in reducing the incidence of *G. oryzae*. Inoculation with *T. harzianum* resulted in the highest percentage of seeds without signs of fungal infection, being the only biological treatment with similar results as fungicide treatment for this evaluation.

Table 3. Incidence (%) of fungi in fungicide-treated and untreated rice seeds inoculated with biological agents

Biological agent	*Aspergillus flavus*		*Pyricularia oryzae*	
	Fungicide-treated seeds	Untreated seeds	Fungicide-treated seeds	Untreated seeds
Control (distilled water)	5.5 Ba	16.5 Aa	0.0 Ba	13.0 Aa
Azospirillum brasilense	1.5 Ba	14.5 Aa	0.0 Ba	12.0 Aa
Bacillus subtilis	1.0 Aa	3.5 Ab	0.0 Ba	6.0 Aab
Pseudomonas fluorescens	0.0 Aa	2.0 Ab	0.0 Aa	2.0 Ab
Trichoderma harzianum	0.0 Aa	2.0 Ab	0.0 Aa	1.0 Ab
Biological agent	*Gerlachia oryzae*		*Phoma sorghina*	
	Fungicide-treated seeds	Untreated seeds	Fungicide-treated seeds	Untreated seeds
Control (distilled water)	5.0 Ba	66.0 Aa	1.0 Ba	45.5 Aa
Azospirillum brasilense	7.0 Ba	25.0 Ac	12.0 Aa	13.5 Ab
Bacillus subtilis	2.0 Ba	42.5 Ab	1.0 Aa	6.0 Ab
Pseudomonas fluorescens	6.0 Ba	33.0 Abc	0.0 Aa	8.5 Ab
Trichoderma harzianum	0.0 Aa	2.0 Ad	0.5 Aa	6.5 Ab
Biological agent	Seeds without visual signs of fungal infection			
	Fungicide-treated seeds		Untreated seeds	
Control (distilled water)	90.0 Aa		17.0 Bc	
Azospirillum brasilense	87.0 Aa		35.0 Bbc	
Bacillus subtilis	99.5 Aa		42.0 Bb	
Pseudomonas fluorescens	89.0 Aa		54.5 Bb	
Trichoderma harzianum	85.0 Aa		88.5 Aa	

Note. Means followed by the same lowercase letter in each column or uppercase letter in each row are not significantly different (Tukey's test, $p < 0.05$).

4. Discussion

Several studies reported the beneficial effect of fungicide treatment on seed quality (Pereira, Oliveira, Rosa, Oliveira, & Costa Neto, 2009; Pereira et al., 2011; Silva, Lucca Filho, Zimmer, & Bonini Filho, 2011; Hossen, Corrêa Junior, Guimarães, Nunes, & Galon, 2014). Ramos, Marcos Filho, and Galli (2008) working with supersweet corn, observed that fungicide treatment improved seed vigor under accelerated aging conditions. Pereira et al. (2011) found that treatment of soybean seeds with fungicide increased seedling emergence by 44.3%, confirming the importance of controlling naturally occurring pathogens in seeds even under optimal germination conditions. Similar beneficial effects of chemical treatment were observed on rice seed vigor and viability (Table 1).

Initial seed quality determines the response of seeds to biological and chemical treatment; that is, seeds with high physiological quality do not benefit as much from treatments as do medium-quality seeds (Carvalho & Nakagawa, 2012). This fact probably explains the little effect of biological inoculation on rice seeds viability and seedling growth in the current study.

Another observation of the present study was the reduction of seed vigor after the use of some biological treatments. These results differ from those found in literature. There are evidences showing that seeds biological treatment improves (Brotman et al., 2013) and accelerates seed germination, increases seedling vigor and ameliorates water, osmotic, salinity, chilling and heat stresses (Mastouri, Björkman, & Harman, 2010). The interaction of the biological control agent with seeds/seedlings is complex and depends on factors such as the gene constitution of the plant (Simon et al., 2001; Smith, Tola, de Boer, & O'Gara, 1999), the production of organic acids by it is essential for establishing plant/bacterial interaction (as reviewed by Bloemberg & Lugtenberg, 2001), for example, and the presence of other microorganisms in the environment (Bloemberg & Lugtenberg, 2001). The germination test is carried out on paper substrate, changing the interaction conditions that the seed and the biological treatment would find in the soil. Therefore, in the mentioned test the absence of several factors which interfere in establishing the interaction, could contribute to alter the energy balance and consequently could contribute to reduce seed vigor.

However, biological treatment on seeds can be responsible for several other advantageous effects as it will be discussed further. Some characteristics of *B. subtilis* make it a particularly effective seed inoculant, such as its

sporulation ability, tolerance to desiccation, and improved survival in polymer formulations (Choudhary & Johri, 2009). These properties probably explain the high shoot and root lengths observed in rice plants inoculated with *B. subtilis* (Table 2). Bacteria of the genus *Bacillus* are known for their versatile defense mechanisms and antagonistic activities against plant pathogens, which are required for their survival and maintenance in specific ecological niches (Lanna Filho, Ferro, & Pinho, 2010). A study showed that inoculation of rice, common bean, chickpea, soybean, and cotton seeds with *B. subtilis* improves seed vigor, seedling emergence, and seedling dry weight (Custódio et al., 2013). In the same study, corn and cotton plants inoculated with the bacterium were found to have higher phosphorus concentrations in leaves than control plants, and inoculated corn plants were shown to absorb significantly higher amounts of phosphorus even in phosphorus-deficient soil (Araujo, 2008). *B. subtilis* is also beneficial to peanut seeds, improving plant vigor and harvest yield (Abd-Allah & Didamony, 2007).

Additionally, other microorganisms were found to exert positive effects on plants. *A. brasilense*, for instance, improved plant nutrition, water and mineral absorption, tolerance to drought and salinity stress, and root growth by increasing the resistance of plants to pathogen attack (Hungary, 2011). According to Gava and Menezes (2012), when *T. harzianum* is able to colonize the endosperm, it protects the seed and radicle from infection. Interestingly, *Trichoderma* colonization was found to occur preferentially at specific sites of the rhizoplane, mainly at regions of secondary root emergence and at points of contact between soil and roots, where abrasion occurs. Although a certain degree of root damage by soil is considered normal during development of the root system, it makes plants more susceptible to infection. *Trichoderma* inoculation may be a solution to this problem.

Inoculation with *B. subtilis*, *P. fluorescens*, or *T. harzianum* was able to control *A. flavus* in rice seeds (Table 3). The same effect was observed by Reddy, Raghavender, Reddy, and Salleh (2010), who reported growth inhibitions of 72, 74, and 65% by *B. subtilis*, *P. fluorescens*, and *T. harzianum*, respectively. Yang, Zhang, Chen, Liu, and Lu (2017) found growth inhibitions above 80% in the control of *A. flavus* by *P. fluorescens*.

Pseudomonas and *Bacillus* isolates were reported to control the rice blast fungus *P. oryzae* (Suryadi, Susilowati, Riana, & Mubarik, 2013). Inoculation with *P. fluorescens* and *Bacillus* isolates via seed treatment was effective in controlling the brown spot fungus *B. oryzae* and the leaf scald fungus *G. oryzae* in rice (Moura et al., 2014). Another study found that rice seeds inoculated with *B. subtilis* DFs422 and infected with *G. oryzae* showed low-severity symptoms of the disease for the first 21 days and became resistant to the fungus after this period (Ludwig, Moura, Santos, & Ribeiro, 2009).

Biological agents differ in their mechanisms of action. Some exert beneficial effects during seed germination, others during seedling growth, and others may be effective during all stages of the plant life cycle. It is interesting to note that *A. brasilense* has been gaining popularity in recent years as a nitrogen-fixing rhizobacterium (Fibach-Paldi, Burdman, & Okon, 2012), but its effects are not limited to plant growth promotion. In a pioneering work, Russo et al. (2008) demonstrated that *A. brasilense* is an excellent biological control agent against *Rhizoctonia* spp. and, since then, efforts have been made to elucidate its mechanisms of action. The bacterium was shown to produce and secrete phenylacetic acid, which has antimicrobial action against phytopathogenic fungi and bacteria (Somers, Ptacek, Gysegom, Srinivasan, & Vanderleyden, 2005), and siderophores, which are iron-chelating compounds shown to reduce the incidence of *Colletotrichum acutatum* in strawberry (Tortora, Díaz-Ricci, & Pedraza, 2011).

B. subtilis produces a variety of antibiotic substances, including iturine and fengycin, which are inhibitory to *Fusarium*, *Penicillium*, *Aspergillus*, *Colletotrichum*, and *Rhizoctonia solani* (Nagórska, Bikowski, & Obuchowski, 2007); surfactin, which has synergistic effects with fengycin against *A. flavus* (Farzaneh, Shi, Ahmadzadeh, Hu, & Ghassempour, 2016); and bacillomycin, a compound with fungicidal activity (Gong et al., 2014). *P. fluorescens* produces 2,4-diacetylphloroglucinol, a prominent antimicrobial that inhibits the growth of several phytopathogenic bacteria, oomycetes, and fungi (Couillerot et al., 2011). *T. harzianum* secrets hydrolytic enzymes, produces fungistatic compounds (Contreras-Cornejo, Macías-Rodriguez, Del-Val, & Larsen, 2016), and parasitizes other fungi (Silva et al., 2017). All these data support our findings regarding fungi control on rice seeds using biological control (Table 3).

Different pathogens induce different responses in biological control agents. For instance, *R. solani* alters the expression of genes associated with secondary metabolite detoxification and metabolism in *P. fluorescens*, whereas *Pythium aphanidermatum* does not (Hennessy, Glaring, Olsson, & Stougaard, 2017).

Overall, these findings suggest that inoculation of rice seeds with more than one microorganism might be an effective strategy for the control of pathogens (Babalola, 2010). Coinoculation of tomato leaves with

Trichoderma spp. and *B. subtilis*, *Trichoderma* spp. and *P. fluorescens*, or the three microorganisms combined was more effective against the pathogen *Ralstonia* spp. than inoculation with a single microorganism or chemical control (Yendyo, Ramesh, & Pandey, 2018). Similar effects are expected for coinoculation of seeds.

In this study, biological inoculation was found to be more effective in promoting seed vigor and viability and protecting rice seeds from fungi when combined with fungicide treatment (Tables 1 and 3). In the absence of fungicide treatment, microbial inoculation was more effective than the control (Table 3). These results indicate that chemical treatment had a much greater effect on seeds than biological treatment, thereby precluding observation of the beneficial effects of biological agents. Fungicide application might have decreased the positive effects of *T. harzianum* inoculation and might even have affected plant responses to *A. brasilense*, *B. subtilis*, and *P. fluorescens*. A previous study showed that fungicides can have deleterious effects not only on fungi but also on bacteria and are not compatible with *A. brasilense* inoculated via seed treatment (Munareto et al., 2018).

Fungicide treatment increased rice seed vigor, viability, and resistance to fungi. Microbial inoculation did not improve the physiological quality of seeds. *A. brasilense*, *B. subtilis*, *P. fluorescens*, and *T. harzianum* inoculated via seed treatment were effective in controlling *P. sorghina*, *B. subtilis*, *P. fluorescens*, and *T. harzianum* were effective against *A. flavus*, *P. fluorescens* and *T. harzianum* successfully controlled *P. oryzae* and *T. harzianum* was effective against *G. oryzae*.

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Random Forests in the Supervised Classification of Multidimensional Images of the Tetrazolium Test

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Abstract

The quality of the soybean seed can be influenced by several factors that may occur at any stage of production. Mechanical damage, deterioration by humidity and the damage caused by bed bugs are among such problems. The tetrazolium test is adopted by the seed industry, especially for testing soybeans, due to its accuracy, fast result, and the large amount of information it provides. Digital processing and image analysis can be used to aid the extraction and classification of standards for minimizing the subjectivity implicit in the test, thus allowing more credibility to the information. The aim of this work is testing the effectiveness of Random Forests in the supervised classification of soybean embryos images submitted to the tetrazolium test. In order to do so, we used the Trainable Weka Segmentation plugin to perform the segmentation process, and the WEKA software to evaluate the quality of the classifier model obtained. During the process, 222,646 instances among 230,388 instances were correctly classified (96.7%), with Kappa index of 0.95, showing the classifier excellent performance regarding the proposed dataset. The supervised classification, combined with pixel-based segmentation, proved to be efficient in extracting more coherent visual information on seed damage. Also, we conclude that the choice of image attributes, along with the algorithm used in the work, showed to be competent in the classification process of high dimensionality samples.

Keywords: seed technology, seed vigor, machine learning

1. Introduction

Soybeans are one of the species most in need of consideration, given the degree of collection by producers. Seed technology has as main objective to develop efficient mechanisms for the productive chain, using lots of seeds of high quality. This set of knowledge, which it is based on practical experience and scientific experimentation, begins with the work of genetic improvement and proceeds to the harvesting, processing and distribution of high-quality lots, bringing together the genetic, physiological and sanitary attributes (Marcos Filho, 2015).

The quality of soybean seed, especially in tropical regions, may be influenced by several factors, which may occur at any stage of production (field, harvest and post-harvest). Among these problems they bring, mechanically caused damages in the harvesting and processing operations, damages caused by deterioration due to humidity, arising from drought period, temperature extremes, during maturation, and fluctuations of ambient humidity conditions, and damage caused by bed bugs stand out (França Neto, Krzyzanowski, Henning, & Costa, 2000).

The tetrazolium test has been outstanding among the quality analysis tests adopted by the seed industry, especially for soybeans, not only because of its accuracy and speed, when compared to other tests, but also because of the large number of information it provides, such as the diagnosis of possible causes of quality

reduction (mechanical damage, deterioration by moisture, and bedbug damage), and the possibility to evaluate the quality and vigor of seed lots (França Neto & Krzyzanowski, 2018).

Even without using expensive equipment and reagents, the accuracy of the test depends on a well-trained seed analyst, who knows all the techniques and procedures involved in the test, so the analyst's ability to recognize typical patterns of the various types of damage that can be visualized in the seeds is essential to obtain a correct diagnosis of the causes of viability loss (França Neto & Krzyzanowski, 2018; Moore, 1985).

Computational tools that employ digital processing and image analysis to aid the extraction and classification of patterns in information that minimize or nullify the subjectivity implied in the accomplishment of some tests contribute to a greater information credibility and guarantee of the results, besides reducing the classification time of the test.

The ImageJ platform is a free distribution software, licensed under the GNU (General Public Licenses), and is used by an active community, composed by researchers from various knowledge fields. Its use allows a range of applications, from data visualization to advanced image processing and statistical analysis. Due to its extensibility, it attracts biologists and computer scientists who efficiently implement specific image processing algorithms (Schindelin, Rueden, Hiner, & Eliceiri, 2015).

Image segmentation is generally defined as the decomposition process in non-intersecting regions, where a label is assigned to these regions (pixel set), which share certain visual characteristics. Most traditional segmentation methods are based on pixel intensity information only. However, humans use other information when performing segmentation naturally. For this reason, recently, trainable segmentation methods emerged as an important alternative to improve the accuracy of the region labeling process (Arganda-Carreras et al., 2016).

Recently, a new family of algorithms based on machine learning has been recognized as being successful for image classification, by using computational intelligence paradigms, which studies the development of inference techniques from samples. These techniques, based on mathematical models, present the ability to “learn” from the samples and generalize the knowledge generated for the whole image (Andrade, Francisco, & Almeida, 2015).

Learning-based algorithms have been developed to obtain more accurate and reliable information as an alternative to the usual pixel-based approaches and objects. Random Forest (RF), Bagging, Boosting, Decision Tree, Artificial Neural Network, Supported Vector Machine (SVM) and K-Nearest-Neighbor are among the most commonly used learning-based algorithms. These algorithms are also known as machine learning methods, which look for the best model for the data, using a set of data with sufficient size and parameters, and decision rules created from the input data (Breiman, 2001; Akar & Güngör, 2012).

Breiman (2001) has proposed Random Forests, that include an additional layer of randomness to the bagging procedure. Therefore, in addition to constructing each tree using a bootstrap sample different from data, Random Forests change the way classification or regression trees are built. By default, in trees, each node is divided according to the best combination of all variables. In a Random Forest, each node is divided using the best among a subset of randomly chosen predictors on that node. This classification strategy is efficient when compared to other classifiers, such as discriminant analysis, support vector machines, and neural networks, besides being robust against overfitting (Breiman, 2001; Liaw & Wiener, 2002).

Random Forests are composed of a set of decision trees, where the prediction of the class for new values is based on a voting system, in which, after generating a large number of trees (forest), the class is chosen, based on the majority of tree votes, being formally described as h(x, Θk), where h is the decision tree, x is the input to be sorted, and Θk is the k^{th} random vector sampled independently (Breiman, 2001). Hence, each tree votes for the most popular class for the x entry to be sorted.

These forests are obtained through a method to generate multiple versions of a predictor, known as bagging (bootstrapping aggregating) Breiman (1996), according to which the final forecast is performed by the average of predictions B (Equation 1) or by the majority vote (Equation 2) (Goldstein, Polley, & Briggs, 2011).

$$\hat{f}_{av}(x) = \frac{1}{T}\sum_{t=1}^{T} \hat{f}^{t}(x) \quad (1)$$

Where, $f^{t}(x)$ is the function with the features to be studied; and T is the number of training samples.

$$\hat{f}_{bag}(x) = \frac{1}{B}\sum_{b=1}^{B} \hat{f}^{*b}(x) \quad (2)$$

Where, $f^{*b}(x)$ is the function with the features to be studied; and B is the number of bootstrap samples.

Figure 1 exemplifies the classification of an image by Random Forest, where each decision tree recursively classifies the input patch into the root node until a leaf node (class) is reached, being the patch classified according to the class that obtains most votes.

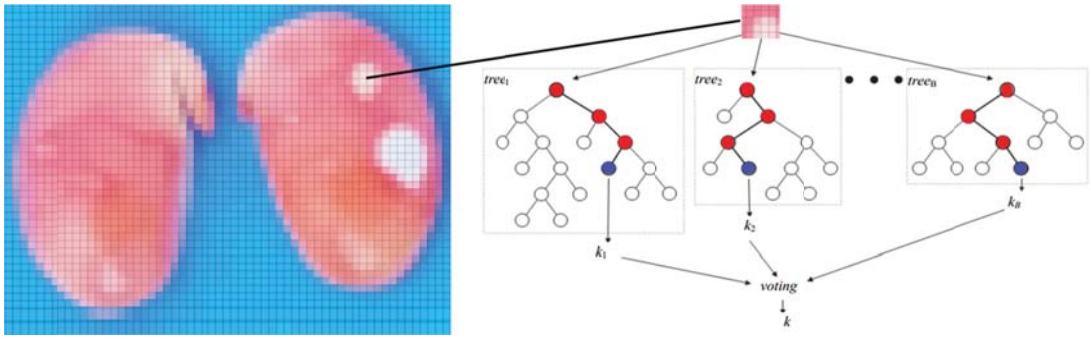


Figure 1. Example of a Random Forest classifying an input patch, and assigning it the class from the majority vote of the trees. Adapted from (Huang, Siu, & Liu, 2015; C. Nguyen, Wang, & H. Nguyen, 2013)

According to Akar and Güngör (2012), Random Forest is known for being one of the most efficient methods of classification and for attracting researchers from different areas of knowledge, due to its intrinsic interdisciplinary character. Therefore, this work aims to investigate the performance of the Random Forest algorithm using multidimensional images of the tetrazolium test.

2. Materials and Methods

This work was carried out at the Laboratory of Seed and Plant Evaluation of Western Paraná State University (UNIOESTE), Cascavel campus, and also at the Biology and Chemistry Teaching Laboratories at Federal Technological University of Paraná (UTFPR), Santa Helena campus. Soybean seeds of several cultivars from agricultural properties, as well as seeds known to be carriers of specific damages, provided by an official seed analysis laboratory in the region, were used. For performing the tetrazolium test, approximately 500 g of seeds were selected. The stock solution at 1.0% was prepared by mixing 10.0 g of tetrazolium salt in 1.0 L distilled water. As suggested by Association of Official Seed Analysts [AOSA] (1983) 100 seeds of each sample were used (two subsamples of 50 seeds each). The seeds were packed in moisture germination paper and kept under these conditions for 16 hours in a BOD (Biochemical Oxygen Demand) type oven at 25 °C. After this period, the seeds were put in plastic bags to receive 0.075% tetrazolium solution to stay completely submersed. Afterwards, the temperature was between 35 and 40 °C for 150-180 minutes (2.5 to 3 hours) until the staining.

In the process of image analysis, the libraries of the FIJI software, which are a distribution of ImageJ software that adds several functionalities that facilitate the analysis of scientific image, were used. Such software was proposed as a productive collaboration platform between Computer Science researchers and Biology research groups (Schindelin et al., 2012).

To perform the segmentation of the images, the Trainable Weka Segmentation (TWS) plug-in was employed, since it is integrated with the FIJI software. This plug-in works as a link between machine learning fields and digital image processing, providing the framework needed to use and compare classifiers that perform image segmentation. It combines a collection of machine learning algorithms with one set of image characteristics, to produce pixel-based segmentations. The TWS provides a set of methods for extracting statistical properties from an image, based on pixel samples, and then, from this information, segment the rest of the pixels. Waikato Environment for Knowledge Analysis (WEKA) is open source software. It consists of a range of machine learning algorithms for data mining, which includes tools for: data preprocessing, classification, regression, clustering, association and visualization rules. All WEKA classification, regression and clustering algorithms can be used by the TWS (Hall, Frank, Holmes, Pfahringer, Reutemann, & Witten, 2009; Arganda-Carreras, Cardona, Kaynig, Rueden, & Schindelin, 2011; Arganda-Carreras et al., 2016).

The acquisition of the tetrazolium test images was performed by using a Sony HX200V 18.2 Mega Pixels camera, with Charge-Coupled Device (CMD), Exmor R™ CMOS sensor, without flash. The images were preprocessed (enhancement and restoration operations) to improve information or suppress the irrelevant ones. The purpose of the process was to facilitate the subsequent operations in the search for better results (Awcock &

Thomas, 1996). The image generated for replication I has resolution of 2304 pixels wide, 1329 pixels high, in PNG format. Replication II has a resolution of 2307 pixels wide, and 1350 pixels high, in PNG format.

The characteristics of analyzed image are color in HSB pattern and Entropy with $r_{max} = 8$ and $r_{min} = 1$. The used classifier was the FastRandomForest beginning with 200 trees and two random characteristics per knot. FastRandomForest is a multitasking reimplementation of Random Forest, created by Fran Supek, which optimizes speed and memory usage (Arganda-Carreras et al., 2016). This process aims at extracting the characteristics of image used in the system and building the decision trees, based on the characteristic vector constructed by the previous process.

After training the classifier, it is important to evaluate its ability to perform generalizations, that is, its performance with a new set of data with the same features. For this purpose, the cross-validation technique with n-fold (Equation 3) was used (Geisser, 1993).

$$VC_{(n)} = \frac{1}{n}\sum_{i=1}^{n} E_{y_i, \hat{y}_i} \quad (3)$$

Where, n is the validation data number; $E_{y_i, \hat{y}_i}$ is the residue from the difference between the actual output value and the predicted value.

This technique consists of stratifying the database into n subsets (folds), in which n – 1 are used in training, and one validates the model. This process is repeated n times, so that each stratification is used once as a set of tests for model validation. In each training, the classification error of the subsets is calculated, and the final result of this process is the average accuracy of the classifier in the n tests. In this way, an estimation of the classifier quality is obtained, allowing the analysis to be performed (Dias, Sanches, & Alves, & Nogueira 2012).

To evaluate the performance of the classifier on the dataset, the WEKA software was used, and the Cross-validation 10 folds method was applied to the data.

The confusion matrix was generated (Figure 2), which shows the samples that were not correctly classified, based on the reference classes. From this matrix, the following Kappa (Cohen, 1960) (Equation 4), Precision (Equation 5), Sensitivity (Equation 6), and Accuracy (Equation 7) indices were calculated (Kohavi & Provost, 1998).

		Predicted Class	
		ω1	ω2
Class	ω1	TP	FN
	ω2	FP	TN

Figure 2. Adaptation of a confusion matrix formation; TP-True positive; FN-False negative; FP-False positive; TN-True negative. Adapted from (Santra & Christy, 2012)

The Kappa (K) index (Equation 4), which ranges from 0 to 1, provides a measure that is the difference between the concordance examined in the precision of the method employed and the randomized values. In this index, the values are considered excellent when K > 0.81 (Cohen, 1960; Landis & Koch, 1977).

$$K = \frac{\sum_{i=1}^{c} \omega_{ii} - \sum_{i=1}^{c} (\omega_{i+} \cdot \omega_{+i})}{n^2 - \sum_{i=1}^{c} (\omega_{i+} \cdot \omega_{+i})} \quad (4)$$

Where, ω_{ii} is the total number of correctly sorted samples; ω_{i+} is the total number of samples sorted for category i; ω_{+i} is the total of samples collected from category i; n is the total number of samples; c is the number of categories.

The precision (Equation 5) is the rate of correct predictions performed by the classification model on the dataset, that is, it is the proportion of instances that legitimately belong to a class by the total of cases that is classified into such category.

$$Pr = \frac{Tp}{Tp + Fp} \quad (5)$$

Where, Tp is the total of the category classified as true positive; Fp is the total of categories classified as false positive.

Sensitivity (Equation 6) is the ratio of true positives, that is, the model ability to perfectly predict the true class.

$$S = \frac{Tp}{Tp + Fn} \quad (6)$$

Where, Tp is the total of the category classified as true positive; Fn is the total of categories classified as false negative.

The accuracy (Equation 7) is defined as the ratio of correct classifications, without false positives and negatives.

$$Ac = \frac{Tp + Tn}{T} \quad (7)$$

Where, Tp is total of the classifier category as true positive; Tn is the total of the classes categorized as true negative; T is the total dataset.

3. Results

The set of seeds used presents several damages, which can be found in the same embryos. For example, some typical patterns can be observed, such as mechanical damage (DM), characterized by abrasions (Figures 3a and 3b); characteristic damages of moisture deterioration (DU), such as intense red or white lesions on the tissues (Figures 3c to 3k); whitish circular lesions, typical of bedbug bites (DP) (Figure 3k to 3o), and also embryos without apparent lesions (SL) (Figure 3p).

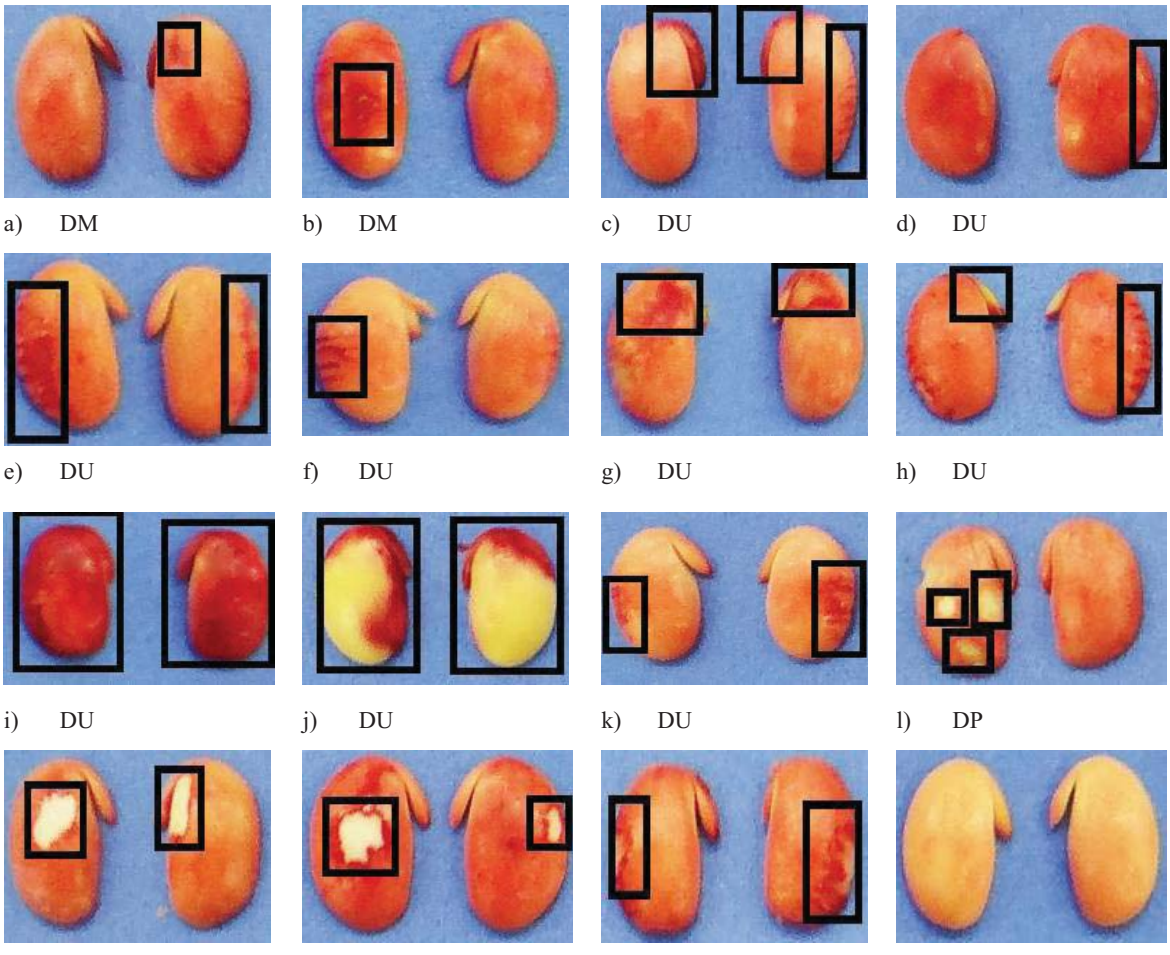


Figure 3. Example of damage present in the training set; MD-Mechanical damage, DH-Deterioration by humidity, BD-Bed bug damage, ND-No damage

The image that shows the training set (Figure 4) was obtained from the composition of an image showing 16 soybean embryos, selected from the images of replication I and II, which satisfactorily describe the standards contained in the tetrazolium test, covering mechanical damage, moisture, bed bugs, and healthy embryos.

Through the interface of extraction of characteristics and training of the classifier model, we identified in the training images the regions with the background patterns (marked in gray), bedbug damage (marked in green), deterioration due to humidity (marked in cyan), and healthy embryos (marked in yellow) (Figure 4). In the process, 39 regions belonging to the background class of the image (68,236 pixels) were detected, also, 141 regions were classified as no damage (48,005 pixels), 2 regions were classified as mechanical damage (470 pixels), 59 regions were classified as deterioration by humidity (30,354 Pixels), and 8 regions were classified as bedbug damage (2,775 pixels). They create a training dataset with 143,291 instances to describe the classes.

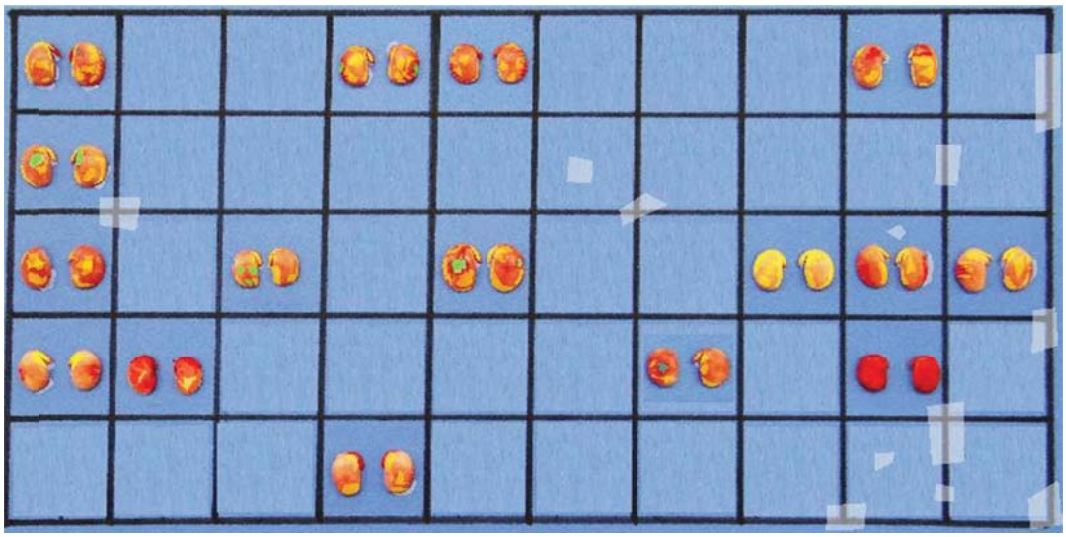
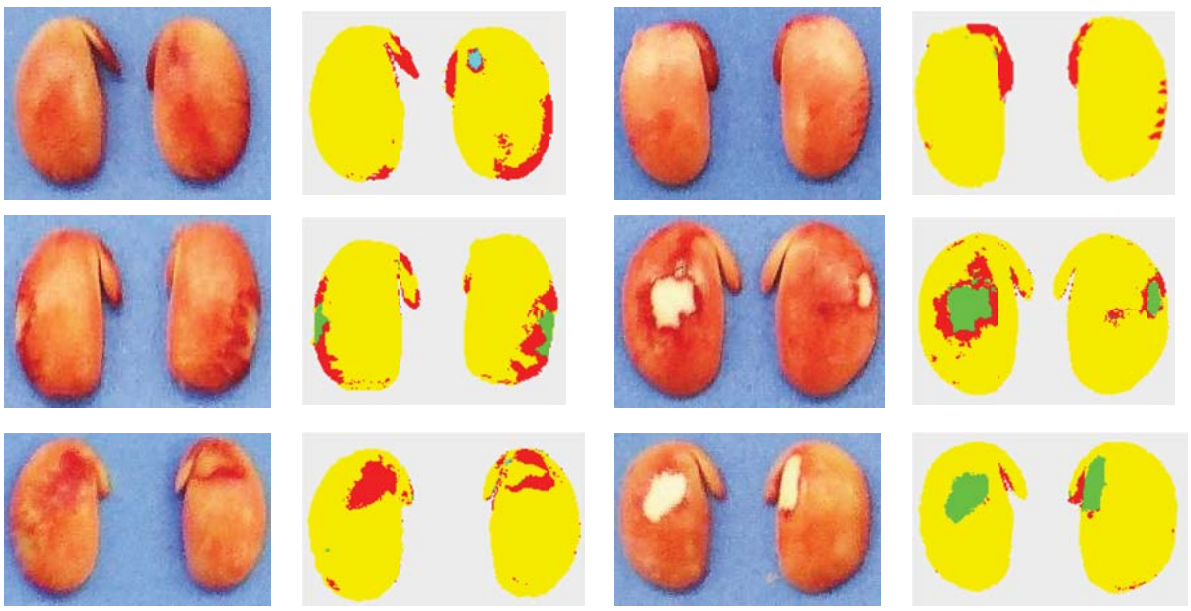


Figure 4. Regions belonging to each class

Once the regions of interest were delimited, the classifier training was performed and, as a result, the classified training image was obtained (Figure 5).



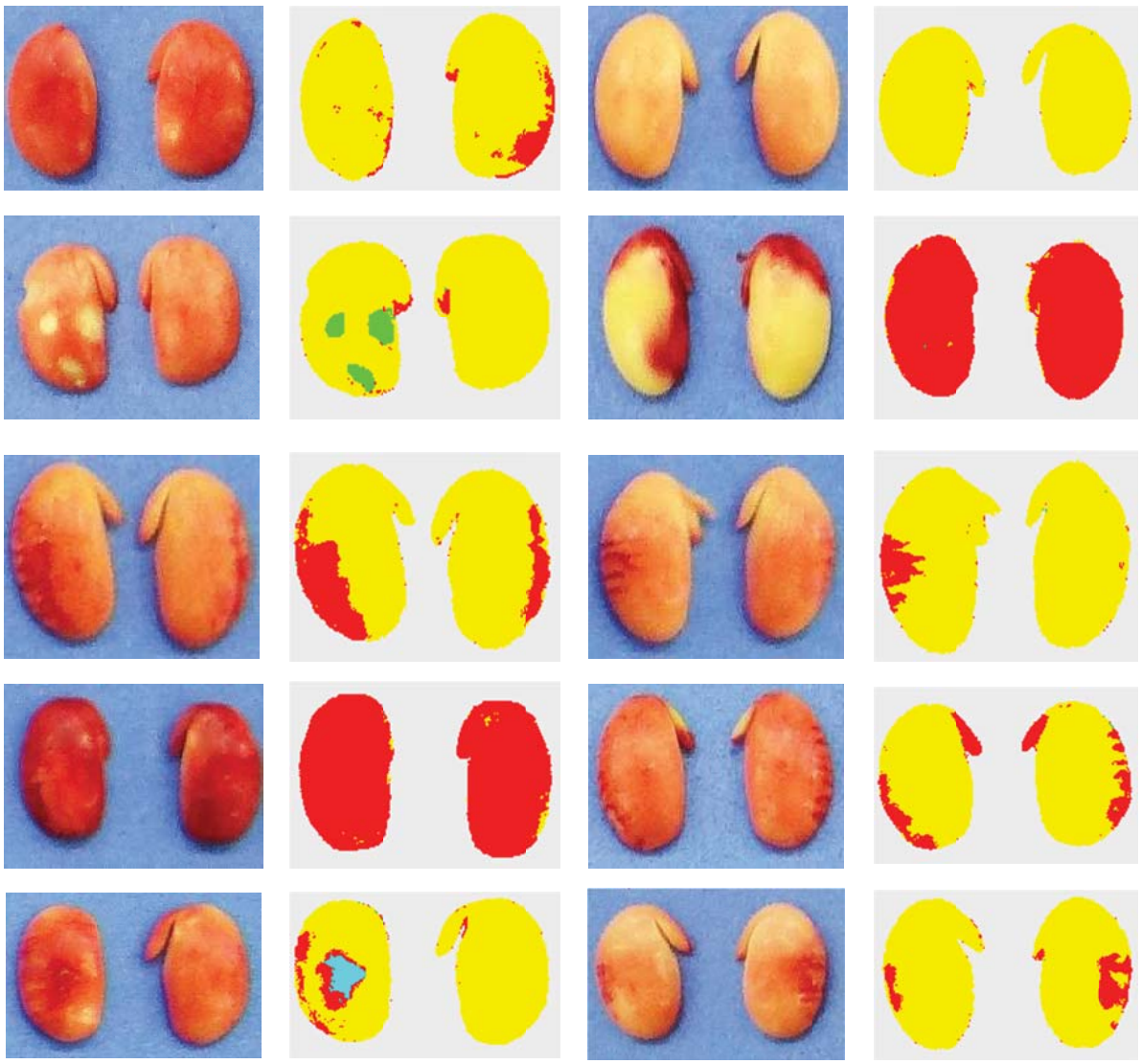


Figure 5. Training images and their sectioned classification; Color legend for segmented images: Gray-background; Red-deterioration by humidity; Green-bed bug damage; Cyan-mechanical damage; Yellow-vigorous tissue

After the image analysis, we realized that a model that would adequately classify the patterns contained in the training image was obtained. This model was the study object to test its own performance.

Table 1 presents the confusion matrix generated from the results obtained by the classifier for the classification data set.

Table 1. Confusion matrix

Class	Predicted class					Σ
	Background	ND	MD	DH	BD	
B	79.255	80	0	121	3	79.459
ND	119	89.019	32	1.592	81	90.843
MD	1	174	1.060	483	0	1.718
DH	201	3.421	60	48.531	77	52.290
BD	16	794	0	487	4.781	6.078
Σ	79.592	93.488	1.152	51.214	4.942	230.388

Note. B-Background; ND-No-damage; MD-Mechanical damage; DH-Deterioration by humidity; BD-Bed bug damage.

We observed that 80 instances among the 79,459 instances of the background class were erroneously classified as healthy embryos, 121, as deterioration by moisture, and three, as bedbug damage. Therefore, the accuracy of the class was 99.7%, with sensitivity of 99.7% and precision of 99.6%.

Regarding no-damage class, 119 instances among 90,843 were misclassified as background, 32 as mechanical damage, 1,592 as deterioration by moisture, and 81 as bedbug damage. The accuracy was 98%, with sensitivity of 96.6%, and accuracy of 95.2%. The mechanical damage class was the one with the lowest correctness, since, among 1,718 instances, one instance was wrongly classified as background, 174 as healthy embryos, and 483 as deterioration by humidity. Its accuracy was 61.7%, with sensitivity of 73.9%, and precision of 92%.

The class of deterioration by humidity presented 201 instances classified as background, 3,421 classified as undamaged, 60 as mechanical damage, and 77 as bedbug damage, obtaining accuracy of 92.8%, sensitivity of 93.8% and precision of 95.2%. Also, the class of bedbug damage presented 16 instances that were mistakenly classified as background, 794 as no-damage, and 487 as deterioration by humidity. Its accuracy was 78.7, with sensitivity of 86.8%, and precision of 96.7%.

The accuracy, sensitivity, precision, and Kappa index values for the model were calculated in relation to data, based on the values of the confusion matrix and cross-validation (Table 2).

Table 2. Classifier performance results

	Ac (%)	Pr (%)	S (%)	Icc (%)	Iic (%)	K
B	99.7	99.6	99.7	96.6	3.4	0.95
ND	98	95.2	98			
MD	61.7	92	61.7			
DH	92.8	94.8	92.8			
BD	78.7	96.7	78.7			
x_p	96.6	96.6	96.6			

Note. B-Background; ND-No-damage; MD-Mechanical damage; DH-Deterioration by Humidity; BD-Bed bug damage; Ac-Accuracy; Pr.-Precision; S-Sensitivity; Icc-Instances correctly classified; Iic-Instances incorrectly classified; K-Kappa Index; x_p-Weighted average.

By the analysis of the results, we observed that, 222,646 instances among 230,388 instances were correctly classified (96.7%), and 7,742 were incorrectly classified (3.4%), with a Kappa index of 0.95. Kulkarni and Lowe (2016), reached similar results studying RF algorithm for land cover classification concluded that their performance was better than all other studied classifiers in terms of overall accuracy and kappa coefficient. Chan and Paelinck (2008), evaluating Random Forest and Adaboost classification for ecotope mapping using hyperspectral imagery concluted that in terms of accuracy performance, RF have outperformed a neural network classifier.

4. Conclusion

Supervised classification combined with pixel-based segmentation has proved to be efficient at extracting information from the tetrazolium test, allowing more accurate evaluations with less subjectivity. Moreover, the image attribute choices, along with the Random Forests algorithm, were efficient in the process of sample

classification with high dimensionality, which leads the development of new alternatives technologies facilitating to perform exhaustive visual tests.

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Influence of Pulp, Sugar and Maltodextrin Addiction in the Formulation of Kiwi Jellies With Lemon Grass Tea

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Abstract

The jellies constitute an important alternative for the processing of fruits, adding greater economic and nutritional value. The objective of this study was to evaluate the effects of different concentrations of pulp, sugar and maltodextrin on the physical-chemical and textural characteristics of kiwifruit jelly with lemon grass tea. Factorial design 2^3 was used with 3 replicates at the central point, resulting in 11 experiments with variation of sugar percentages (30, 40 and 50%), pulp (50, 60 and 70%) and maltodextrin (5, 10 and 15%). Water content, moisture content, total soluble solids (TSS), total titratable acidity (TTA), ashes, pH, reducing sugars, non-sugars were evaluated for the following physico-chemical parameters: reducers, total sugars, lipids and vitamin C. Regarding the texture profile, the following parameters were evaluated: hardness, cohesiveness, chewing, gummy and adhesiveness. It was found that among the analyzed variables, the ones that were considered as significant and/or predictive according to ANOVA and the F test were: (moisture, total solids, carbohydrates and vitamin C), through the graphs of the surfaces of responses observed that the percentage of pulp and maltodextrin used was proportional to the increase in moisture content, vitamin C, total solids and carbohydrates. The G2 experiment presented the lowest values of moisture and water activity, and higher carbohydrate contents, total solids and cohesiveness, in which it was formulated with the sugar concentration (-1) and pulp and maltodextrin (+1). The development of kiwi jelly with lemon grass tea is an excellent alternative for the use of the raw material, since it is a product with high nutritional value, stability during storage and potential for consumer acceptance.

Keywords: *Actinidia deliciosa*, new products, texture

1. Introduction

The kiwi (*Actinidia deliciosa*) is a fruit that has great economic importance, mainly in China, Italy and New Zealand, that are its main producers. It has high content of vitamin C and bioactive compounds, such as phenolic compounds, insoluble fiber, carotenoids, flavonoids and minerals. The nutritional quality and TTAractive taste are responsible for the good acceptance of kiwi worldwide and despite the numerous qualities described above, kiwi fruit is highly perishable due to its sensitivity to mechanical damage and high water content, which makes it possible the development of microorganisms and the occurrence of biochemical reactions that cause their rapid deterioration and consequently many losses in the post-harvest stages (Lyu et al., 2018; Móran et al., 2018).

The purpose of the processing is to increase the stability of the product, to allow its storage for a long period of time, allowing its consumption in the off-season. Jellies constitute an important alternative for fruit processing and is defined as the product obtained from cooking fruits, pulp or juice, mixed with sugar, water and other optional raw materials such as pectin and acidulants. The stabilization of the jellies is obtained through the heat treatment applied, associated to the increase of the soluble solids content, increase of the acidity and decrease of the water content. These parameters are responsible for the inhibition of the growth of microorganisms and enzymatic activity, besides being of great importance for the texture, structure and general quality of fruit sweets, since the adequate gelation of pectins of high methoxylation occurs only in narrow bands of pH (2.8 to 3.5) and high sugar content (600 to 800 g/kg), making it possible to obtain a product with high added value, easy handling

and greater stability. The final product must have a semi-solid consistency and be packaged in a way to guarantee its perfect preservation (Oliveira et al., 2014; Garrido et al., 2015; Sousa et al., 2015; Silva et al., 2018).

During the process of producing jellies, the heat treatment is applied until the desired solids content is reached by evaporation of the water. The concentration of the mixture enables the reduction of water activity, allowing greater microbiological stability to the product and reduction of the microbial load, which allows the increase of the shelf life. In addition to microbiological safety, other aspects of quality in the production of jellies, such as color, texture and physical and chemical parameters that may be affected during storage, should be considered, making it necessary to study the chemical and physical stability of new food products to optimize the process and obtain a product that is well accepted in the market and safe for the consumer (Oliveira et al., 2014).

Maltodextrin is a type of complex carbohydrate, classified as an oligosaccharide with a high glycemic index, low osmotic value and neutral taste, besides being highly soluble in water and easily absorbed by the body. For athletes, the ingestion of maltodextrin before, during and after exercise is very common because its consumption is associated with the rapid and significant increase in glycemia in healthy individuals, providing maintenance of blood glucose levels and avoiding a decrease in performance during the exercise associated with hypoglycemia, in addition to helping to increase muscle glycogen stores during intense and prolonged exercises (Cardoso et al., 2017).

In the food industry, maltodextrin is used in the production of various products and more recurringly it is used as adjuvant in the convective drying process or in the microencapsulation process in spray dryer drying. Maltodextrin has high emulsification capacity, low cost and gives the product greater stability, retention of volatile compounds and reduction of hygroscopicity (Carmo et al., 2015; Cardoso et al., 2017; Freitas et al., 2019).

The objective of the present study was to elaborate and evaluate kiwifruit jellies flavored with holy grass tea, checking the influence of the variables (pulp, sugar and maltodextrin) on their physico-chemical and textural properties, development of new products that meet consumer demand.

2. Material and Methods

For the accomplishment of this research, the fruits of kiwi cv. Hayward (*Actinidia deliciosa*) and lemon grass (*Cymbopogon citratus*) were purchased at the local commerce of the municipality of Campina Grande and conducted to the Laboratory of Storage and Processing of Agricultural Products, belonging to the Federal University of Campina Grande, Brazil.

The fruits were selected for uniformity and maturation stage. Initially the fruits were washed in running water and sanitized in sodium hypochlorite solution at 200 ppm for 15 min. Subsequently, the kiwifruit were peeled with a stainless steel knife and processed in a blender to obtain the pulp.

For the preparation of the lemon grass tea, the ratio 1:1 (lemon grass:water) was used, the water after boiling was placed on the lemon grass and allowed to infuse until the mixture cooled. Thereafter, the mixture was sieved and the tea obtained was used in the preparation of the jellies.

2.1 Processing of Jellies

The kiwi pulp was mixed with the crystal sugar and the maltodextrin, then the mixture was brought to the open pan cooking under heating with continuous manual stirring. To make gel formation possible in the formulations, 1% pectin with high methoxylation content was used, during cooking the same was added previously dissolved in the holy grass tea and the pH of the mixture was corrected to 3.2 by addition of citric acid.

When the jellies reached solids content higher than 65 °Brix, the cooking process was completed, then they were hot packed in pre-sterilized glass containers (100 °C for 30 min) and stored under refrigeration at 5 °C until the moment of the analyzes.

2.2 Physico-Chemical Analysis

The jellies produced were submitted in triplicate, the following physical-chemical analysis: Moisture and total solids in a vacuum oven at 70 °C to constant weight; Ash by muffle incineration; Total protein content was quantified by the Micro-Kjeldahl method, which consisted of the determination of total nitrogen; Total Soluble Solids (SST) in refractometer; Titratable Total Acidity (TTA) determined by titration; Relationship SST/TTA (ratio); pH measured directly in digital potentiometer according to the methodology described by Brazil (2008); The lipid content was determined by the method of Bligh and Dyer (1959); The total carbohydrate content was calculated by difference to obtain 100% of the total composition (FAO, 2003); Water activity (a_w) was

determined using the Decagon® Aqualab CX-2T device at 25 °C. Non-reducing sugars (NR), reducing sugars (RS) and total sugars (TA) were determined by the method described by Lane and Eynon (1934); the content of ascorbic acid (Vitamin C) was determined according to the methodology proposed by Adolfo Luttz Institute (Brazil, 2008) and the results expressed in mg of ascorbic acid/100 g sample.

2.3 Texture Profile

To obtain the parameters of the jelly's instrumental texture profiles, the TPA test in the TAXT Plus Texturometer (Stable Micro Systems) equipped with the ExponentStable Micro Systems software, using the P/36R probe, was used. In the texture profile, the studied TTAributes were firmness, cohesiveness, adhesiveness, guminess and chewing.

2.4 Statistic Analyzes

Kiwi jellies were processed using the factorial design method 23 with 3 replicates at the central point, resulting in a matrix with 11 experiments (Table 1), in order to evaluate the influence of the independent variables (concentrations of sugar, pulp and maltodextrin) on the variables responses (physical-chemical and textural characteristics, as well as the interactions between them). The effect of the independent variables on the dependent variables was evaluated by statistical analysis, using the Statistica® software version 7.0.

Table 1. Planning matrix for the elaboration of kiwifruit jams with sacred grass tea, with their respective independent variables and their actual and codified levels

Experiments	Independent variables		
	Sugar (%)	Pulp (%)	Maltodextrin (%)
G_1	+1(50)	+1 (70)	+1 (15)
G_2	-1 (30)	+1 (70)	+1 (15)
G_3	+1(50)	-1(50)	+1 (15)
G_4	-1 (30)	-1(50)	+1 (15)
G_5	+1(50)	+1 (70)	-1 (5)
G_6	-1 (30)	+1 (70)	-1 (5)
G_7	+1(50)	-1(50)	-1 (5)
G_8	-1 (30)	-1(50)	-1 (5)
G^9 (C)	0 (40)	0 (60)	0 (10)
G^{10} (C)	0 (40)	0 (60)	0 (10)
G^{11} (C)	0 (40)	0 (60)	0 (10)

Note. G1, G2 ... G11: Jelly kiwi with lemon grass tea; (C) Central point.

3. Results and Discussion

Table 2 presents the mean values of the variable responses for the physical-chemical characteristics of kiwifruit jelly with lemon grass tea.

Table 2. Results of physical-chemical analysis of kiwifruit jelly with lemon grass tea

Experiments	Moisture content (%)	a_w	TTA (% citric acid)	pH	SST (°Brix)	Ratio	ANR (%)	AR (%)
G_1	35.45	0.829	0.617	3.59	63.33	97.75	46.69	36.31
G_2	32.55	0.805	0.495	3.49	62.67	104.70	44.69	34.08
G_3	35.81	0.822	0.682	3.47	64.33	79.74	40.57	29.55
G_4	34.49	0.815	0.649	3.50	68.33	105.24	38.44	28.95
G_5	35.11	0.819	0.637	3.56	65.33	87.00	44.91	35.29
G_6	33.64	0.818	0.540	3.57	66.33	117.31	44.17	21.91
G_7	36.79	0.857	0.862	3.53	63.33	61.91	39.69	27.91
G_8	33.90	0.818	0.655	3.51	64.33	105.24	40.18	25.80
G_9	34.57	0.809	0.682	3.50	72.33	98.30	39.76	22.15
G_{10}	34.62	0.809	0.696	3.49	72.67	104.38	40.31	21.23
G_{11}	34.34	0.807	0.693	3.49	72.33	104.45	40.35	21.17
Experiments	AT (%)	ST (%)	Proteins (%)	Lipidis (%)	Carbohydrates (%)	Ashes (%)	Vitamin C[1]	
G_1	83.00	64.55	0.25	0.35	63.64	0.31	20.24	
G_2	79.98	67.45	0.23	0.29	66.61	0.32	14.04	
G_3	70.13	64.19	0.24	0.36	63.23	0.36	18.77	
G_4	66.36	65.51	0.20	0.29	64.71	0.31	20.03	
G_5	78.99	64.89	0.22	0.37	63.98	0.33	17.68	
G_6	76.07	66.36	0.24	0.28	65.56	0.29	15.54	
G_7	68.64	63.21	0.22	0.36	62.25	0.37	20.46	
G_8	65.98	66.10	0.24	0.25	65.32	0.29	18.05	
G_9	61.91	65.43	0.25	0.17	64.63	0.38	17.84	
G_{10}	61.54	65.38	0.24	0.17	64.58	0.39	17.92	
G_{11}	61.52	65.66	0.24	0.17	64.86	0.40	17.82	

Note. [1] Results expressed in mg of ascorbic acid/100 g sample.

The moisture content of kiwifruit jams with lemon grass tea ranged from 32.55 (G2) to 36.79% (G7), lower values of this parameter were observed in the experiments at the lower level (-1) of pulp and maltodextrin. It can also be stated that the values of moisture content obtained are presented according to the quality standard established by the Brazilian legislation (Brazil, 1978), which indicates that the maximum moisture content for fruit jellies should be lower to 38%. In relation to the water activity (a_w) there was a variation of 0.805 to 0.857, this parameter presented a behavior similar to that observed with respect to the moisture content, in which lower values were observed in samples containing less percentage of pulp.

According to Barros et al. (2019a), reduced values of moisture content and water activity indicate higher stability of the product during storage and foods that have a moisture content of more than 20% and a higher water activity of 0.60 are subject to deterioration processes caused by molds and yeasts.

Titratable total acidity (TFA) values expressed as citric acid ranged from 0.495 (G2) to 0.862% (G7). These percentages are similar to those observed by Oliveira et al. (2019) in achachairu jellies (0.500 to 0.690%) and by Barros et al. (2019a) in pineapple jellies with cinnamon (0.47 to 0.99% citric acid), the authors stated that although the legislation does not indicate the range of TTA suitable for jellies, values lower than 0.3% or higher than 0.8% may cause loss of elasticity of the jelly due to pectin hydrolysis.

The hydrogenation potential (pH) of the samples varied from 3.47 (G3) to 3.59 (G1), the samples were adequate to Brazilian legislation for fruit products (Brazil, 2005), which establishes that the maximum limit for this parameter is 4.5. Teles et al. (2017) when developing graviola jelly with pepper obtained pH values close to the present study (3.69 to 3.93), however, Garcia et al. (2017) stated that the ideal pH range for gel formation to occur is 3.0 to 3.2. While for Bolzan and Pereira (2017), the ideal pH range for gel formation is 3.0 to 3.5.

The jellies presented total soluble solids content (TSS) ranging from 62.67 (G2) to 72.67 °Brix (G10) and are in accordance with the legislation, which establishes a minimum SST content for common 62 °Brix jelly (Brazil, 1978). Values similar to those were obtained by Oliveira et al. (2016) in oat-enriched orange jellies (62 to 66 °Brix). High levels of total soluble solids (TSS) associated to low water content and pH are capable of minimizing the development of microorganisms and may favor the formation of crystallization of sucrose, which is responsible for improving the viscosity and texture of the product (Oliveira et al., 2019; Barros et al., 2019a).

Regarding the Ratio parameter, a variation from 61.91 (G7) to 117.31 (G6) was observed, indicating that the G6 sample has a higher degree of sweetness. For, according to Sousa et al. (2018), the Ratio parameter is a technological index used to indicate the relationship between SST and TTA of the product and is able to evaluate the taste of the product, also indicating the degree of sweetness.

The content of reducing sugars varied from 21.17 (G11) to 36.31% (G1) and the non-reducing sugars varied from 38.44 (G4) to 46.69% (G1), as expected, the highest values were verified in the samples with the highest percentage of sugar. The total sugars obtained in the present study presented a variation from 61.52 (G11) to 83% of glucose (G1), values higher than that observed by Martins el al. (2015) in mixed jelly of pineapple peel and peach pulp (44.56%).

In relation to the total solids content, a variation from 63.21 (G7) to 67.45% (G2) was observed, values obtained were directly proportional to the percentage of maltodextrin and kiwi pulp used in the formulations, similar values were obtained by Barros et al. (2019b) in blackberry jelly.

Kiwifruit jams with low-salt tea have low protein values (0.20 to 0.25%) and are slightly lower than those observed by Silva et al. (2018) in sweet orange jelly (0.58 to 0.62%). The jellies also presented low lipid content (0.17 to 0.37%), being slightly higher than that found by Souza et al. (2015) in blackberry jellies (0.09 to 0.15%). Regarding the carbohydrate content, a variation of 62.25 to 66.61% was observed, values similar to those observed by Souza et al. (2018) in umbu jelly and mango (67.29 to 70.03%).

As regards ash content, low values (0.29 to 0.40%) were observed, as verified by Oliveira et al. (2019) in achachairu jelly (0.28 to 0.80%). The vitamin C content (ascorbic acid) ranged from 14.04 to 20.46 mg. This parameter was found to correlate with the percentage of sugar and maltodextrin used. These values are similar to those quantified by Azevedo et al. (2018) in manuring jellies (7.40 to 14.19 mg).

Table 3 presents the mean values of the variable responses for the textural characteristics of kiwifruit jelly with lemon grass tea.

Table 3. Results of the texture profile of kiwifruit jelly with lemon grass tea

Experiments	Firmness (N)	Cohesiveness (N.m)	Adhesiveness (N.m)	Gumminess (N)	Chewiness (J)
G_1	0.263	0.8404	0.285	0.2210	0.2210
G_2	0.228	0.8876	0.184	0.2023	0.2024
G_3	0.216	0.8769	0.113	0.1894	0.1894
G_4	0.247	0.8457	0.268	0.2089	0.2089
G_5	0.228	0.8833	0.185	0.2036	0.2037
G_6	0.224	0.8838	0.179	0.1979	0.1980
G_7	0.256	0.8332	0.302	0.2133	0.2133
G_8	0.233	0.8594	0.229	0.2002	0.2002
G_9	0.242	0.8527	0.251	0.2063	0.2063
G_{10}	0.231	0.8667	0.223	0.2002	0.2002
G_{11}	0.234	0.8607	0.231	0.2014	0.2014

It can be seen from Table 3 that the firmness parameter presented a variation from 0.216 to 0.263 N, the highest value obtained for the sample (G1). According to Garrido et al. (2015), firmness is defined as the force required to reach a given deformation, in the context of sensory analysis, represents the force required to compress the food between the molars at the first bite.

With respect to cohesiveness, small variations (0.8404 to 0.8933 N.m) were observed, in which the highest value was verified in sample G5. Besbes et al. (2009), when evaluating the cohesiveness of the date jelly, obtained values ranging from 0.51 to 0.77 N.m. According to Atallah and Morsy (2017), this parameter is often discussed in terms of adhesion forces and is responsible for the deformation occurring in the material prior to rupture, indicating its structural integrity.

The samples presented a variation from 0.113 (G3) to 0.302 N.m (G7) in the adhesiveness parameter, values similar to that obtained by Abid et al. (2018) in pomegranate jellies using different types of gelling agents (0.158 to 0.807 N.m). According to Guiné et al. (2015), adhesiveness is the force required to remove the material adhering to a specific surface and during food intake corresponds to adherence to the lips, mouth and teeth.

The guminess of the jellies varied from 0.189 (G3) to 0.221 N (G1), similar values were observed by Curi et al. (2017) in physalis jellies (0.033 to 0.476 N). For Bolzan and Pereira, (2017), gum is a parameter associated with firmness and cohesiveness, its variation being the reflection of these.

Superior chewability value was obtained in the formulation G1 (0.221 N), similar to that obtained by Curi et al. (2018) in physalis jams with brie cheese (0.08 to 0.58 N). According to Curi et al. (2017) chewability is the parameter that represents the energy needed to chew a solid food to the point of ingestion. Therefore, it can be stated that the G1 sample has greater resistance to chewing when compared to the others.

Several factors can be associated with the variation in texture between kiwifruit jams with lemon grass, such as the percentage of raw materials (sugar, kiwi pulp and maltodextrin) and chemical parameters such as pH, acidity and humidity (Curi et al., 2018).

Table 4 shows the analysis of variance (ANOVA) and the F test with 95% confidence only for the variables that were significant and/or predictive (moisture, vitamin C, total solids and carbohydrates) in the processing of kiwi jelly with tea of lemon grass.

Table 4. Analysis of variance (ANOVA) for moisture, total solids, carbohydrates and vitamin C of kiwifruit jelly with holy grass tea

Source of variation	Quadratic sum	DF	Average Quadratic	Fcal	Ftab	Fcalculated/Ftabulated	R^2
Moisture							
Regression	12.735	7	1.819	39.24 (1)	6.16(3)	6.37	98.92
Waste	0.1391	3	0.0463				
Lack of adjustment	0.0922	1	0.0921	3.93(2)	19(4)	0.21	
Pure error	0.0469	2	0.0234				
Total	12.874	10					
Total solids							
Regression	12.734	7	1.819	39.241(1)	6.16(3)	6.37	98.92
Waste	0.1391	3	0.0463				
Lack of adjustment	0.0922	1	0.0921	3.931(2)	19(4)	0.21	
Pure error	0.0469	2	0.0234				
Total	12.874	10					
Carbohydrates							
Regression	13.890	7	1.984	27.524(1)	6.16(3)	4.47	99.69
Waste	0.2162	3	0.0721				
Lack of adjustment	0.1718	1	0.1718	7.736(2)	19(4)	0.41	
Pure error	0.0443	2	0.0222				
Total	14.106	10					
Vitamin C							
Regression	37.575	7	5.3678	119.341)	6.16(3)	19.373	99.64
Waste	0.1349	3	0.0449				
Lack of adjustment	0.1287	1	0.1287	41.39(2)	19(4)	2.18	
Pure error	0.0062	2	0.0031				
Total	37.709	10					

Note. (1) MS Regression/MS Residue; (2) MS No adjustment/MS Pure Error; (3) F95%, 7.3; (4) F95%, 1.2.

From the analysis of the results obtained with respect to the parameters of moisture, vitamin C, total solids and carbohydrates, it was verified that the coefficients of determination were superior to 98%, indicating a better fit to the experimental data. For all the studied parameters, the calculated F was superior to the F tabulated for the regression. The inverse occurred for the lack of adjustment, except for the parameter of vitamin C. These data show the statistical significance of the models.

The individual effects of the independent variables (sugar, pulp and maltodextrin) as well as the interaction between them on the response variables (physical, chemical and textural analysis) presented a statistically significant model (Fc ≥ Ftab). It can be verified in the pareto diagrams (Figure 1) the factors that had the greatest influence on the processing of the jelly.

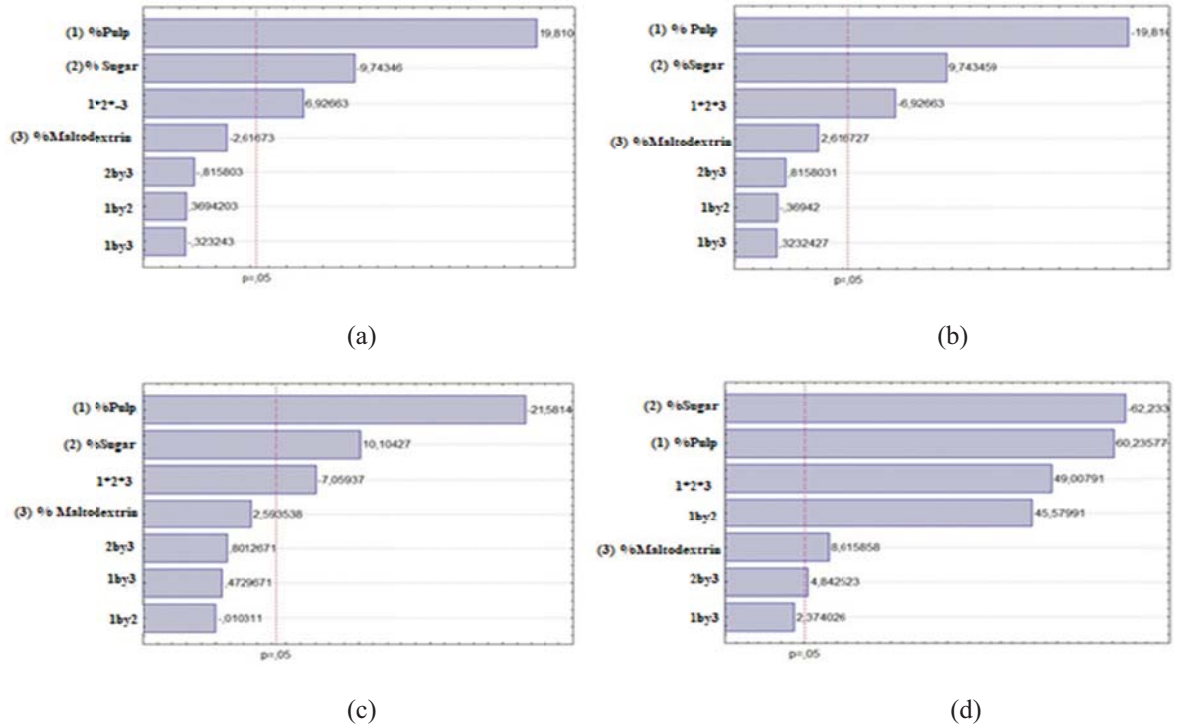

Figure 1. Pareto diagram for influence of sugar, pulp and maltodextrin concentration factors for the parameters: a) Moisture; b) Total solids; c) Carbohydrates; d) Vitamin C

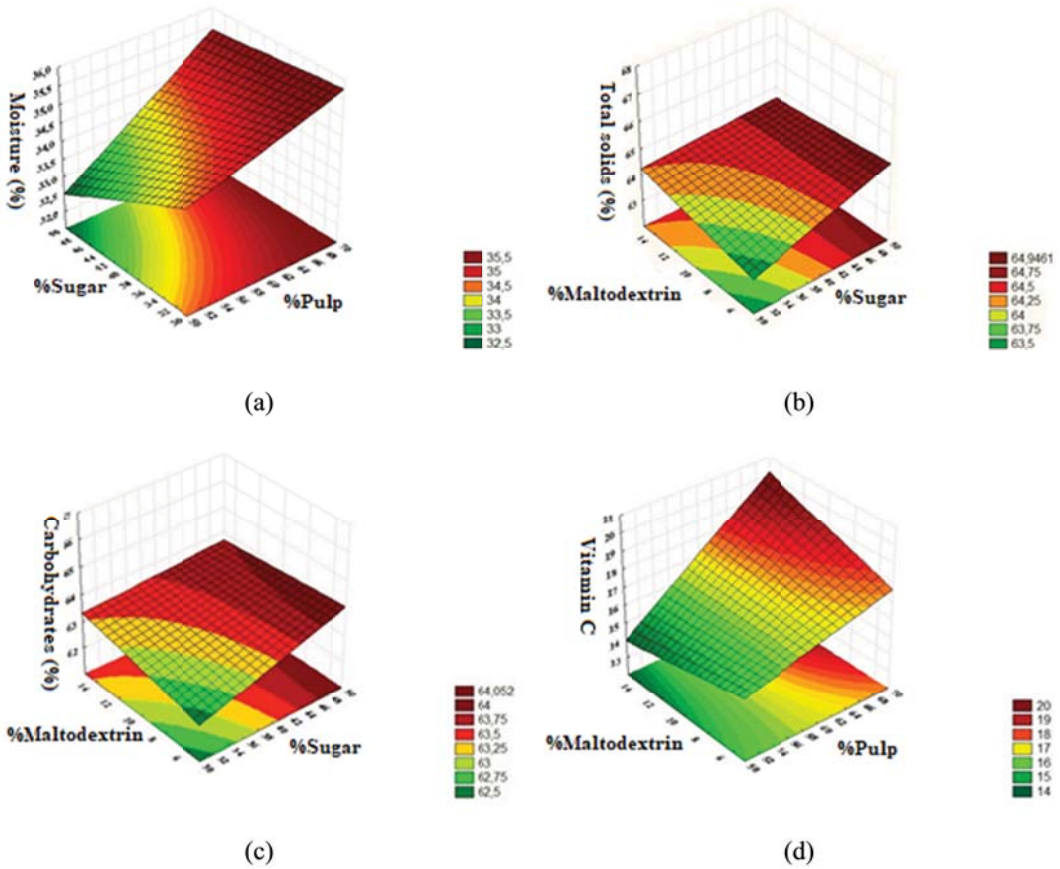

Figure 2. Response surfaces for the parameters of: a) moisture; b) total solids; c) carbohydrates; and d) vitamin C of the kiwifruit jelly with lemon grass, according to the percentages of sugar, pulp and maltodextrin

From the pareto graphs with a significance level of 95%, it was possible to observe that the percentage of sugar, pulp and the interaction between the percentage of sugar, pulp and maltodextrin were the factors that most influenced significantly the variables responses (moisture, total solids, carbohydrates and vitamin C). It is also verified that the percentage of maltodextrin only showed significant effect for the content of vitamin C.

The response surfaces obtained for the physical and chemical analyzes (response variables) presenting statistically significant models (Fc ≥ Ftab) are represented in Figure 2.

According to the graphs of the response surfaces, it is possible to infer that as the percentage variables of pulp and maltodextrin increase, parameters of moisture, total solids, carbohydrates and vitamin C tend to grow.

4. Conclusion

Through the present study, it was verified the viability of the use of kiwi and lemon grass in the elaboration of jelly, which is a product of high nutritional quality. It was verified that all the samples are in agreement with the standards of quality established by the Brazilian legislation, presenting in this way potential of commercialization. The independent variables that most influenced the formulation of the jellies were: percentage of sugar, pulp and the interaction between the percentage of sugar, pulp and maltodextrin. It was verified that the increase in the percentage of pulp and maltodextrin, provided the increase of moisture content, total solids, carbohydrates and vitamin C. The experiment G2 presented the lowest values of moisture and water activity, and higher carbohydrate contents, solids total and cohesiveness, in which it was formulated with the sugar concentration (-1) and pulp and maltodextrin (+1).

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Reactive Natural Phosphate in Safflower Fertilization in Cerrado Oxisol

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Abstract

Fertilizer management has a direct influence on crop productivity, especially phosphorus, which is most limiting to the development of crops in tropical soils due to the genesis of these soils. In this sense, it is necessary to use nutrient sources that are agronomically efficient at reduced costs compared to conventional sources. Thus, the objective of the present study was to evaluate the effects of reactive natural phosphate as a source of phosphorus on the development, growth, and yield of safflower in Cerrado Oxisol. The experiment was carried out in a greenhouse at the Federal University of Mato Grosso, Campus of Rondonópolis. The completely randomized design consisted of the following treatments: 0, 100, 200, 400 and 600 mg dm^{-3} of reactive natural phosphate (Bayóvar reactive phosphate), with 6 replicates, consisting by pots with 2 dm^3 of capacity. To the Oxisol used to fill the plots was incorporated dolomitic limestone to increase base saturation to60%. Safflower cultivar used was IMA 0213 with a final population of three plants per plot. Plant height, number of leaves and chlorophyll index were evaluated at 15, 30, 45 and 55 days after emergence. In the last evaluation plants were cut and the number and diameter of the chapters, shoot and chapters dry mass, volume and root dry mass were also evaluated. The results were submitted to analysis of variance and regression up to 10% probability. In general, safflower crop shows a positive response to application of reactive natural phosphate. Doses between 389 and 600 mg dm^{-3} promoted best results for development and safflower production in an Oxisol.

Keywords: *Carthamus tinctorius* L., Bayóvar, oilseed, fertilizer management, alternative crops

1. Introduction

The increased demand for biofuels has now been the main reason for the growth of research on alternative energy. Faced with the need for new energy sources, the large-scale use of biomass energy is seen as an option that could contribute to more sustainable development. Thus, the importance of oilseed crops, especially safflower (*Carthamus tinctorius* L.), has increased, especially with interest in the production of biofuels (Dordas & Sioulas, 2008).

According to Coronado (2010), safflower is an oleaginous crop cultivated for more than two millennia, and its raw material is destined for the production of oil both for human consumption and for the industry with diverse uses (Shahrokhnia & Sepaskhah, 2016). Seeds have high oil content (27 to 47%), presenting potential as raw material for biodiesel production, as well as minerals (Zn, Cu, Mn and Fe), vitamins (thiamine and β-carotene) and tocopherols (α, β and γ), of a high quality oil (Velasco et al., 2005).

Safflower cultivation presents great productive and adaptable potential, as well as agronomic desirable characteristics, such as high quality and quantity of oil produced, tolerance to water deficit, soil salinity, high temperatures and low relative humidity air (Kizil, 2008), these characteristics justify their cultivation in regions with a hot climate and irregular rainfall, such as the Brazilian Cerrado in the dry period, diversifying the production in these areas, is therefore an alternative for crop rotation in Brazil (Anicésio et al., 2015; Bonfim-Silva et al., 2015).

Phosphorus (P) is the most limiting nutrient for agricultural production in tropical soils, characterized by the high degree of weathering and low levels of available phosphorus in the plants (Novais & Smyth, 1999; Bonser et al.,

1996; Rocha et al., 2005), as well as low phosphorus availability in the Oxisols, these present high phosphorus fixation capacity due to high acidity, low base saturation and cation exchange capacity, with predominance of iron and aluminum oxides in their mineralogy and high exchangeable aluminum content (Guedes et al., 2009; EMBRAPA, 2006).

In order to supply the required amount of phosphorus in agricultural crops in Cerrado soils where there is a predominance of Oxisols, the use of soluble phosphorus sources, such as superphosphates, promotes the immediate availability of the nutrient to the soil due to its greater agronomic efficiency, but their sources presents higher costs due to their process of industrialization, the deficit of reserves of quality phosphate rocks, and that part of the nutrient will be subject to fixation in the soil, reducing its availability to the plants (Lima et al., 2007; Horowitz & Meurer, 2004).

An alternative to reducing costs with soluble phosphate fertilizers has been the use of less soluble phosphorus sources, such as reactive natural phosphates (Raij, 2011). The reactive natural phosphates have a lower acquisition cost and can reduce the fixation of phosphorus in the soil, since they react in a gradual way, presenting a residual effect that, when estimated for long periods, can have its effect equal to the soluble phosphates (Freire et al., 2005).

In this context, the objective was to evaluate doses of reactive natural phosphate as a source of phosphorus in the development, growth, and yield of safflower cultivation in Cerrado Oxisol.

2. Material and Methods

2.1 Overview and Experimental Design

The experiment was carried out in a greenhouse at the Federal University of Mato Grosso, Campus of Rondonópolis-MT, located geographically at latitude 16º27′49″ S, longitude 54º34′47″ W and altitude of 284 m. The region climate is classified as Aw according to Köppen, characterized by a warm and humid climate, with two defined seasons: one rainy in the summer, and another dry coinciding with the winter. The average temperature and relative humidity of the air during experiment were 27 °C and 81%, respectively.

2.2 Soil Characterization and Preparation

Soil used in the experiment was collected in an area under Cerrado vegetation in the 0-0.20 m depth layer of an Oxisol with a sandy-loam texture (EMBRAPA, 2013), sieving of 4 mm of opening to fill the experimental plots. A soil sample was sieved in a 2 mm aperture mesh for the chemical and granulometric characterization according to Embrapa (2011).

The analysis of Oxisol presented the following chemical and granulometric characteristics: pH ($CaCl_2$) = 4.0; P = 1.1 mg dm^{-3}; K = 43 mg dm^{-3}; Ca = 0.5 cmolc dm^{-3}; Mg = 0.3 cmolc dm^{-3}; Al = 1.2 cmolc dm^{-3}; H + Al = 7.4 cmolc dm^{-3}; O.M. = 28.9 g kg^{-1}; sand = 425 g kg^{-1}; silte = 100 g kg^{-1}; e clay = 475 g kg^{-1}; bases sum of 0.9 cmolc dm^{-3}; CEC = 8.3 cmolc dm^{-3}; V = 11%.

The soil acidity was corrected with the incorporation of dolomitic limestone (Relative Power of the Total Neutralization (PRNT) = 80.3%), with the objective of increasing base saturation at the level of 60% (Anicésio et al., 2015). After liming, the soil was moistened and maintained at 60% of the maximum water retention capacity, remaining incubated for a period of 30 days.

The statistical design was completely randomized with five treatments and six replications, totaling 30 experimental plots, represented by plastic pots with a capacity of 2 dm^3 of soil. The treatments were constituted by the application of 0, 100, 200, 400 and 600 mg dm^{-3} of reactive natural phosphate (29% of total P) to the soil. It was used as a plant species, safflower (*Carthamus tinctorius* L.), cultivar IMA 0213, obtained from the germplasm bank of the Mato Grosso Cotton Institute (IMA), Brazil.

After the incubation period of the limestone, the soil was removed from the plastic bags and packed in the pots composing the experimental units. Ten seeds were sown per pot at a depth of 0.02 m and thinned at 7 and 15 days after plant emergence based on vigor, homogeneity and size criteria, establishing a final population of three plants per pot.

Planting fertilization was carried out simultaneously with the sowing of safflower. All treatments were also fertilized with nitrogen (N), potassium (K_2O) and micronutrients, with sources as urea, potassium chloride and FTE-BR 12, respectively.

Nitrogen fertilization was carried out in three equal rates, at 15, 30 and 45 days after emergence, with both applications performed as a solution. The potassium recommendation was 200 mg dm^{-3} of K_2O applied in the solid and granular form. Planting fertilization consisted of applying also 15 mg dm^{-3} of the formulated FTE-BR

12 (Sulfur: 3.9%, Copper: 0.85%, Boron: 1.8%, Manganese: 2.0% and Zinc: 9.0%).

The reactive natural phosphate used comes from Bayóvar (Peru) and has 29% phosphorus (P_2O_5), 14% citric acid and 32% calcium in its composition (Farias, 2012), and the doses (0, 100, 200, 400 and 600 mg dm^{-3}) were added to the soil at the time of planting.

Irrigation management was the gravimetric method. The maximum water retention capacity of the soil was determined in the laboratory, the pot capacity (maximum soil water retention capacity) was determined according to the methodology described by Bonfim-Silva et al. (2011). During the conduction of the experiment, the humidity was maintained at 60% of the maximum water retention capacity of the soil.

2.3 Response Variables

Plant growth and development evaluations were performed at 15, 30, 45 and 55 days after emergence (DAE), in which: plant height (unit measure) was obtained by measuring the distance from the plant collar to the soil to the apex of each plant present in the pot with the aid of a graduated ruler, with an average to compose the height of the plot; number of leaves (unit measure) by manual counting and their values expressed in units and chlorophyll index (SPAD), obtained from average of six readings performed on random sheets in the middle third of the plants, using the portable chlorophyll meter model Minolta SPAD-502.

At the time of the 55 DAE cut-off, the number of chapters (unit measure) obtained by means of counting and the diameter of chapters (unit measure) with a measurement made in the middle and cross section of the chapter was evaluated with the aid of a digital caliper.

The plants were cut close to the ground and separated into leaves + branches and chapters and packed in paper bags, identified and transferred to a drying oven with forced air circulation at 65 ºC for 72 hours. After drying, material was weighed to obtain dry mass of the shoot and of chapters (unit measure).

The roots of the safflower were collected and washed on a 4 mm opening sieve, and to obtain the root volume, in cm^3, the volume was measured in a graduated cylinder and the difference found corresponded to the root volume. The roots were conditioned in paper bags and oven dried at 65 ºC for 72 hours, after which the material was weighed to obtain root dry mass (unit measure).

The results were submitted to statistical analyses using the statistical program SISVAR (Ferreira, 2011) with analysis of variance and regression test with significance level at 1, 5 and 10% probability.

3. Results and Discussion

Plant height at 15, 30 and 55 days after emergence (DAE), responded to the linear regression model as a function of phosphorus doses. The maximum height of 11.13, 24.28 and 27.16 cm plants was observed in the highest dose of the natural reactive phosphate used, with an increase of 37.30, 45.72 and 31.75%, respectively, when compares the highest dose (600 mg dm^{-3}) with the control (Figure 1).

For the third evaluation performed at 45 DAE, plant height varied significantly with soil phosphorus doses, adjusted to the quadratic regression model, with maximum height (26.84 cm) at the dose of 556.95 mg dm^{-3} (P_2O_5) (Figure 1).

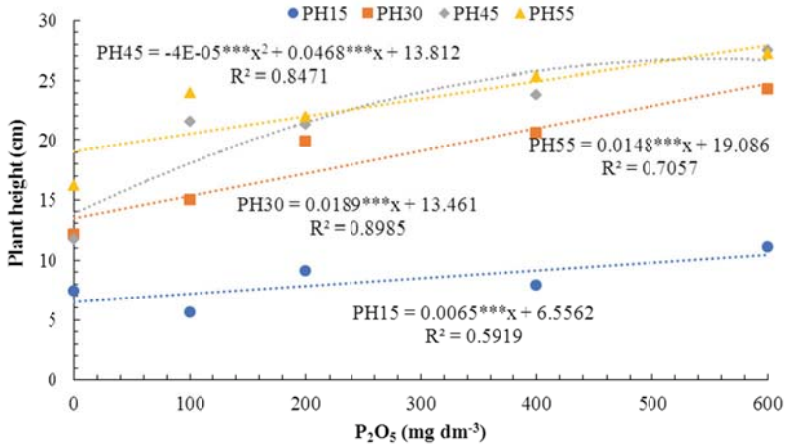

Figure 1. Plant height as a function of phosphorus (P_2O_5) doses, with a natural phosphate source reactive at 15, 30, 45 and 55 days after emergence (DAE). *** Significant at 1%

Phosphorus acts directly in the process of cell division in addition to energy generation of the plants, consequently, it is induced that, plants that have developed in insufficient levels of phosphorus in the soil, have lower heights that have developed in higher levels of that element (Machado, 2011), such us observed in this research.

According to Malavolta (2006), the sources of natural phosphate possess significant amounts of calcium in its composition, neutralizing the aluminum present in the soil and helping in the reduction of the concentration of hydrogen ions, making the soil propitious to the development of the roots favoring the growth of the plants.

Similar behavior to plant height was observed when the number of leaves of the safflower plants was analyzed, adjusting to a linear regression model always except the 45 DAE. When comparing the highest dose of phosphorus with the absence of fertilization, the number of leaves presented increases of 26.53, 35.40 and 30.95% for the evaluations performed at 15, 30 and 55 after the emergence of the plants respectively (Figure 2). At 45 DAE, the number of leaves was significantly increased with the phosphorus doses, adjusting to the quadratic regression model, with the maximum value (16.46 leaves) provided by the dose of 500 mg dm^{-3} of P_2O_5, presenting an increase of 45.56% of production in relation to the absence of phosphate fertilization (Figure 2).

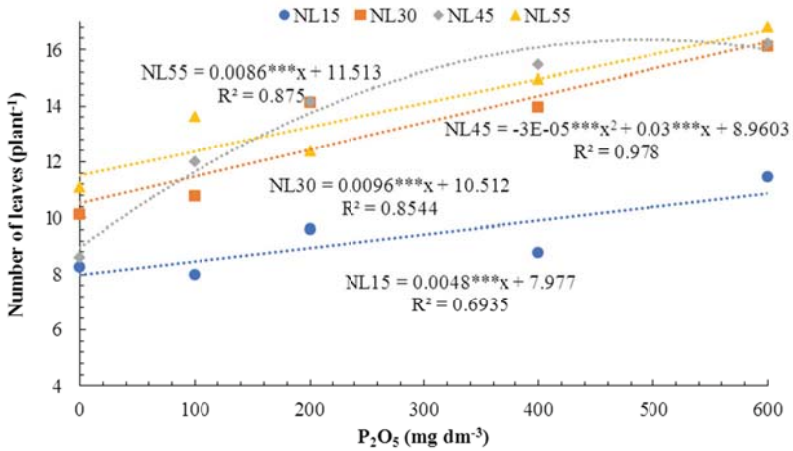

Figure 2. A number of leaves as a function of phosphorus doses (P_2O_5) with a natural phosphate source reactive at 15, 30, 45 and 55 DAE. *** Significant at 1%

The linear increase in the number of leaves due to the addition of phosphorus is mainly due to the dynamics of this element, than in highly weathered soils, composed mainly of clays of the 1:1 type, which has high affinity with the soil phosphate ions end up disabling a good part of the nutrient for the solution of the soil, consequently its absorption (Raij, 1991; Novais, 1999).

Bonfim-Silva et al. (2017), analyzed the development of safflower cultivated in the same soil of the present experiment in phosphorus levels up to 540 mg dm^{-3}, using triple superphosphate source and noticed a linear increase in the number of safflower leaves. This increase may have occurred mainly due to the alteration of the source used, since when fertilized with natural phosphate, the release of phosphorus to the soil solution occurs more slowly and gradually when compared to other sources of faster solubilization.

Phosphate fertilization influenced the chlorophyll content of safflower leaves, adjusting to the quadratic model of regression in the evaluation performed at 30 DAE, demonstrating that the phosphorus dose of 389.17 mg dm^{-3} was responsible for the highest chlorophyll index (50.87) with an increase of 17.86% when compared to the absence of phosphate fertilization (Figure 3).

For the evaluation performed at 45 DAE, the highest dose of natural reactive phosphate used allowed a 15.04% increase in the chlorophyll index when compared to the highest dose (600 mg dm^{-3}) with the control (Figure 3).

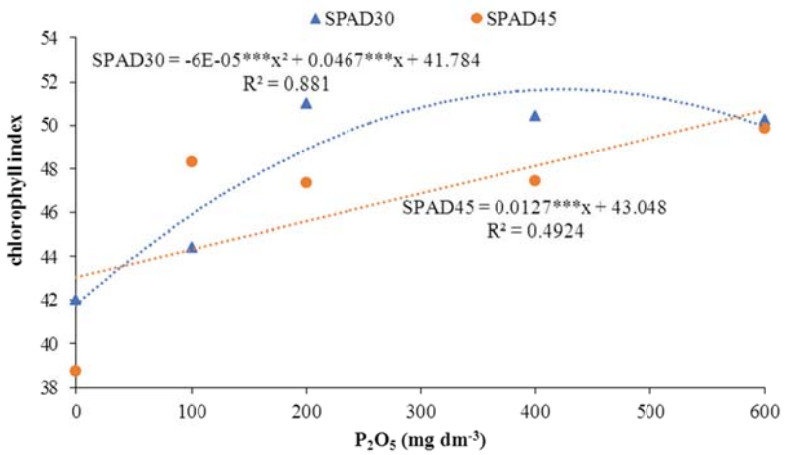

Figure 3. Chlorophyll index content (SPAD) as a function of phosphorus doses (P_2O_5) with a natural phosphate source reactive at 30 and 45 DAE. *** Significant at 1%

The chlorophyll index increased in response to phosphorus supplementation. This increase may be due to the importance of phosphorus in plant nutrition, which participates in the beneficial ATP molecule the active process of nitrogen absorption by plants (Malavolta et al., 1997; Taiz & Zeiger, 2010), making it clear that higher phosphorus concentrations contribute to higher nitrogen uptake and higher chlorophyll content (Haim et al., 2012; Hurtado et al., 2011).

The results found in the present study corroborate with those observed by Silva et al. (2010) and Souza et al. (2011), which verified that the chlorophyll content increased with the phosphorus doses applied to the soil, suggesting that the absorption of phosphorus also favors the nitrogen absorption by the plant.

The diameter of the chapters presented a significant difference as a function of the phosphorus doses, adjusting to the quadratic model of regression in the evaluation performed at the time of the cut at 55 DAE (Figure 4A), demonstrating that the phosphorus dose of 457.17 mg dm^{-3} was responsible for the largest chapter diameter (10.35 mm) of safflower plants.

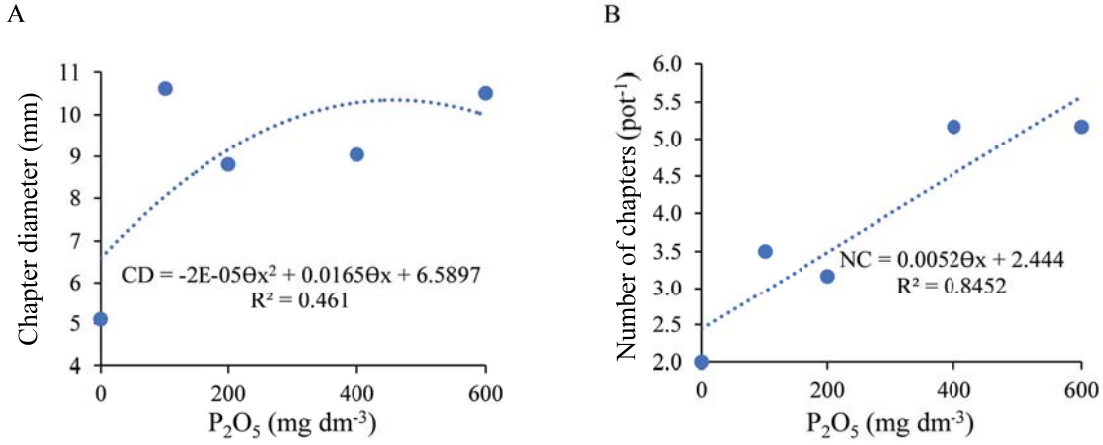

Figure 4. The diameter of chapters as a function of phosphorus doses (P_2O_5) with natural phosphate source reactive at 55 DAE. Significant at 10%

A good development of the chapter characterizes a good production of the safflower culture, the bigger the diameter of the chapter the greater the number of flowers and according to the quantity of inflorescence will be the yield of grains of the culture, with consequent increase in the oil content of the grains (Paludo et al., 2017). At the time of cutting at 55 DAE, the efficiency of the reactive natural phosphate could be perceptible, contributing to the increase in the diameter of chapters of safflower plants. Soares and Macedo (1988), that, over time, soluble phosphorus sources tend to increase their reactivity, as they have a higher residual effect.

Abbadi and Gerendas (2011), verified that the safflower culture responds positively to the increase of phosphorus to the soil, presenting greater development and better responses in the productive variables of the safflower culture, as well as a greater number of chapters per plant, a larger diameter of chapters and a greater number of chapters in the main and secondary.

At the 55 DAE cut, there was a linear increase in the number of chapters of safflower plants with increasing doses of phosphorus (Figure 4B), whose dose of 600 mg dm^{-3} provided the highest number of chapters (5.17) with an increase of 56.15% when compared to the absence of phosphate fertilization.

The number of chapters per safflower plant is one of the most important yield components since it is directly related to the final production of the crop. According to Paludo et al. (2017), the number of chapters per plant and the number of seeds per chapter is strongly correlated with safflower productivity, as well as related to each other.

The results found in the present study corroborate those of Abbadi and Gerendas (2011), which evaluated the development of safflower and sunflower, cultivated at phosphorus doses of 8 to 150 mg of P dm^{-3}, found that when grown with the highest dose of the element, the plants showed an increase of 83.3% on average when compared to the lower dose of calcium phosphate. Abbadi et al. (2011) found that the safflower plants are extremely demanding in phosphorus since productive variables were influenced when cultivated in low levels of both phosphorus and potassium.

The chapter dry mass of the and the shoot dry mass were significantly increased with the phosphorus doses, adjusting to the linear regression model (Figures 5A and 5B), with higher yield (0.72 and 0.78 g pot^{-1}) in the dose of 600 mg dm^{-3} of P_2O_5 with an increase of 68.10 and 44.79% respectively, when compared to the higher dose of the experimental interval with the control.

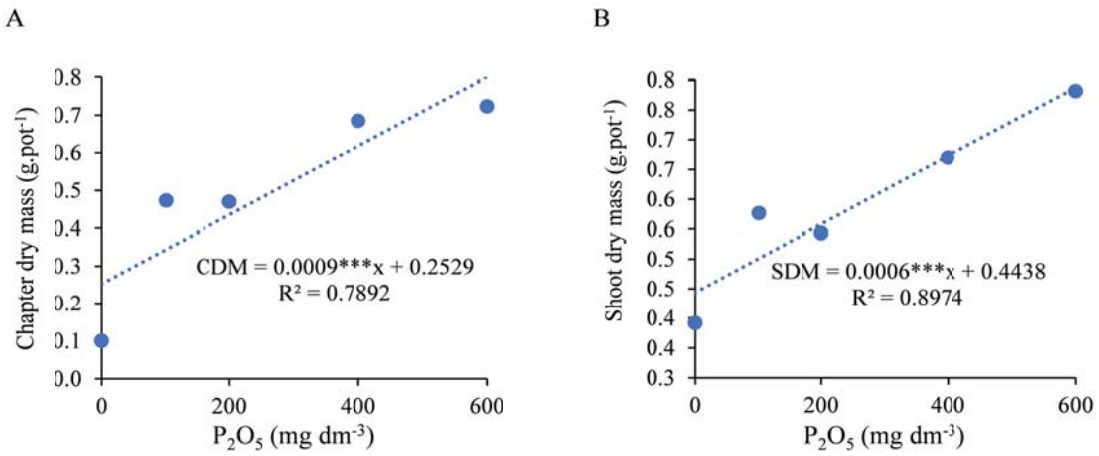

Figure 5. Chapters dry mass (A) and shoot dry mass (B) of safflower plants (*Carthamus tinctorius* L.) as a function of phosphorus (P_2O_5) doses with natural phosphate source reactive at 55 DAE. *** Significance of 1%

The positive effect of phosphatic fertilization on safflower cultivation reflects an increase in the production of dry mass of the crop, because phosphorus is directly involved in the metabolic processes of plants, playing an important role in cell energy transfer, respiration and photosynthesis as well as a constituent of several protein complexes (Malavolta et al., 1997; Zobiole et al., 2010; Tomich et al., 2003).

These results demonstrate that the root system is more developed there is a greater contact of the roots with the natural reactive phosphate incorporated in the soil, causing a greater absorption of phosphorus, favoring the growth and development of the plant, promoting greater height and as a consequence, higher production of dry mass (Chien & Menon, 1995a, 1995b, Oliveira Junior et al., 2008).

The root volume showed a significant difference as a function of the phosphorus doses, adjusting to the linear regression model in the evaluation performed at the 55 DAE cut (Figure 6), demonstrating that the phosphorus dose of 600 mg dm^{-3} was responsible for the highest root volume (3.57 ml) of safflower plants, increasing by 57.39% in relation to the absence of phosphate fertilization.

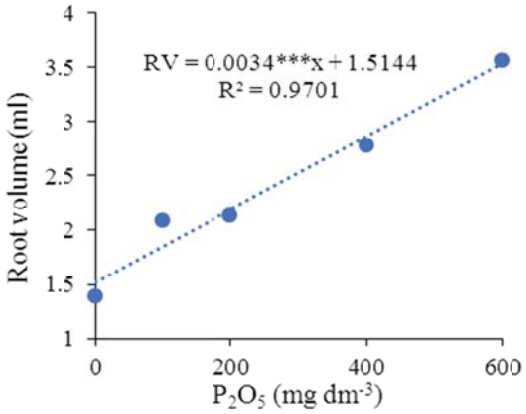

Figure 6. Root volume of safflower (*Carthamus tinctorius* L.) as a function of phosphorus (P_2O_5) doses with a natural source of phosphate reactive in the cut. *** Significance of 1%

A well-developed root system is essential for plant growth, nourishment and sustenance and, as a result, a good development and increase in grain yield. According to Guedes et al. (2009), plants well-nourished mainly in phosphorus ensure a greater root development, consequently an increase in the production of photoassimilates that are redistributed to the plant, causing root growth in length and volume.

4. Conclusions

The natural phosphate fertilization promotes significant changes in the morphological and productive characteristics of the safflower crop.

Fertilization with Bayóvar reactive phosphate (P_2O_5) between 389.17 and 600 mg dm^{-3} yields the best responses for the development and production of safflower cultivar IMA 0213 in Cerrado Oxisol.

The safflower presents positive responses to the application of the reactive natural phosphate as a source of phosphorus and can be an alternative for phosphate fertilization in Cerrado Oxisol.

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Seed Technology of *Myrcianthes pungens* (Berg) Legr: An Approach to Biometry and Germination

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Abstract

Myrcianthes pungens, native fruit with medicinal, ornamental and ecological potential, lacks information on biometry and technology for seed germination. Thus, the aim of this study was to characterize the fruits and seeds of *M. pungens*, determining the appropriate substrate for laboratory tests, as well as to evaluate the effect of different concentrations of gibberellic acid (GA_3) on the germination of the species. In the biometry, the mensuration of the fruits and seeds was made with the aid of a digital caliper. In the laboratory, three substrates (between filter paper, between vermiculite and between sand) were used, with five concentrations of GA_3 (0; 125; 250; 375 and 500 mg L^{-1}) and the tests were conducted in a germination chamber. The percentage of germination, hard seeds, germination speed index and seedling length root and shoot were evaluated. The average fruit length and width were 17.38 and 16.05 mm, respectively. The fruits presented one or two seeds with 8.10; 9.56 and 6.49 mm in length, width and thickness. The germination test is more efficient between fine sand, and vermiculite may also be used. The wetting of the substrate with gibberellin in the concentration of 125 to 274 mg L^{-1} optimizes the percentage and speed of germination.

Keywords: native fruit, guabijú, gibberellin, substrate

1. Introduction

Myrcianthes pungens (BERG) LEGR, which belongs to the family Myrtaceae, is popularly known as guabijú, guabijuzeiro, among others. It occurs naturally in South of Brazil (Backes & Irgang, 2009) and also in Argentina, Uruguay, Paraguay and Bolivia (Tropicos, 2019). Its fruits are edible globose type berry and the seeds are reniform, measuring from 6 to 7 mm (Lorenzi, 2002).

The species, which is characterized as late secondary (Vaccaro, Longhi, & Brena, 1999), stands out among the native fruit of Brazil, and it is indicated for ornamental purposes, honey producer, as well as for the production of fruits for consumption in natura or in industry (Sarmento & Villela, 2010). The guabijú also presents a medicinal potential, with analgesic and antiseptic properties (Nessello, Campos, Capistrano, Buzzi, & Cechinel Filho, 2016), antioxidant activity (Dalla Nora et al., 2014), and also presents positive effects in Alzheimer's treatment (Silveira et al., 2011). Besides those indications, it presents a great environmental value, being used for reforestation of degraded areas and permanent preservation because its flowers are nectariferous and its fruits are attractive to bird fauna (Backes & Irgang, 2009).

In Brazil, studies in the field of forest seed technology follow the recommendations of Seed Testing Rules (RAS) (Brasil, 2009), and the Instructions for Testing of Forest Seed Specimens (Brasil, 2013). However, given the great diversity of species, there is still missing information about the vast number of native species, especially those that belong to Myrtaceae family, which have seeds whose tolerance to dissection and the behavior in storage characterize themselves as recalcitrant or intermediate (Mayrinck, Vaz, & Davide, 2016). Thus, studying

procedures for conducting trials with species that are not yet in official records is of great importance for enabling the certification and commercialization of seed lots (Oliveira, D. C. F. Dias, Hilst, Silva, & L. A. Dias, 2014), as in the case of *M. pungens* (Fior et al., 2010).

Plants produce fruits and seeds of uneven sizes due to genetic, nutritional and environmental reasons, which can influence seed physiological quality. Thus, the biometric characterization of fruit and seeds is a main tool that can be employed in conservation and genetic improvement studies of populations, identification and differentiation of species of the same genus, as well as laboratorial testing standardization (Silva, Santos, Lima, & Morais, 2014).

Besides the biometric characteristics, germination of seeds can also supply important information about the quality of the analyzed seed lot, being a complex process, because it involves several factors, among them we highlight quantity of water, lighting, temperature, oxygen, substrates, presence or not of dormancy and pathogen incidence. One of the basic components used on germination tests is the substrate (Sorana et al., 2019), whose choice must consider the size of the seed, its water demand, photoplasty and facility for the development and evaluation of seedlings (Figliolia, Oliveira, & Piña-Rodrigues, 1993; Brasil, 2009) besides material availability.

Additionally, to support fast germination, with homogenous seedlings development, phytoregulators promoting growth such as the gibberellic acid (GA_3) are being used in fructiferous seed species technology (Lata, Sharma, Garg, & Joshi, 2018) and more recently in forest species (Cabello, Espinoza, Espinoza, Cabrera, & Santelices, 2019). When it comes to seeds, the exogenous application of GA_3 can increase the potential growth of the embryo (Pipinis et al., 2015), promoting the cellular stretching that induces the radical development, increasing the germination percentage (Campos, Abreu, Guimarães, & Seleguini, 2015).

Because of the great importance of *M. pungens* and the lack of information about the species seed technology, the objectives of this present work was: a) characterize biometrically fruits and seeds of *M. pungens*; b) determinate the proper substrate for species germination; and c) evaluate the effect of using different concentrations of GA_3 on *M. pungens* seed germination.

2. Material and Methods

2.1 Collection of Fruits and Seeds Lot Formation

M. pungens fruits where collected when they showed a dark-purpled coloring, in five remaining trees located in remnants of Subtropical Seasonal Forest, in the Central region of Rio Grande do Sul State (29°38′14.79″ S and 53°27′44.11″ W) in Southern Brazil. The local weather, accordingly to Köppen classification, is humid subtropical, with average annual precipitation between 1400 and 1760 mm, with fair distribution along the year with matching temperatures between -3 ºC and 30 ºC (Alvares, Stape, Sentelhas, Gonçalves, & Sparovek, 2013). According to the Brazilian System of Soil Classification, the soil of the region is Lithic Neosol type, physically flat and undeveloped (Embrapa, 2013).

After being collected, the fruits were taken to Forestry and Forest Nursery Laboratory (Laboratório de Silvicultura e Viveiro Florestal) belonging to Forest Science Department (Departamento de Ciências Florestais) from Universidade Federal de Santa Maria (UFSM), in Santa Maria, RS. The fruits were submerged in water for 24 hours to ease the extraction process. Then, pulping was made in streaming water, extracting the seeds, which were dried with shady environment and ventilated for two days, manually homogenized, forming the lot under studying.

2.2 Biometry of Fruits and Seeds

Initially the length and width of the fruits were determined, and also the length, width and thickness of the seeds, based on a sample of 100 units for each attribute. A digital pachymeter was used to determine dimensions (0.001 mm), being subsequently calculated the maximum, minimum, average and standard deviation, for each biometric characteristic.

Besides that, it was determined the number of seeds for each fruit, the weight of thousand seeds by using eight samples of one hundred seeds, and the degree of humidity by greenhouse method for 24 hours at 105±3 °C (Brasil, 2009).

2.3 Germination Test

The germination test was performed, right after the lot formation. Prior to the test installment, the seeds were disinfested, being plunged in distilled water solution with 2% of sodium hypochlorite for two minutes and after washed with distilled water.

The testing was performed with "gerbox" transparent containers by using a fully casual design, with factorial scheme (5 × 3). The treatment consisted in five concentrations of GA_3 (0; 125; 250; 375 and 500 mg L^{-1} of distilled water) and three substrates (BFP: between filter paper, BV: between vermiculite and BS: between sand), totaling 15 treatments with four repetitions of 25 seeds each. The dilution of GA_3 concentrations in distilled water followed the recommendations of Brasil (2009).

In BFP substrate two "Germitest" paper sheets were used, one in base and one at the top, dampened with the solution (represented by the different GA_3 concentrations), knowing that the utilized volume matched 2,5 times the paper weight. In relation with the other substrates, sand (0.84 mm of sifting mesh) and vermiculite (thin granulometry), both were dampened at 60% capacity of the solution retaining capacity (Brasil, 2009).

The test was conducted in the germination chamber, Mangelsdorf type, with constant lighting and 25±2 °C of temperature. Counts were made every three days, in order to obtain normal seedlings percentages (NS), according to technological criterion (Gui-Ferreira & Borghetti, 2004), and the number of firm seeds (hard) (HS) in accordance with Brasil (2009). In each evaluation, the values evaluated were the length of shoot (LS) and length of root (LR) of the NS, with graduated millimeter ruler aid. According to the observations, it was possible to determine the germination percentage (G%) and germination speed index (GSI) (Maguire, 1962).

The data were submitted to normality residual presumptions by Shapiro-Wilk and variances homogeneity by Barlett, by Action software means (Team Estatcamp, 2014). When it was necessary, the data transformation was proceeded with Box-Cox, for further variances analysis, Tukey average comparison (0.05) and regression analysis with SISVAR software aid (Ferreira, 2011).

3. Results and Discussion

3.1 Biometry of the Fruits and Seeds

The fruits of *M. pungens* had an average length of 17.38 mm and a width of 16.05 mm, and one to three seeds, and 77% of the fruits had one seed and 20% two units. The seeds presented on average 8.10; 9.56 and 6.49 mm in length, width and thickness, respectively. In general, a small variation in the fruit and seed dimensions of *M. pungens* was observed, whose coefficient of variation was below 10% for most of the observed variables.

The results obtained in the biometric characterization of the fruits and seeds of *M. pungens* in the present study, differ from the values evidenced by Assumpção, Dalmaso, and Bragança (2017) evaluating the same species. The variation in the dimensions of the fruits and seeds between individuals of the same species can be influenced by environmental factors as rainfall, temperature and photoperiod, during the flourishing and development, as well as for the genetic variability and age among the main trees (Macedo et al., 2009; Pereira et al., 2017).

Table 1. Values (maximum, minimum, average, standard deviation and variation coefficient) regarding the biometric characterization of fruits and seeds of *M. pungens*, Santa Maria, RS

	Determinations	Maximum	Minimum	Average	Standard Deviation	CV (%)
Fruit	Length (mm)	20.70	14.45	17.38	1.31	7.50
	Width (mm)	18.91	13.48	16.05	1.34	8.30
Seed	Length (mm)	9.80	4.55	8.10	0.75	9.30
	Width (mm)	11.23	7.65	9.56	0.73	7.60
	Thickness (mm)	8.42	4.24	6.49	0.92	14.2

Note. CV: variation coefficient.

The weight of a thousand seeds was 344.11 grams, totaling 2.906 seeds kg^{-1}. These results are in agreement with Fior et al. (2010) who obtained an average of 2.576 and 5.429 seeds kg^{-1} from seeds collected in six trees in the municipality of Cachoeira do Sul and Encruzilhada do Sul, respectively. However, they differ from those described by Lorenzi (2002), who observed 4.000 seeds kg^{-1}. That difference among results confirmthe morphologic variation among the seeds of that species, that can be attributed to the climate and soil conditions, genetic factors, position of the fruit in the plant mother (Fenner & Thompson, 2005) and anthropic modifications in the area where the seed trees are established (Mendonça, Ramos, & Paula, 2001).

The seeds of *M. pungens* presented high humidity degree (38.2%), characteristic of the intolerant species to the dissection. Wielewicki, Leonhardt, Schlindwein, and Medeiros (2006) found average values of 39.6% and proposed a 34.8% humidity degree for seeds of *M. pungens*. L. dos S. de Souza, Fior, P. V. D. de Souza, and Schwarz (2011), by studying this same species, obtained degree of humidity of 38.7% and Fior et al. (2010)

between 41.4% and 43.6%. According to the autors, the variation in the number of seeds and humidity degree verified in the different studies can be related to the time, collection place and degree of maturation of the fruits, as well as to the climatic conditions of the area, and procedures before the performance of the analysis.

3.2 Germination Test

The germination of the seeds of *M. pungens* was slow and lasted longer. The germination was first attained on the 35th day and lasted for 71 days from first day of experiment. Interaction was evinced among the factors (substrate x concentrations of GA_3) just for the length of the shoot (LS). For the other attributes, effect was just verified for the isolated factors ($p < 0.05$).

Maximum seed germination (87%) was attained when the substrates were moistened with 125 mg L^{-1}solution of GA_3. However, favourable germination was observed to be higher up to concentrations of 274 mg L^{-1}, suggesting a positive effect in the germination at doses within the 125 to 274 mg L^{-1} interval. Minimum germination was observed seeds without pre-germination treatment (79.67%) (Figure 1A).

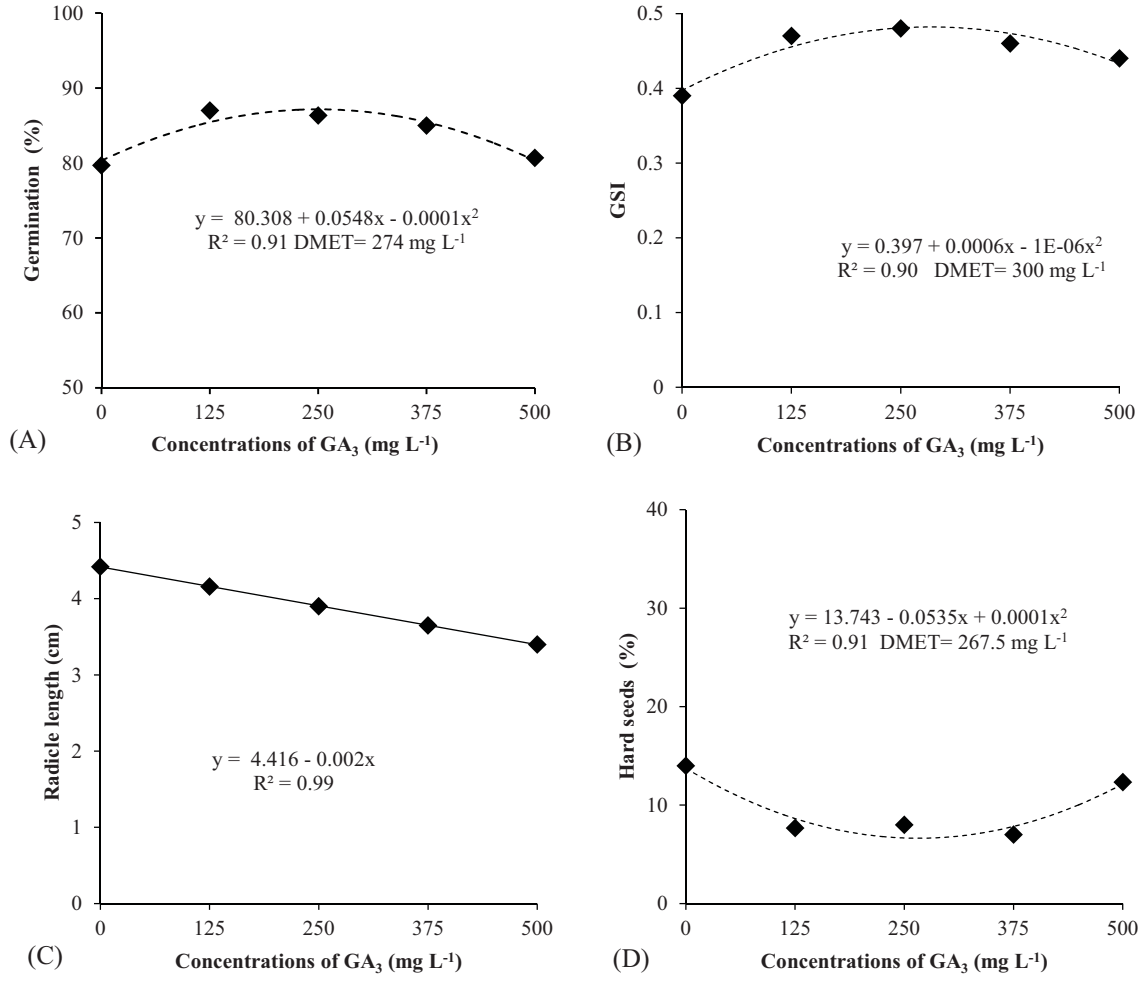

Figure 1. Effect of different concentrations of gibberellic acid about the germination (A), germination speed index (GSI) (B), radicle length (C) and hard seeds (D) of *M. pungens*

Similarly, a higher index of germination speed (GSI) was obtained in the same foregoing interval, with 0.47 (Figure 1B). GSI is calculated by the amount of germinated seeds divided by the number of days resulting from the germination test, and the higher the GSI, the greater the batch vigor (Nakagawa, 1994).

The beneficial effect of GA_3 in the germination of seeds of forest species was also observed by Saldias and Velozo (2014) in seeds of *Myrcianthes coquimbensis* (Barneoud) Lnadrum and Grifo and by Cabello et al. (2019), in seed of *Nothofagus* glauca (Phil.) Krasser. The positive influence of GA_3 on the germination of seeds

is related with the capacity of the phytoregulator to act on the cellular elongation, generating a larger metabolic incentive and larger mobilization of nutritious and energy reservations that are supplied for the development of the embryo (Taiz et al., 2017). Besides, GA_3 stimulates the synthesis and translation of the specific mRNA for the enzyme α-amilase (Muralikrishna & Nirmala, 2005), causing the degradation of the starch and weakness of the layer of the endosperm that involves the embryo, promoting thus, a faster germination and the reduction of the number of hard seeds (Figure 1D).

The use of GA_3 caused linear reduction in the development of the root system of *M. pungens* (Figure 1C), resulting on the contrary to what was observed by Campos et al. (2015), which showed a linear increase in the length of *Rollinia mucosa* (Jacq.) Baill radicle in increasing concentrations of gibberellic acid (0, 125, 250, 500 and 1000 mg L^{-1}). According to Macedo et al. (2009), application of GA_3 can effect germination among species. There is a probability that the concentrations of GA_3 promoted an increase of the auxins synthesis in the developing radicle (Fang et al., 1960; Michelwolwbrtz & Sjronval, 1963), provoking inhibitory effect in the elongation of the root system.

In relation to the tested substrate, treatments between vermiculite (BV) and between sand (BS) presented the largest germination averages (86.4 and 88.2%), and a smaller number of HS (7.4 and 4.6, respectively) (Table 2). In that sense, those substrates were the most appropriate to express the vigor of *M. pungens* seeds.

Wielewicki et al. (2006) supported use of paper roll as a substrate for *M. pungens* seed germination. Although in the literature there are no reports about the use of the substrates BS and BV in the test of germination of *M. pungens* seeds, however, use of BS and BV in this study was favourable for germination. This observation is in agreement with the Instruction for Analysis of Seeds of Forest Species. The substrates BS and BV are recommended for the species belonging to the same botanical family of *M. pungens* (Brasil, 2013).

Table 1. Effect of different substrates in germination (G), germination speed index (GSI), radicle length (RL) and number of hard seeds (HS)

Parameters	Substrates			CV (%)
	BFP	BV	BS	
G (%)	77 B*	86 A	88 A	7.23
GSI	0.4 B	0.4 B	0.5 A	15.31
RL	4.5 A	3.4 B	3.8 B	17.25
HS (%)	17.4 B	7.4 A	4.6A	42.48

Note. BFP (between filter paper); BV (between vermiculite) and BS (between sand). Averages followed by the same letter in the line do not differ among them by the Tukey test at 5% of error probability.

The substrate sand is indicated for every seed type, besides the ones of the most sensitive species to the drying and that demand a prolonged period for completing the germination (Abreu et al., 2005). However, according to Gasparin, Araujo, Tolfo, Foltz, and Magistrali (2013) the substrate BS presents disadvantages due to the largest weight, being necessary additional cares with the sowing depth, for not harming the germination. Additionally, the substratum vermiculite, according to Figliolia et al. (1993), is used for germination of seeds of forest species due to good absorption capacity and retention of water, being also indicated for seeds with slow germination and emergency.

The largest values of GSI were observed in the substrate BS, possibly due to larger contact area that it offers to the seeds, favoring the absorption of water and GA_3, corroborating with what was evinced by Gasparin et al. (2013) for the species *Parapiptadenia rigida* (Benth.) Brenan. Additionally, according to Flores et al. (2014) the contact area of the substrate moistened with the seed is very important, because in spite of not being limiting to germination, it influences the germination speed.

Through the analysis of length of shoot (LS) of the seedlings, it was verified that for the substrate BS and BFP the maximum efficiency was reached when the concentrations of 225 mg L^{-1} and 270 mg L^{-1} were used, respectively. However, seedlings that grew in the substrate BV presented larger LS when this was only moistened with water (Figure 2).

In the conditions of BS or BV and about 274 mg L^{-1} of gibberellins, the first counting can be accomplished at the 35 days and the last with 50, being necessary intermediate counting, avoiding compromising of the results by the pathogens presence.

It was verified that more vigorous seedlings are produced when the seeds of *M. pungens* are submitted to the action of GA_3, generating earnings of time in the formation of seedlings, for the fastest and uniform emergency. In spite of the obtained verifications, other studies related to the species should be accomplished seeking to accelerate the speed of the germination, reducing thus the pathogens presence. Studies are suggested to evaluate the possible presence of a non deep or combined physiologic dormancy of *M. pungens* seeds (Baskin & Baskin, 2004), bearing in mind that the same presents appropriate soak, however with non-uniformity and expressive time for germination.

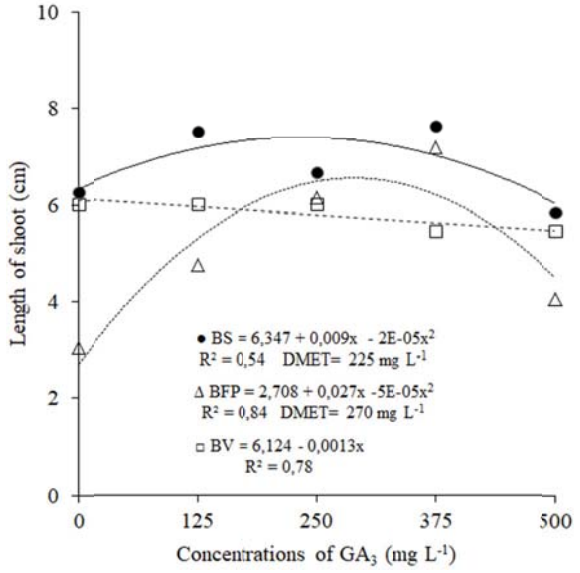

Figure 2. Effect of five different concentrations of gibberellic acid in three types of substrate, in the length of shoot of seedlings (LS) of *M. pungens*

Note. BS = between sand, BFP = between filter paper, and BV: between vermiculite.

4. Conclusions

The length and the width of the *Myrcianthes pungens* fruits are uniform (17.38 and 16.05 mm, respectively), usually presenting, one or two seeds, with 8.10, 9.56 and 6.49 mm length, width and thickness.

The germination test is more efficient among sand, and vermiculite can also been used. The first germination counting should be accomplished at the 35th day, closing up the test on the 71st day after the sowing.

The moistening of the substrate with gibberellins is recommended in the concentration of between 125 and 274 mg L^{-1} for obtaining larger percentage and speed of germination of *M. pungens*.

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Seed Germination and Initial Growth of Quinoa Seedlings Under Water and Salt Stress

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Abstract

Excessive amounts of salts and soil water deficiency interfere on seed germination and the full development of several crops. The objective of this research was to evaluate the effect of water stress and salinity on the germination process and initial growth of quinoa (*Chenopodium quinoa* Willd.) seedlings. In the first experiment, two quinoa seed lots with different physiological conditions were distributed on paper soaked in aqueous solution containing polyethylene glycol PEG-6000 in osmotic potentials corresponding to 0.0; -0.1; -0.2; -0.3 and -0.4 MPa and held at 20 °C under 8 hours of light exposition. In the second experiment, solutions of sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) were used to simulate the effect of salinity using the osmotic potentials, temperature and light conditions previously described. Assessed parameters were the germination percentage, first count, length and dry mass of seedlings. There was a reduction in quinoa germination percentage, first seed count and seedling length as the osmotic potential decreased in $CaCl_2$, NaCl, KCl, $MgCl_2$ and PEG-6000 solutions. The quinoa seeds exhibited higher tolerance to NaCl and KCl salts in the germination process and initial seedling growth. The progressive reduction of the osmotic potential induced by salts NaCl, KCl, $CaCl_2$, $MgCl_2$ and PEG-6000 negatively affects seed germination and initial growth of quinoa seedlings.

Keywords: *Chenopodium quinoa* Willd., germination process, salinity

1. Introduction

Quinoa (*Chenopodium quinoa* Willd.) belongs to the Amaranthaceae family and is native from the Andean region of South America, being recently introduced to Brazil in the 1990s (Ceccato, Berleto, & Batlla, 2011). The crop exhibits high yield potential under adverse climatic and soil conditions. In addition, the grains present enhanced nutritional properties, arising as a complement in human and animal diets, besides being used as a forage and cover crop (Strenske, Vasconcelos, Egewarth, Herzog, & Malavasi, 2017).

Seed germination is a critical stage in plant life cycle, depending on the genetics of each species and environmental conditions that seeds are exposed. In arid and semi-arid regions with recurrent adversities such as salinity and water deficiency, the water absorption by the seed during the germination process is hampered by the negativity of the soil matrix potential (Santos, N. V. Silva, Walter, E. C. A. Silva, & Nogueira, 2016).

Excessive amounts of salts and soil water deficiency are abiotic factors that directly interfere on seed germination and full crop development, limiting the maximum yield performance (Torres, Vieira, & Marcos Filho, 2000; Moterle, Lopes, Braccini, & Scapim, 2006). In addition to the water absorption restriction due to the reduction of soil potential, toxic effects in seeds undergoing the germination process have been observed. These toxic effects may lead to cell metabolism alterations, reduction in germination percentage and speed, and changes in the development and growth of seedlings (Pelegrini, Borcioni, Nogueira, Koehler, & Quoirin, 2013; Santos et al., 2016).

Movement of the water present in the solution through the seed tissues is necessary for the proper trigger of the seed germination process. This movement is dependent on the presence of a water potential gradient, which is reduced when there is presence of salts and eventually restricts seed water absorption (Pereira, C. C. Martins, D. Martins, & Silva, 2014). Besides the negative interference caused by plant toxicity, the presence of salts in the

solution, such as sodium chloride (NaCl), calcium chloride ($CaCl_2$) and potassium chloride (KCl) can act as an inducer of plant water stress (Souza & Cardoso, 2000).

In this sense, evaluation of the germination process and seedling development under conditions of salinity and water deficiency are substantial since they may be related to crop sensitivity or tolerance at subsequent development stages (Taiz & Zeiger, 2013). Thus, aiming to identify quinoa tolerance to cropping in saline or water deficient environments, the objective of this research was to evaluate the effect of water stress and salinity on the germination process and initial growth of quinoa seedlings.

2. Material and Methods

The research was carried out by means of two completely randomized design experiments during 2018 at the Didactic and Seed Research Laboratory, belonging to the Federal University of Santa Maria (UFSM).

2.1 Water Stress

For experiment 1, two lots of quinoa seeds of the Q 13-31 line were used to evaluate the water stress on the quinoa germination process. Seeds were obtained through cultivation in the Experimental Area of the Plant Science Department (UFSM) during the 2017 agricultural year. After the characterization of the seed lots, lot 1 was classified with germination of 86% and vigor of 83%, while lot 2 exhibited germination of 70% and vigor of 64%. Both lots exhibit minimum germination value required by MAPA (Ministry of Agriculture, Livestock and Supply) for the marketing as seed (> 60%) (Brasil, 2011).

The water deficiency was simulated with aqueous solutions containing polyethylene glycol (PEG-6000) concentrations required to obtain osmotic potentials corresponding to 0.0; -0.1; -0.2; -0.3 and -0.4 MPa. The control treatment (0.0 MPa) corresponded to the solution containing only distilled water. The amount of PEG-6000 required to obtain the different osmotic potentials was determined in Table 1 and was obtained from Villela et al. (1991).

Table 1. Amount of salts calcium chloride ($CaCl_2$), sodium chloride (NaCl), potassium chloride (KCl) and magnesium chloride ($MgCl_2$) and polyethylene glycol (PEG-6000) in g L^{-1} to obtain solutions with different levels of osmotic potential

Levels of osmotic potential (MPa)	Salts (g L^{-1})				PEG-6000 (g L^{-1})
	$CaCl_2$	NaCl	KCl	$MgCl_2$	
0	0.00	0.00	0.00	0.00	0.00
-0.1	6.03	1.33	3.06	8.34	72.48
-0.2	12.06	2.67	6.12	16.68	112.23
-0.3	18.09	4.00	9.17	25.02	143.18
-0.4	24.12	5.33	12.23	33.36	201.32

2.2 Salt Stress

For experiment 2, quinoa seeds of the Q 13-31 line with germination of 90% and vigor of 86% were used to evaluate the effect of salinity on the quinoa germination process. Seeds were obtained by field cropping in the Experimental Area of the Plant Science Department (UFSM) during the 2017 agricultural year. The experiment was arranged in a 4x5 factorial scheme, being the first factor levels constituted of salts calcium chloride ($CaCl_2$), sodium chloride (NaCl), potassium chloride (KCl) and magnesium chloride ($MgCl_2$), and the second by the osmotic potentials corresponding to 0.0; -0.1; -0.2; -0.3; -0.4 MPa. The control treatment (0.0 MPa) corresponded to the solution containing only distilled water. The salt concentration required to obtain the osmotic potentials was determined in Table 1 and was obtained by the Van't Hoff equation cited by Taiz and Zeiger (2013), as follows:

$$\Psi os = -RTC \quad (1)$$

where, Ψos: osmotic potential (atm); R: ideal gas constant (8.32 J mol^{-1} K^{-1}); T: temperature (K); C: concentration (mol L^{-1}). The NaCl concentrations were calculated by means of the calibration curve established by Braccini et al. (1996).

For the evaluation of water and saline stress (experiments 1 and 2), the standard germination test (SGT) was carried out with 4 replicates of 100 seeds distributed in transparent plastic boxes (gerbox) on three sheets of germitest paper soaked with distilled water or solution corresponding to the experiment, in the proportion of 2.5

times the dry paper weight. After sowing, plastic boxes were accommodated in the B.O.D. (Biological Oxygen Demand) germination incubator and maintained at a constant temperature of 20 °C under 8 hours of light exposure. The first and second seedling counts were performed at four and six days after the beginning of the test. Results were expressed as a percentage of normal seedlings (Brasil, 2009). Normal seedlings were considered those that presented more than 1.5 cm and well-developed shoot and root system.

Normal seedlings deployed to assess seedling length and dry mass were obtained by sowing four replicates of 20 seeds arranged in two rows on the upper third of the germination paper. The paper was soaked on distilled water or the solution corresponding to the experiment and submitted to the same conditions of the SGT. At four days after sowing, the length of shoot and primary root of ten normal seedlings of each replicate was measured by means of a millimeter ruler. Dry mass determination was performed by drying the plant material in a forced ventilation oven at 65±5 ºC for 48 h (Nakagawa, 1999) and weighing it in a digital scale (0.001 g).

Data obtained in the experiments and expressed as a percentage was transformed using arc-sine $\sqrt{\%/100}$ and subjected to analysis of variance (ANOVA). When significant, means were compared using the Scott-Knott test for the qualitative factor and regression analysis was performed for the quantitative factors, with 5% error probability, using the statistical software SISVAR (Ferreira, 2011). The significant higher order model (R^2) was selected using the equation that best fitted the data.

3. Results and Discussion

3.1 Water Stress

There was no significant interaction between the factors lots and osmotic potentials (Table 2). Therefore, the factors acted independently on the variables germination, first count, shoot, root and total length of quinoa seedlings. Analyzing the simple effects of the factors, there was a significant difference ($p < 0.05$) between the lots and the osmotic potentials of the solution containing PEG-6000 (Table 2).

Table 2. Summary of the analysis of variance for the variables germination (G), first count (FC), total length (TL), shoot length (SL), root length (RL) and dry mass (DM) of quinoa seedlings exposed to different concentrations of PEG-6000

Source of variation	DF	Mean square					
		G	FC	TL	SL	RL	DM
Lot	1	2624.40*	2624.40*	22.72*	13.57*	1.14*	0.006
Concentration	4	1272.25*	2717.65*	14.75*	5.37*	2.90*	0.104
Concentration*Lot	4	69.65	108.15	0.59	0.75	0.03	0.046
Residue	30	89.60	104.60	0.54	0.30	0.07	0.065
CV (%)	-	13.72	18.07	14.32	18.17	13.35	17.11

Note. * Significant at 5% probability by the F test. CV = Coefficient of variation.

Analyzing the means of the lots, it was verified that lot 1 was superior to lot 2 in all analyzed variables, except for dry mass, which theren't was significance (Table 3). In the control treatment with absence of PEG-6000, seeds from lot 1 and 2 presented a germination of 86 and 70%, with a significant decrease in the other concentrations of PEG-6000, reaching values respectively of 52 and 43% at the -0.4 MPa osmotic potential (Table 3). The vigor results obtained in the germination first count also indicated reduction of the percentage of normal seedlings with decreased water potential of the solution.

Table 3. Mean results of the percentage of normal seedlings in the first and final count of the germination test, shoot, root and total length, and dry mass of quinoa seedlings subjected to four levels of osmotic potential in solution of PEG-6000

Lot	Osmotic Potential (MPa)					Mean *
	0.0	-0.1	-0.2	-0.3	-0.4	
Germination (%)						
Lot 1	86	85	82	82	52	77 A
Lot 2	70	68	67	57	43	61 B
CV %	13.72					
First count (%)						
Lot 1	83	74	70	68	30	65 A
Lot 2	64	64	51	42	23	49 B
CV %	18.07					
Shoot length (cm)						
Lot 1	3.53	4.64	4.64	3.01	2.29	3.62 A
Lot 2	2.94	3.28	2.55	1.86	1.67	2.46 B
CV %	18.17					
Root lenght (cm)						
Lot 1	3.03	2.68	2.22	2.00	1.46	2.28 A
Lot 2	2.51	2.52	1.94	1.63	1.10	1.94 B
CV %	13.35					
Total length (cm)						
Lot 1	6.56	7.33	6.86	5.01	3.75	5.90 A
Lot 2	5.45	5.80	4.48	3.49	2.76	4.39 B
CV %	14.32					
Dry mass (mg)						
Lot 1	1.38	1.50	1.63	1.45	1.45	1.48 A
Lot 2	1.43	1.55	1.63	1.68	1.25	1.51 A
CV %	17.11					

Note. * Means followed by upper case letters in the same column do not differ among each other at 5% error probability by the Scott-Knott test.

The results of this study corroborate with the findings of Sousa et al. (2018), which observed that water stress reduced seed germination and vigor of *Bidens subalternans* L. and the species did not tolerate osmotic potentials greater than -0.4 MPa. Moreover, Stefanello et al. (2018) concluded that thyme (*Thymus vulgaris* L.) seed germination and seedling performance were negatively affected under PEG-6000-induced water stress starting from -0.3 MPa.

Distinctive modes of assimilation of water stress are observed when plants are submitted to water scarcity conditions. As a stress response, there may be a decrease in seed metabolism, limited digestion of reserves and translocation of metabolized products, which can reduce germination percentage. There is a wide range of response variation between species, from very sensitive to more resistant ones (Gordin, Scalon, & Masetto, 2015; Feng, Lindner, Robbins, & Dinnenya, 2016).

Shoot, root and total length of quinoa seedlings decreased as the water potential of the substrate decreased in both lots (Figures 1C, 1D, and 1E). Total seedling length reduction from 6.56 cm (0.0 MPa) to 3.75 cm (-0.4 MPa) was found in lot 1 and from 5.45 cm (0.0 MPa) to 2.76 cm (-0.4 MPa) was observed in lot 2 (Table 3). On the other hand, the seedling dry mass was not influenced by the different osmotic potentials in the two studied lots (Figure 1D).

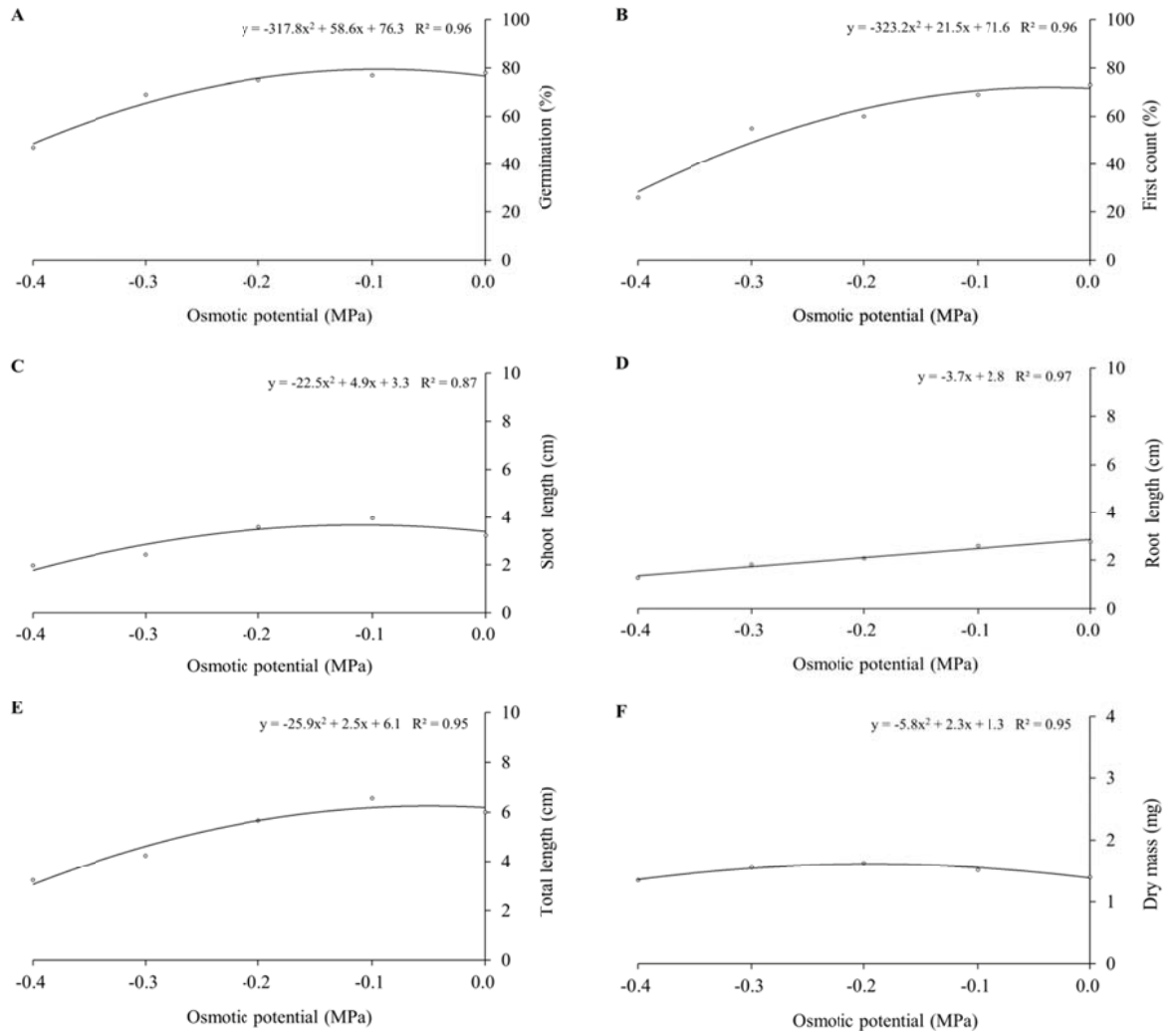

Figure 1. Germination percentage (A), germination first count (B), shoot length (C), root length (D) and total length (E), and seedling total dry mass (F) of quinoa seed subjected to four levels of osmotic potential in polyethylene glycol solution (PEG-6000)

Similar results to this study were obtained by Arcoverde et al. (2017), with decreased shoot length of niger (*Guizotia abyssinica* Cass.) as a function of decreased substrate water potential. Similarly, Hellal et al. (2018) found that the presence of increased PEG-6000 concentrations during growth of barley (*Hordeum vulgare* L.) seedlings inhibited developmental and survival traits, with decreased seedling vigor index occurring with increased stress levels.

3.2 Salt Stress

The analysis of variance evidenced interaction between the studied factors (salts and osmotic potentials) for the variables germination percentage, first count, root, shoot and total length, and dry mass of quinoa seedlings (Table 4). This result indicates that the osmotic potentials influenced the salt action on the seed germination process and initial growth of quinoa seedlings.

Table 4. Summary of the analysis of variance for the variables germination (G), first count (FC), total length (TL), shoot length (SL), root length (RL) and dry mass (DM) of quinoa seedlings exposed to different concentrations of sais

Source of variation	DF	Mean square					
		G	FC	TL	SL	RL	DM
Sal	3	5703.51*	6080.98*	41.24*	11.72*	12.28*	1.61*
Concentration	4	6626.92*	7778.42*	59.43*	6.97*	26.23*	1.82*
Concentration*Salt	12	793.22*	771.35*	3.47*	1.37*	0.94*	0.90*
Residue	60	30.75	37.78	0.52	0.13	0.12	0.04
CV (%)	-	8.59	10.93	15.74	15.64	15.21	16.89

Note. * Significant at 5% probability by the F test. CV = Coefficient of variation.

For all salts, decreased germination percentage and vigor at the first count were observed as the osmotic potential of studied solutions became more negative (Figures 2A and 2B). Nevertheless, $CaCl_2$ and $MgCl_2$ salts promoted greater negative interference in these variables when compared to NaCl and KCl salts, *i.e.*, quinoa seeds presented greater tolerance to these salts in the germination process (Table 4). Different germination and first count results can be found due to chemical differences between the elements that constitute the solutions, even in similar osmotic potentials (Souza & Cardoso, 2000).

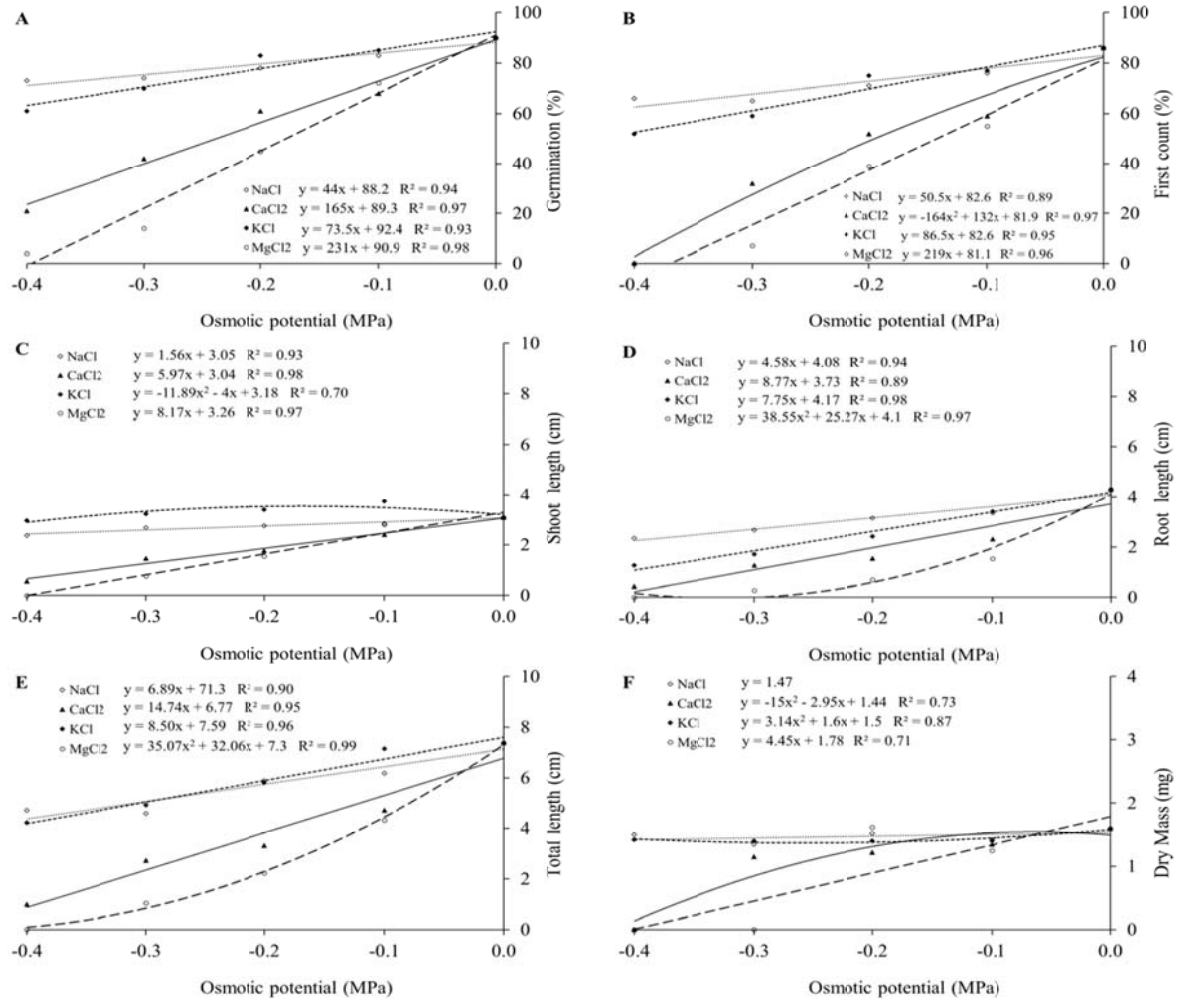

Figure 2. Germination percentage (A), germination first count (B), shoot length (C), root length (D) and total length (E), and total dry mass of quinoa seedlings subjected to four levels of osmotic potential in calcium chloride solution ($CaCl_2$), sodium chloride (NaCl), potassium chloride (KCl) and magnesium chloride ($MgCl_2$)

In the control treatment with absence of salts (0.0 MPa), the seeds presented mean germination of 90% (Figure 2A). When subjected to the NaCl and KCl solutions, germination percentages greater than 80% were observed in the osmotic potentials of -0.1 and -0.2 MPa, whereas germination percentages of 70 and 60% were observed in the solutions containing respectively the same salts in the osmotic potential of -0.4 MPa (Table 4). Nunes et al. (2009) and Bernardes et al. (2015) evaluated the effect of NaCl and KCl on germination of respectively sunn hemp (*Crotalaria juncea* L.) and cabbage (*Brassica oleracea* L. var. capitata) seeds and verified germination greater than 80% with salt solutions at the osmotic potential of -0.2 MPa. Germination percentage was 80% when quinoa seeds were subjected to the $CaCl_2$ and $MgCl_2$ salts at the osmotic potential of -0.1 MPa and reached respectively 21 and 4% germination at the osmotic potential of -0.4 MPa.

First count of quinoa seeds under saline solutions with NaCl and KCl displayed respectively 76 and 77% of normal seedlings at the osmotic potential of -0.1 MPa (Figure 2B). However, when the seeds were subjected to the same salts at the osmotic potential of -0.4 MPa, there was a reduction respectively of 20 and 24 percentage points in the first count of normal seedlings in relation to the control treatment (absence of salts). Meanwhile, 32% and 7% of normal seedlings were recorded respectively with the solutions of $CaCl_2$ and $MgCl_2$ in the osmotic potential of -0.3 MPa. Furthermore, null values of normal seedlings at the osmotic potential of -0.4 MPa were observed for both salts.

The shoot, root and total length were negatively affected by the salt treatments, being the most severe effect observed for $CaCl_2$ and $MgCl_2$ solutions in all osmotic potentials (Figures 2C, 2D, and 2E). The $MgCl_2$ solution caused the greatest reduction in total length of quinoa seedlings at the maximum tested osmotic potential (-0.4 MPa), promoting 100% inhibition of root and shoot growth of quinoa seedlings. Negative osmotic potentials obtained in saline solutions promote decreased seed vigor and can be evaluated by seedling length, which is negatively affected (Dickmann, Carvalho, Braga, & Sousa, 2005).

In general, a more drastic effect of salt solutions was observed on the root length of quinoa seedlings when compared to the shoot length. In an investigation of the influence of saline solutions on the expression of the physiological quality of zucchini seeds (*Cucurbita pepo* L.), Harter et al. (2014) observed a decreasing linear effect for root and shoot length as the NaCl concentration increased, with the most pronounced effect on the seedling root portion, a similar result to those found in our research.

For shoot and root length, 2.4 and 2.4 cm; 0.6 and 0.4 cm; 3.0 and 1.3 cm; 0.0 and 0.0 cm were obtained respectively for the seedlings under solutions with NaCl, $CaCl_2$, KCl and $MgCl_2$ at the maximum tested osmotic potential (-0.4 MPa). Meanwhile, 3.1 cm of shoot and 4.3 cm of root length were found in the control solution (0.0 MPa). The accumulation of ionic species in plant tissues, such as Na^+, Cl^-, Mg^{2+} and Ca^{2+}, existing in the environment where root growth occurs can cause toxic effects to plants, affecting the absorption capacity, transport and use of ions required for their adequate growth (Nobre, Gheyi, Correia, Soares, & Andrade, 2010).

In the treatment containing $MgCl_2$ solution, there was a reduction in the dry mass of quinoa seedlings at the osmotic potential of -0.1 MPa in relation to the control and dry mass values equal to the control in the potential of -0.2 MPa. In the osmotic potentials of -0.3 and -0.4 MPa, the dry mass of quinoa seedlings was null. For NaCl, KCl and $CaCl_2$, smaller seedling dry mass values than the control were verified when under the different tested osmotic potentials.

Therefore, this generated informations aid to understand the effect of water stress and salinity of soils on the germination process and initial growth of quinoa seedlings, contributing with new knowledge about the physiology of this culture and as a way to indicate, in the future, this culture for cultivation in environments with these characteristics.

4. Conclusion

The progressive reduction of the osmotic potential induced by salts NaCl, KCl, $CaCl_2$, $MgCl_2$ and PEG-6000 negatively affects seed germination and initial growth of quinoa seedlings.

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Compatibility Test and Agronomic Performance of Coffee Genotypes (*Coffea canephora* Pierre ex Froehner) in the State of Rondônia, Brazil

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Abstract

The objective of this work was to evaluate the degree of compatibility and agronomic performance in clonal genotypes of canephora coffee plants (*Coffea canephora* ex Froehner) for cultivation in the state of Rondônia, Brazil. The study was conducted with nine genotypes with three replications of *Coffea canephora* Arranged in the field: UFRO-60; UFRO-31; UFRO-61; UFRO-25; UFRO-03; UFRO-08; UFRO-21; UFRO-05 and UFRO-138 In adulthood and in two years of harvest (2013/2014 and 2014/2015). We evaluated: productivity, profitability, conversion index fruit cherry/grain benefited, mass of one hundred grains benefited, average diameter of the fruits, number of rosettes per branch, average distance between rosettes and number of fruits per rosette. Additionally, the degree of compatibility between the clones of clonal. The genotypes of *Coffea canephora* with the best agronomic performances evaluated for the edafoclimatic conditions of the State of Rondônia in this study were UFRO-08, UFRO-25, UFRO-03 and UFRO-138. According to the compatibility tests, the sequence of correct disposition in the field of *Coffea canephora* among the genotypes studied in this study are: UFRO-138; UFRO-31; UFRO-25; UFRO-08; UFRO-60; UFRO-21; UFRO-61; UFRO-03 and; UFRO-05. Close to 74% of the tests Were and self-pollination tests resulted in low fruiting, evidencing the characteristic of self-incompatibility.

Keywords: coffee growing in Rondônia, genetic materials, gametophytic auto incompatibility

1. Introduction

Worldwide, it is estimated that 500 million people are involved in the coffee production chain (Damatta et al., 2007; Ferrão et al., 2017). Brazil stands out as the world's largest producer of coffee and has one of the most efficient coffee growers in the world with a planted area of approximately 2.2 million hectares in 2018 (CONAB, 2018). The species *Coffea arabica* L. and *Coffea canephora* Pierre Ex Froehner are widely cultivated in the country due to the edaphoclimatic conditions favorable, mainly in areas of Southeastern and Northern Brazil (Ferrão et al., 2017). In areas of the Amazon Region, the species is mainly cultivated *Coffea canephora,* due to specific altitude and rainfall requirements (Partelli et al., 2014).

In the State of Rondônia, located in the southern part of Western Amazonia, coffee (*Coffea canephora*) is configured as the most planted perennial culture and contributes to the formation of family income in rural areas (CONAB, 2018).It is estimated, according to CONAB (2018), that the planted area is about 63.900 hectares and approximately 2 million of benefited sacks produced annually. Although the average productivity is still low, with about 31 sacks/ha, there is an annual increase in productivity influenced by the technological package that

involves fertilization, liming, irrigation and pest control in addition to the expressive use of new materials production for renewal of clonal crops (Dubberstein et al., 2017; CONAB, 2018).

Being fundamental for the increase of coffee productivity in the state of Rondônia, the genetic improvement, among other benefits, allows for uniformity of maturation, resistance to periods of drought, decrease of the effect of coffee bienality and greater Yield of the benefited fruits (Ivoglo et al., 2008). However, as the *Coffea canephora* is an alodic species, that is, dependent on other plants so that there is flowering and fruiting, the genetic compatibility among coffee genotypes becomes essential so that the crop can be productive (Marcolan & Espindula, 2015). The self-incompatibility between genotypes is the rejection of their own pollen or of clone plants that carry allelic characteristics that prevent self-fertilization (Nettancourt, 1997). According to Castric & Vekemas (2004), the capacity of the *Coffea canephora* in avoiding self-fertilisation is a vital characteristic of the species in order to avoid the deleterious effects caused by endogamy depression.

For this reason, it is indicated that there is a sufficient quantity of clones or genotypes in the planting area, in order to increase the chances of compatibility and production. Therefore, it is imperative that genetic materials undergo compatibility tests that ensure sufficient pollination, flowering and fruiting capacity, since clones not sufficiently compatible compromise these phenological phases, causing miscarriage and defective grains and consequently decreasing the productivity and quality of the final beverage (Lopes, 2015; Ferrão et al., 2017). Thus, the present study aimed to evaluate the degree of compatibility and agronomic performance in clonal genotypes of canephora coffee plants (*Coffea canephora*) for cultivation in the state of Rondônia, Brazil.

2. Material and Methods

2.1 Location and Experimental Design

The work was carried out in 'Ouro Verde', in the rural area of Novo Horizonte do Oeste, in the State of Rondônia, Brazilian Amazon, Brazil. The climate is predominantly the hot and humid tropical type Aw, according to Köppen, with a well-defined dry period, occurrence of water deficit from June to September, average annual temperature of 25 ºC, average annual precipitation of 2,400 mm (Ageitec, 2019). A randomized block design was used, containing nine treatments, nine genotypes of Conilon coffee (*Coffea canephora*): UFRO-60; UFRO-31; UFRO-61; UFRO-25; UFRO-03; UFRO-08; UFRO-21; UFRO-05 and UFRO-138, arranged in lines, within the field of cultivation, as recommended by Ferrão et al. (2017a) and three repetitions. Except for the evaluation of the 2014/2015 crop, which did not count on the UFRO-61 clone, thus limiting itself to eight treatments. Adult plants were used, producing in the second and third commercial harvest, which were conducted with all the technical recommendations recommended by Ferrão et al. (2017). It was adopted a spacing of 3m x 1,3m, between lines and between plants, respectively, totaling approximately 2.560 plants ha^{-1}, conducted with 3 to 4 rods.

2.2 Experimental Conditions and Variables Analyzed

The harvest of coffee genotypes in the 2013/2014 and 2014/2015 was carried out in April and May when the plants had 80% of the cherry grain stage fruits, ideal harvest point as determined (Nunes et al., 2005). The experimental variables, except for productivity, were estimated from twelve plagiotropic branches taken from the median portion of the canopy of the useful area of the plot, being one branch for each cardinal point in each plant that composed the experimental plot. The evaluations that involved the obtaining of the grain mass were corrected to 13% of moisture, using the drying at full sun on the yard covered by canvas, and performed the periodic turning, according to the orientations of Pinheiro et al. (2012).

Plants were evaluated in open pollination: grain yield (Bags ha^{-1}) determined according to average production per plant, multiplying by the number of plants per hectare; beneficiation bield, consisting of the conversion of fruits into 'coco' type in benefited grains; cherry/grain benefit ratio; mass of 100 grains; average diameter, measured using a caliper, determined from the arithmetic mean obtained from 50 fruits. The morphological characteristics were also evaluated: number of rosettes per plagiotropic branch; average distance between rosette, determined by the distance between the first and last rosette, and divided by the number of rosette and the average number of fruits per rosette for each genotype.

2.3 Genetic Compatibility Test

The compatibility test was installed in the main florade of the genotypes of *Coffea canephora*, a stage resulting from temporary water deficit, Following the procedures and steps proposed by Ferrão et al. (2017b) and Teixeira et al. (2011). The plants that would serve as pollen receptors were determined and constituted in an experimental plot. Each parcel received nine crosses, consisting of manual pollination with pollen from the other varieties and a self-pollination test. In each branch plagiotrópico selected himself four rosettes from the apex of the branch,

and in these, counted all the floral buds contained. Subsequently, the apical meristems were cut to avoid the emission of new floral buds, being protected with Kraft paper sad the branches that would serve as receptors and pollen donors in order to avoid the fertilization of uncontrolled plants in opening of the flowers. The branches were protected from the sun's rays, being chosen those that were on the side of the shadow, seen the potentiality of the burning of the floral buds.

One day after these procedures, the flowers were opened, and artificial pollination was performed at the beginning of the day according to the recommendations of Ferrão et al. (2017b). The pollen donor branches were cut off and with the removal of protective sacks from the receptor branches, pollination was performed. After four days, after the period of receptibility of the flowers to pollen, he withdrew all the paper sacks and the normality of the flowers was verified, identifying the crosses performed in each branch. During the whole fruiting period, monitoring was performed weekly for the emission of new buttons. At the end of 21 weeks the initial date, when there were no greater manifestations of incompatibility and to prevent the environment from interfering in the final number of fruits, carried out the final count of the fruits that they aved, to determine the compatibility among the genotypes (Figure 1).

Figure 1. Protection of the branches of the genotypes of *Coffea canephora* (left) and monitoring of the floral buttons (right)

Source: Authors.

2.4 Analysiss Statistics

The agronomic assessments and compatibility testing were submitted to determination of the confidence interval ($P < 0.05$), based on the difference between the upper confidence limit of the mean (95%) and the arithmetic mean, the coefficient of variation and the Scott-Knott test, at a 5% probability level.

3. Results and Discussion

The yield of the genotypes (Clones) of *Coffea canephora* studied did not differ statistically. However, the average productivity in 2013/2014 harvest of the clones was about 101 sacks ha^{-1}, around three times higher than the current average productivity of Rondônia, of 30.97 sacks ha^{-1} (CONAB, 2018). In The 2014/2015 harvest, the clones UFRO-25, UFRO-03, UFRO-08 and UFRO-138 presented the highest yields (76 sacks ha^{-1}), however, were 24.4% of the previous crop (2013/2014) (Table 1). Teixeira (2014) also verified the decrease in productivity and subsequent brothers and according to Ferrão et al. (2017) and Rocha et al. (2015) this fact correlates with the productive variation of each genotype or to non-measured climatic variations. The conversion rate of the cherry fruit to benefited grain was 4.44 in the Harvest 2013/2014, with the clones UFRO-60 and UFRO-61 with the highest indices and 4.36 in the Harvest 2014/2015 with the clones UFRO-60 and UFRO-61 with the highest indexes. Similar values were obtained by Ferrão et al. (2013abc) for varieties of *Coffea canephora* "Diamond Incaper 8112", 'Jequitibá Incaper 8122' and 'Centenary Incaper 8132' of 4.27, 4.22 and 4.23, respectively.

The highest indices reflect proportionally the lowest profitability in coffee cultivation (Ferrão et al., 2013a). The clones UFRO-25, UFRO-08 and UFRO-138 showed the best profitability in the 2013/2014 crop, however, in the 2014/2015 crop, it did not present significant differences. The mean yield values are above those observed by Gaspari-Pezzopane et al. (2004), which ranged from 48.4% to 61.9% for *Coffea canephora*. According to these authors, the yield directly influences the final cost of coffee production, and when the lower these values, the

costlier is the production. Clonal crops, such as the ones in this study, present higher grain maturation uniformity, increasing the yield in relation to the seminiferous crops (Partelli et al., 2006).

The mass of 100 grains was significantly more in several clones, except in the genotype UFRO-61 (15.13 g). As can be seen in Table 1, the lower weight of the grains seems to be directly related to the Yield of this genotype, which was also the smallest. In general, the mean mass of 100 grains observed were higher than those observed by Kameyama et al. (2016) for several coffee genotypes in the state of São Paulo, Brazil, between 7.1 and 13.2 G. The genotypes studied by Ferrão et al. (2013a) obtained weights similar to the Obtained by the genotypes studied in this study. According to Medina Filho & Bordignon (2003) the mass of 100 grains is an excellent indicator of pollination and compatibility, because the emergence of 'Moca' grains indicates that only one of the grain compartments was fertilized, decreasing the weight of the grains and the productivity.

Table 1. Evaluation of the components: productivity, beneficiation yield (BY), cherry fruit/grain benefit ratio (CF/GB), mass of 100 grains benefited (M100) and fruit diameter (FD), in clonal genotypes of Canephora Coffee (*Coffea canephora* Pierre ex Froehner)

	Productivity (bags ha^{-1})		BY (%)		CF/GB		M100 (g)		FD (mm)	
	--------- Harvests ---------		---- Harvests ----		--- Harvests ---		---- Harvests ----		---- Harvests ----	
	1º	2º	1º	2º	1º	2º	1º	2º	1º	2º
UFRO-60	85.16a	73.76b	64.40b	59.45a	4.92a	5.04a	22.20a	16.70a	11.11b	10.96a
UFRO-31	87.74a	59.30b	63.95b	63.44a	4.40b	4.30b	19.37b	17.00a	11.98a	10.55a
UFRO-61	95.73a	-	59.64c	-	4.83a	-	15.13c	-	10.62b	-
UFRO-25	113.84a	86.78a	67.28a	64.22a	4.36b	4.05b	25.17a	18.27a	11.23b	9.96a
UFRO-03	107.54a	95.44a	63.33b	64.51a	4.50b	3.86b	23.30a	18.77a	10.47b	11.23a
UFRO-08	93.82a	81.99a	66.48a	62.57a	4.29b	4.17b	25.07a	18.97a	12.40a	11.33a
UFRO-21	109.17a	66.20b	64.79b	62.26a	4.48b	4.79a	19.20b	16.80a	11.21b	10.28a
UFRO-05	109.08a	69.21b	63.69b	58.41a	4.18b	4.39b	21.93a	16.27a	12.25a	11.04a
UFRO-138	104.97a	80.62a	67.18a	68.20a	4.04b	4.31b	22.83a	19.97a	10.40b	10.65a
Average	100.7	76.67	64.53	62.88	4.44	4.36	21.58	17.84	11.30	10.75
Standard Deviation	±6.71	±6.38	±1.06	±2.25	±0.14	±0.18	±1.41	±0.84	±0.43	±0.29
C.V.	13.79	12.09	2.54	8.32	6.02	5.47	9.45	9.96	7.01	5.30

Note. Averages followed by the same letter in the column do not differ from each other by the Scott-Knott test at a 5% probability level. C.V.: Coefficient of Variation. Harvests: 1st (2013/2014) and 2nd (2014/2015).

Regarding the fruit diameter, there were statistical differences only for the clones in the 2013/2014 harvest in which the clones UFRO-31, UFRO-08 and UFR0-0 were better (Table 2). According to Rocha et al. (2013), the largest grain size has a direct reflex in the final beneficiation of coffee with a positive correlation between this factor and the yield. Both the weight of the grains and the size are genetic characteristics controlled by several genes and may undergo variation among the genotypes and suffer with environmental factors such as water availability and fertilization management (Ferrão et al., 2017b). According to Silva et al. (2008), the decrease in the mass of 100 grains of the harvest of 2013/2014 for the harvest of 2014/2015 is due to the bienality of coffee, characterized by the decrease of the plant reserves due to the high yield crop of the previous year.

The Clones UFRO-31, UFRO-25, UFRO-08 and UFRO-21 presented the highest number of rosettes per stem (NR) in the harvest of 2013/2014 and clones UFRO-03 and UFRO-31 in the Harvest 2014/2015 (Table 2). According to Rocha et al. (2013) the number of rosettes by branches favors the increase in the productivity of *Coffea canephora* and the selection of genotypes with superior characteristics. Verifying cultivars of *Coffea canephora* in the state of Rondônia, Rocha et al. (2013) observed a positive correlation between plant height and the number of rosettes per stem, allowing the selection of genotypes with lower heights, which facilitate treatment cultural, but with higher numbers of rosettes. The average number of rosettes obtained in this study are higher than those obtained by Ricci et al. (2013) in different shading conditions. According to the authors, the increase in luminosity influences the highest number of rosettes in the stem.

For the distance between the rosettes in the branches, the UFRO-05 clone showed the longest distance in the two harvests (2013/2014 and 2014/2015) (Table 2). The longest distances between the rosettes are unfavorable in a selection program of superior genotypes, considering that it is related to the decrease in the number of rosettes that a plagiotropic branch may contain, decreasing the overall productivity of the coffee (Marcolan & Espindula, 2015). According to Charrier and Eskes (2004), the distance between rosettes is correlated to smaller grains, but a larger number of grains per rosettes. Ricci et al. (2013) verified distances lower than this work. In this respect,

the number of grains per rosette (NFR) was higher for the clones UFRO-08, UFRO-21 and UFRO-25 in the Harvest 2013/2014 and UFRO-08 in the Harvest 2014/2015. Second Ferrão et al. (2017) both the spacing when the luminosity but limit the number of grains in the rosette, however, intrinsic characteristics of the plants can directly influence this item.

Table 2. Number of rosettes per plagiotropic branch (NR), distance between rosettes (DR), and average number of fruits per rosette (NFR) of twelve productive stems, in clonal genotypes of Canephora coffee trees (*Coffea canephora* Pierre ex Froehner)

Genotypes	NR		DR (cm)		NFR	
	--------- Harvests ---------		--------- Harvests ---------		--------- Harvests ---------	
	1º	2º	1º	2º	1º	2º
UFRO-60	12.36b	14.22b	5.01b	4.19c	12.28b	12.45c
UFRO-31	14.33a	15.94a	4.75b	4.06c	11.14b	9.35d
UFRO-61	12.89b	-	4.09c	-	13.57b	-
UFRO-25	15.94a	14.89b	4.87b	4.03c	17.59a	13.06c
UFRO-03	12.94b	16.22a	4.65b	4.45b	9.74b	14.64b
UFRO-08	14.69a	14.06b	4.62b	4.42b	16.67a	20.57a
UFRO-21	15.03a	13.75b	4.90b	4.23c	14.79a	14.31b
UFRO-05	12.75b	13.50b	5.35a	4.76a	13.07b	15.90b
UFRO-138	11.61b	13.92b	4.83b	4.56b	12.48b	12.37c
Average	13.61	14.56	4.78	4.34	13.48	14.08
Standard Deviation	±0.60	±0.52	±0.14	±0.12	±1.15	±1.42
C.V.	5.08	6.19	3.07	3.00	13.13	9.34

Note. Averages followed by the same letter in the column do not differ from each other by the Scott-Knott test at a 5% probability level. C.V.: Coefficient of Variation. Harvests: 1st (2013/2014) and 2nd (2014/2015).

The compatibility test between clonal genotypes differed statistically for receivers (lines) e for donors (columns). There is the presence of at least two compatibility groups, being reserved the lowest level of compatibility for self-pollinations, evidencing the self-incompatibility existing in the coffee plant. Analyzing the interactions between the receptors, it was statistically observed that the UFRO-03 genotype presented a low level of compatibility with the UFRO-21 genotype, which is similar to that observed in the self-pollination test. However, it cannot be affirmed that these genotypes share one or more alleles, characterizing them as "half siblings", as evidenced by these results, since the reciprocal among the clones presents high compatibility. Similarly, it occurs with the UFRO-08 clone, which for the UFRO-21 and UFRO-05 clone, presented the aforementioned occurrence (Table 3).

These facts are justified by the loss of pollen viability, which suffers oscillations and the germination of the germinative power due to artificial manipulation that can result in different forms in each genotype (Mendes, 1949). Nevertheless, it is important to emphasize that the donor and receptor branches in this study were covered with sacks at a time that coincides with the warmest period of the year in the state of Rondônia, which may be an additional factor for the reduction of the viability of the pollen, and consequently contributed to the low number of fruits.

As for the clone of *Coffea canephora* UFRO-08, three compatibility groups were presented: the first of lower level, restricted to self-pollination, a second group to which the clones are inserted UFRO-21, UFRO-05 and UFRO-138. With median compatibility and a third group with the other genotypes, composing the high compatibility group. For the clone UFRO-21, four compatibility groups were identified: one of the lowest compatibilities linked to self-pollination, one second of median compatibility, where is the UFRO-138 clone, a third of high compatibility with the UFRO-05 clone and a fourth group with excellent level of compatibility, where all other genotypes are inserted (Table 3; Figure 2).

Table 3. Fruiting percentage in manual pollinations between nine clonal genotypes of Canephora coffee plants (*Coffea canephora* Pierre ex Froehner)

Clone Receiver	Donor Clone											
	UFRO-60	UFRO-31	UFRO-61	UFRO-25	UFRO-03	UFRO-08	UFRO-21	UFRO-05	UFRO-138	Average	Standard Deviation	C. V.
	%											
UFRO-60	16cB	78bA	84aA	86aA	94aA	71aA	85aA	94aA	9aA	77.6	±10.4	16.78
UFRO-31	59bA	14cB	71bA	72bA	57bA	62aA	45bA	48cA	58bA	54.2	±8.1	23.66
UFRO-61	91aA	93aA	10cB	94aA	94aA	83aA	82aA	63bA	93aA	78.2	±11.8	20.07
UFRO-25	67bA	73bA	86aA	9dB	63bA	69aA	68aA	59bA	68bA	62.4	±9.6	15.10
UFRO-03	66bA	73bA	89aA	72bA	10cB	80aA	17cB	57bA	56bA	57.6	±11.3	20.86
UFRO-08	81aA	91aA	79aA	75bA	58bA	7cC	48bB	29cB	45bB	57.1	±11.6	22.78
UFRO-21	92aA	88aA	91aA	89aA	91aA	91aA	13cD	68bB	50bC	74.7	±11.0	12.86
UFRO-05	80aA	77bA	70bA	68bA	71bA	67aA	47bA	12cB	69bA	62.4	±9.3	21.07
UFRO-138	71bA	61bA	55bA	48cA	55bA	43bA	34bA	42cA	6cB	46.1	±8.3	26.83
Average	69.2	72.0	70.6	68.1	65.9	63.7	48.8	52.6	59.4			
Standard Deviation	±9.7	±9.6	±10.5	±10.7	±11.3	±11.0	±10.9	±10.4	±11.2			
C.V.	17.52	14.24	15.76	16.30	21.99	24.51	26.30	29.72	23.65			

Note. Averages followed by the same letter in the column do not differ from each other by the Scott-Knott test at a 5% probability level. C.V.: Coefficient of Variation. Harvests: 1st (2013/2014) and 2nd (2014/2015).

These results were not expected, considering that the literature predicted that the proportions expected for fruiting of the coffee plant, governed by three alleles of the S gene, are 0% for incompatibility, 50% for partially compatible and 100% for Fully compatible (Ferrão et al., 2017b). Other external and internal factors may explain the values outside the recommended margin that interfered in the fruiting percentages. The mechanisms of self-incompatibility usually present in the inhibition of pollen tube elongation as a consequence of protein interactions present in both pollen and stigma. Evidence shows that in the diploid species of the genus *Coffea* as *Coffea canephora*, there is the formation of RNAses in the pistil cells in the gene locus "S" (Asquini et al., 2011; Nowak et al., 2011).

Figure 2. Manifestation of incompatibility in genotype of *Coffea canephora*

Source: Authors.

There is, observing the data in Table 3, a numerical discrepancy for the pollen donor genotypes, allowing the creation of three groups based at the compatibility level, with the exception of the clone UFRO-25. In the first group, interactions with a compatibility equal to or greater than 79% are inserted, except for five interactions, among the clones UFRO-08: Ufro-05, UFRO-08: UFRO-60, UFRO-08: UFRO-31, UFRO-08: UFRO-25, and UFRO-21: UFRO-25 that are inserted in this group and presented compatibility degree of 67%, 71%, 62%, 69% and 68% respectively.

In the second group are the interactions with compatibility between 18 and 78%, except the exceptions and the third group with compatibility between 6 and 17%, except for three interactions, all in the UFRO-05 genotype, between this and the genotypes UFRO-31, UFRO-08 and UFRO-138, which presented fruiting percentage of 48%, 29% and 42% respectively, and which are included in this group. There is an exception to the genotype

UFRO-25 That presented four groups, in which they were inserted in the first ones with a degree of compatibility equal to or above 86%, the second between 68 and 85%, the third between 10 and 67% and the fourth ones with 9% compatibility or less. In four occurrences, when the genotypes were pollens donors, there were statistically significant interactions between genotypes that manifested themselves as similar to the self-pollination test. The interactions between: Ufro-21: Ufro-03, UFRO-05: UFRO-31, UFRO-05: Ufro-08 and UFRO-05: UFRO-138 (Table 3).

Working with compatibility test between genotypes of *Coffea canephora* in the state of Rondônia, Brazil, Lopes (2015) verified compatibility between the tested plants and suggests the existence of more than one compatibility group, confirmed by this work. Some field observations indicated that in the *Coffea canephora*. There are three allelic forms of the "S" gene (S1, S2 and S3) controlling the gametophytic type self-incompatibility characteristic (Conagin & Mendes, 1961) (Figure 2). According to Omolaja and Falzun (2004), they have five allelic forms of the "S" gene can control compatibility and sustain the results of this work. According to the authors, the number of genotypic expressions would reach the ten case, confirming the existence of five alleles (S1S2, S1S3, S1S4, S1S5, S2S3, S2S4, S2S5, S3S4, S3S5, S4S5).

Through descriptive analysis, Monaco and Carvalho (1972) report that cross-matching between cultivars of *Coffea canephora* should present fruiting percentage equal to or greater than 50%, which was obtained in 74% of the tests performed in this study (Table 3). The literature reports the admission of 5 to 10% of self-fertilisation Schifino-Wittmann and Dall'agnol (2002), however, Monaco and Carvalho (1972) and Ferrão et al. (2017b) affirm that this percentage should be 0%, but this fact was not observed in this study. Further studies are needed to technically assess the compatibility between genotype of *Coffea canephora*, in order to identify the interaction of the materials for planting indication to farmers. Knowledge of compatibility or self-incompatibility between genotypes allows for increased productivity and final uniformity in Coffee cultivation (Ferrão et al., 2017; Schifino-Wittmann & Dall'agnol, 2002).

4. Conclusions

According to the compatibility tests, the correct disposition sequence in the field of *Coffea canephora* Among the genotypes studied in this work are: UFRO-138; UFRO-31; UFRO-25; UFRO-08; UFRO-60; UFRO-21; UFRO-61; UFRO-03 and; UFRO-05. The genotypes of *Coffea canephora* with the best agronomic performances evaluated for the edaphoclimatic conditions of the State of Rondônia in this study were UFRO-08, UFRO-25, UFRO-03 and UFRO-138. The results of the compatibility tests were not similar to those proposed for coffee-incompatibility in Brazil.

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Species of *Malva* L. (Malvaceae) Cultivated in the Western of Santa Catarina State and Conformity With Species Marketed as Medicinal Plants in Southern Brazil

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Abstract

The *Malva* genus presents different species with therapeutic potential and inadequate consumption can occur due to the incorrect identification of the plant in the market. The objective of this study was to identify species of the *Malva* genus cultivated in the Western Mesoregion of Santa Catarina State-Southern Brazil, and to verify the conformity of products' labels marketed as dehydrated medicinal plants through the characteristics of the plant parts. The specimens were collected following an unsystematic procedure from households of ten municipalities. The material was identified with the help of specialized bibliography and a specialist. After, they were herborized and deposited in the herbaria of the State University of Santa Catarina (LUSC) and of the Botanical Garden Research Institute of Rio de Janeiro (RB). Five cultivated species of *Malva* were found (*M. nicaeensis*, *M. parviflora*, *M. pseudolavatera*, *M. Sylvestris* and *M. verticillata*). Whereas one species of the Geraniaceae family (*Pelargonium graveolens*) and three species of the Malvaceae family (*Malva sylvestris, M. verticillata* and *Sida cordifolia*) were found in the dehydrated packages. *S. cordifolia* species was present in 80% of the samples, with 65% of them marketed as *M. sylvestris* or under the common name of "mallow". Macroscopic images of the species and illustrations of the mericarps were made and an identification chart along with the morphological description for each species was elaborated based on the morphological results. Descriptions of labels for 60 samples were checked for the determination of the dehydrated *Malva* species marketed. Characteristics of parts of the plant in comparing them with the characteristics described in the specialized literature were performed. The target species of incorrect identifications in the analyzed packages was *M. sylvestris*.

Keywords: *Malva sylvestris*, medicinal plants, morphology, *Sida cordifolia*

1. Introduction

Medicinal plants represent an important therapeutic resource for the Brazilian population and they are part of the country's Public Policies (Brasil, 2006). The use of medicinal plants can be influenced by the economic condition, the high cost of medicines and the difficult access to public consultations. In addition to that, there is a difficulty of access by residents in rural areas to health care units located in urban areas. Moreover, the increase the trend for considering traditional knowledge that supports using natural resources as an alternative to synthetic drugs (Battisti et al., 2013).

The indiscriminate use of plants due to the lack of phytochemical, pharmacological and mainly toxicological knowledge is of great concern for public health. The correct identification of medicinal plant species is necessary, especially when they are processed in order to avoid misuse of medicinal plants (Romitelli & Martins, 2013). The study of the plant's anatomy and morphology can help in the quality control of processed medicinal plants.

The macroscopic and microscopic identifications of parts of the commercialized plants should be part of the current norms applied to drugs of plant origin (Brasil, 2010).

Malvaceae ('the mallows') is a botanical family with a rich diversity of species for textile, medicinal, and ornamental purposes. It consists of 4465 species and about 245 genera (The Plant List, 2013) and mallows present a cosmopolitan distribution, but with a high number of species in the tropics. In Brazil, there are 781 species with 73 genera distributed in all biomes (Flora do Brasil 2020, n.d.). Given its high diversity, its primary genetic center of origin is still discussed, and many species have become generalized over world (Ray, 1995). The *Malva* L. genus, popularly known as mallow, grows spontaneously in almost all of Europe and the Mediterranean region. The vast majority of the mallows genera are native to Brazil, with less than a dozen of which being considered as introduced species.

The *Malva* genus has 25-40 species and it can be considered as an annual and/or biannual herb. Flowers with an epicalyx and 8-15 reticulated mericarps are the typical one (Fryxell, 1988). Ray (1995) and Escobar, Schönswetter, Fuertes Aguilar, Neto Feliner and Schneeweiss (2009) relate their similarity to the *Lavatera* L. genus, where the bractoles of the epicalice are joined at the base, in contrast to in *Malva* where they are totally separated. Although there is inconsistency for some species in relation to the fusion of bracts and other characteristics. Molecular studies have also shown that the separation of these two genera based on this morphology is artificial and unsatisfactory (Escobar et al., 2009).

Malva species are indicated with potential therapeutic as cicatrizing and analgesic by the Ministry of Health. They are part of the National List of Medicinal Plants of interest of the *SUS* (Sistema Único de Saúde-RENISUS), whose objective is to guide the productive chain of medicinal plants and research development. Studies carried out by Alves (2009) and Benson (2015) consider *M. sylvestris* L. as having dental antimicrobial activities in oral treatments. According to the Phytotherapeutic Guidelines of Brazilian Pharmacopoeia, the indications for its use are the leaves and flowers for internal use such as an expectorant, and for external use as an antiseptic for the oral problems (Farmacopeia Brasileira, 2002). Ecker et al. (2015) compiled 56 articles addressing *M. sylvestris* as an herbal medicine for several purposes.

The use of *M. sylvestris* as a medicinal plant in Brazil involves different species, with the consumption of mallow leaves collected from plantations or acquired from commercial/health establishments for medicinal purposes. A survey of the conformity of macroscopic characteristics of parts of leaves, stems, floral buds and fruits of mallows marketed as medicinal plants, and the identification of the species grown near residences can clarify which species are commercialized.

The objectives of this study were to identify species of the genus *Malva* grown in the Western Mesoregion of the state of Santa Catarina, Southern Brazil, and to verify the conformity of the label of commercialized products, such as dehydrated medicinal plants, according to the characteristics of parts of the plant.

2. Method

Species were localized in households of ten municipalities in the Western Mesoregion of Santa Catarina State according to previous indication. The study was made among coordinates 26°35′58.91″ S to 27°22′22.97″ S and 50°40′17.00″ W to 52°36′36.79″ W at an altitude ranging from 450 to 1200 meters (Figure 1, Table 1).

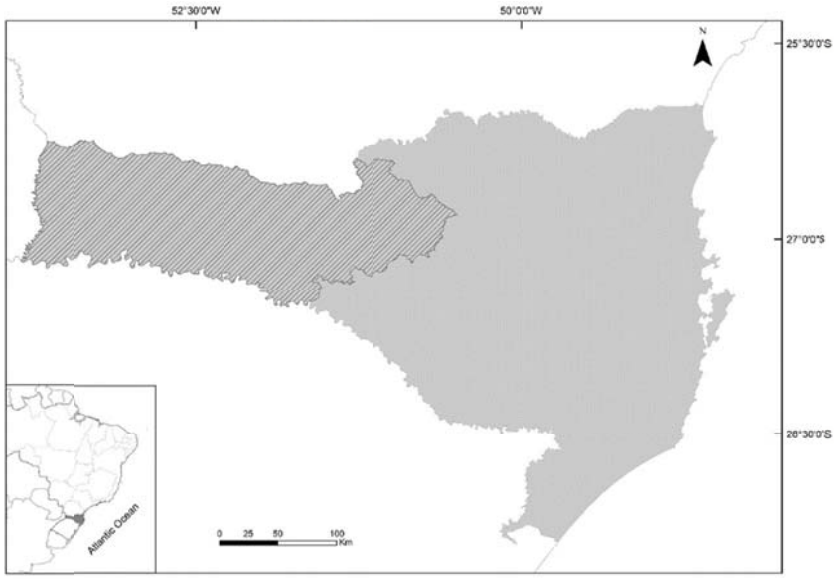

Figure 1. Map of the state of Santa Catarina (Brazil). Oblique lines delimit the Western Mesoregion of Santa Catarina

Malva species were collected in an unsystematic way, meaning without a defined system. The collected material was herborized in accordance with the usual herborization techniques and later incorporated into the Lages herbaria of the State University of Santa Catarina (LUSC) and the Botanical Garden Research Institute of Rio de Janeiro (RB). The morphological study was carried out based on the collected material, and the collections were deposited in the LUSC, RB and R herbaria (acronyms according to Thiers, 2017), as reference standards. Species were identified with the aid of specialized literature as described by Robyns (1965), Baroni (1984), Fryxell (1988), and Ray (1995). Consultation with specialists and often the support of digital platforms such as the Hinsley (2014) and Flora-On (2017) were used. ArcGis 10.5 software was used for preparing the map.

Table 1. Municipalities and coordinates of the sampled sites for *Malva* species grown in the western Mesoregion of Santa Catarina State, Brazil

Municipality	Lat. (S)	Long. (W)	*M. nicaeensis*	*M. parviflora*	*M. pseudolavatera*	*M. sylvestris*	*M. verticillata*
	26°46′44.7″ S	51°0′47.1″ W		X		X	X
	26°44′13.6″ S	50°59′56.9″ W		X		X	
	26°46′59.7″ S	51°0′10″ W		X			
Caçador	26°46′36.2″ S	51°0′49.3″ W		X			
	26°46′44.7″ S	51°0′47.1″ W		X			
	26°46′59.9″ S	51°1′44.0″ W		X			
	26°46′6.3″ S	51°0′22.2″ W				X	
	26°35′58.91″ S	51°6′2.52″ W			X		X
	26°36′15.62″ S	51°5′54.78″ W		X	X		
	26°36′14.61″ S	51°5′54.92″ W		X			
	26°36′15.47″ S	51°5′54.82″ W		X			
Calmon	26°36′15.3″ S	51°5′54.8″ W		X			
	26°36′12.4″ S	51°5′59.1″ W		X			X
	26°36′14.83″ S	51°6′2.80″ W	X				
	26°36′14.83″ S	51°6′2.16″ W	X				
	26°36′13.93″ S	51°5′44.08″ W	X	X			

Catanduvas	27°4′13.98″ S	51°39′ 42.01″ W			X
Cordilheira Alta	26°59′41.10″ S	52°36′36.79″ W	X	X	
Lebon Regis	26°59′59.53″ S	50°43′57.14″ W	X		
	26°55′53.29″ S	50°41′52.65″ W	X		
	26°59′29.65″ S	50°43′28.16″ W	X		
	26°59′6.43″ S	50°42′42.62″ W	X		X
	26°55′54.66″ S	50°41′54.74″ W	X		
	26°51′36.00″S	50°50′18.49″ W	X		
	26°51′33.58″S	50°50′ 19.39″W	X		
	26°55′30.64″ S	50°39′57.59″ W	X		
Peritiba	27° 22′22.97″ S	51°54′14.00″ W			X
Rio das Antas	26°54′9.93″ S	51°4′35.65″ W	X		
	26°52′54.62″ S	51°2′53.7″ W	X		
	26°54′8.62″ S	51°4′34.81″ W			X
	26°52′52.31″ S	51°7′35.56″ W	X		
	26°53′29.07″ S	51°3′36.755″ W	X		
	26°53′39.22″ S	51°4′28.88″ W	X		
	26°53′54.59″ S	51°4′30.86″ W			X
Timbó Grande	26°37′7.60″S	50°40′26.79″ W	X		
	26°37′9.94″ S	50°40′27.04″ W	X		
	26°37′4.18″ S	50°40′17.00″ W	X	X	
	26°37′9.73″ S	50°40′33.6″ W	X		
	26°37′9.73″ S	50°40′33.6″ W	X		
	26°37′8.75″ S	50°40′42.59″ W	X		
	26°37′9.91″ S	50°40′27.01″ W	X		
Vargem Bonita	27°0′24.01″ S	51°44′24″W			X
Videira	27°0′7.02″S	51°9′33.119″ W	X		
	27°0′27.84″ S	51°9′2.55″ W	X		
	27°0′7.34″ S	51°9′33.04″ W	X		
	27°0′0.54″ S	51°7′12.198″ W	X		

Based on the morphological data, an identification chart and a morphological description of each species were elaborated. Macroscopic images of the plants were also performed using a Sony MVC-CD500 digital camera and illustrations of the mericarps were made with ink on butter-paper. The characters used were those that could be observed by the naked eye or with the help of a hand magnifying glass.

Samples of dehydrated *Malva* species marketed as medicinal plants were acquired from commercial establishments and pharmacies in the Southern Region of Brazil, randomly. For this purpose, 60 samples of commercial packages were purchased, in pharmacies (70%), natural products (25%) and supermarkets (5%). Only 85% of the products marketed had the proper batches. The label descriptions of each sample were observed. In addition to that, macroscopic and microscopic characteristics (for exemple trichomes), of parts of the plant by comparing them with the characteristics described in the specialized literature were made.

3. Results

The visits all over the Western Mesoregion of Santa Catarina State could allow observed 180 specimens of *Malva* distributed into five species: *M. nicaeensis* Allioni*, M. parviflora* L.*., M. pseudolavatera* Webb. & Berthel*, M. sylvestris* L., and *M. verticillata* L.

Three species of Malvaceae and one of Geraniaceae were found from 60 samples collected as dehydrated samples labeled under the common name of mallow, *malva-branca* (white-mallow), or *malva-cheirosa* (smelled-mallow). In relation to the *Malvaceae* species, we identified: *M. sylvestris*, *M. parviflora and Sida cordifolia* L.

Material from *Pelargonium graveolens* L'Hér. ex Aiton was found in 1.66% of the packages, which is a Geraniaceae (Table 2); a medicinal and aromatic species originated in South Africa (Arrigoni-Blank, Almeida, Oliveira & Blank, 2011). *P. graveolens* is widely used in the perfume and cosmetic industries (Saxena et al., 2000).

Table 2. Species identified on the labels of the marketed packages with their contents

Botanical name according to the label	Species confirmed on the package contentes			
	Numbers in percentage			
	Sida cordifolia	*Pelargonium graveolens*	*Malva sylvestris*	*Malva parviflora*
M. sylvestris	40.00	0.00	15.00	1.66
*M. crispa**	0.00	1.66	0.00	0.00
Sida cordifolia	15.00	0.00	0.00	0.00
Absent	25.00	0.00	1.66	0.00
Total (%)	80.00	1.66	16.66	1.66

Note. * Current name: *M. verticillata.*

3.1 Key for Identification and Description of Species

3.1.1 Key for the Identification of *Pelargonium graveolens*, *Sida cordifolia* and *Malva* Species From the Western Mesoregion of Santa Catarina

1-Leaves opposite; adaxial surface trichomes simple …… *Pelargonium graveolens*

1-Leaves alternate; adaxial surface trichome fasciculate and simple …….. 2

2-Flowers without involucel; petals not emarginate ……. *Sida cordifolia*

2-Flowers with involucel; petals emarginated ……..... 3

3-Floral bracts linear to lanceolate …….. 4

3-Floral bracts lanceolate to ovate …….. 6

4-Lamina adaxial surface pubescent, trichome fasciculate and simple; mericarps almost smooth ……. *M. pseudolavatera*

4-Lamina adaxial surface glabrescent, trichome simple; mericarps laterally reticulated ……...… 5

5-Mericarps extremely reticulate, with slight winged projections …….. *M. parviflora*

5-Mericarps slight reticulate …….. *M. verticillata*

6-Herbaceous erect; petals 7-20 mm long, pinkish; mericarps gently reticulated …… *M. sylvestris*

6-Herbaceous frequently decumbent. Petals 6-10 mm long, purplish to lavender; mericarps slight reticulated …… *M. nicaeensis*

3.1.2 Description of *Malva* Species Cultivated in the Western Mesoregion of Santa Catarina

Malva nicaeensis Allioni, Fl. Pedem. 2:40. 1785.

Herbaceous decumbent, rarely erect, 0.7-2.1 m tall. Stipule 2-8 × 3-6 mm. Leaves with petioles 4-20 cm long. Lamina 3.3-12 × 4-18 cm, reniform to orbicular, rarely lobate, base slight auricle, margin crenate; glabrescent. Flowers 3-9 in the leaf axils. Bractlets of the involucel lanceolate to ovate, ca. 2 mm wide; calyx 4-7 mm long, spreading in fruit; corolla purplish to lavender, ca. 8 mm diameter; petals 0.6-10 cm long, emarginated. Schizocarp brown when mature, ca. 0.7 cm diameter, 9-12 mericarps, slight reticulated (Figures 2a and 3a).

Malva parviflora L., Demonstr. pl. 18. 1753.

Herbaceous decumbent to erect, 0.4-1.8 m tall. Stipule 2-8 × 3-6 mm. Leaves with petioles 3.8-24.5 cm long. Lamina 2.5-12 x 3.3-18 cm, reniform, lobate, base auriculate, margin crenate; glabrescent. Flowers 2-5 in the leaf axils. Bractlets of the involucel linear to lanceolate, ca. 3 × 2 mm; calyx 2-7 mm long, spreading in fruit; corolla whitish with red center lavender, ca. 0.7 cm diameter; petals 0.4-0.7 cm long, emarginated. Schizocarp brown or gray, when mature, ca. 0.7 cm diameter, 10-11 mericarps, with slight winged projections (Figures 2b and 3b).

Malva pseudolavatera Webb. & Berthel, Hist. Nat. Iles Canaries 3(2(1)):29(-30). 1836.

Herbaceous decumbent to erect, 0.4-2 m tall. Stipule ca. 5 × 3 mm. Leaves with petioles 4 × 14.1 cm long. Leaf blade 3-9.5 × 4-17 cm, reniform, lobate, base truncate to slight subcordate, margin crenate; pubescent. Flowers 2-7 in the leaf axils. Bractlets of the involucel linear to lanceolate, ca. 3 × 2 mm; calyx 5-9 cm long, spreading in fruit; corolla whitish to lavender, maculate, ca. 1 cm diameter; petals 0.6-2 cm long, emarginated. Schizocarp brown, ca. 1 cm diameter, 9 mericarps, almost smooth (Figures 2c and 3c).

Malva sylvestris L., Sp. Pl. 685. 1753.

Herbaceous erect, 0.5-1.5 m tall. Stipule ca. 5 × 3 mm. Leaves with petioles 5-8 cm long. Lamina 3-9 × 3-8 cm, reniform, lobate, base truncate, margin crenate; glabrescent. Flowers 3-8 in the leaf axils. Bractlets of the involucel lanceolate to ovate, ca. 2 mm wide; calyx 3-6 mm long, slight spreading in fruit; corolla purplish, maculate, ca. 2.5 cm diam; petals 0.7-2.2 cm long, emarginated. Schizocarp brown or grey, when mature, ca. 0.7 cm diam., 9-11 mericarps, gently reticulated (Figures 2d and 3d).

Malva verticillata L., Sp. Pl. 2: 689. 1753.

Herbaceous erect, 1-2.5 m tall. Stipule ca. 5 × 3 mm. Leaves with petioles 8-19 cm long. Lamina 8-17 × 8-18 cm, reniform to lobate, lobate, base auriculate, sometimes with imbricate lobes, margin crenate; glabrescent. Flowers 3-10 in the leaf axils. Bractlets of the involucel linear to lanceolate, ca. 3 × 2 mm; calyx 3-9 mm long, spreading in fruit; corolla white, maculate in the apex, ca. 0.8 cm diameter; petals 0.3-0.8 cm long, emarginated. Schizocarp brown, when mature, ca. 0.8 cm diam., 10-11 mericarps, slight reticulated (Figures 2e and 3e).

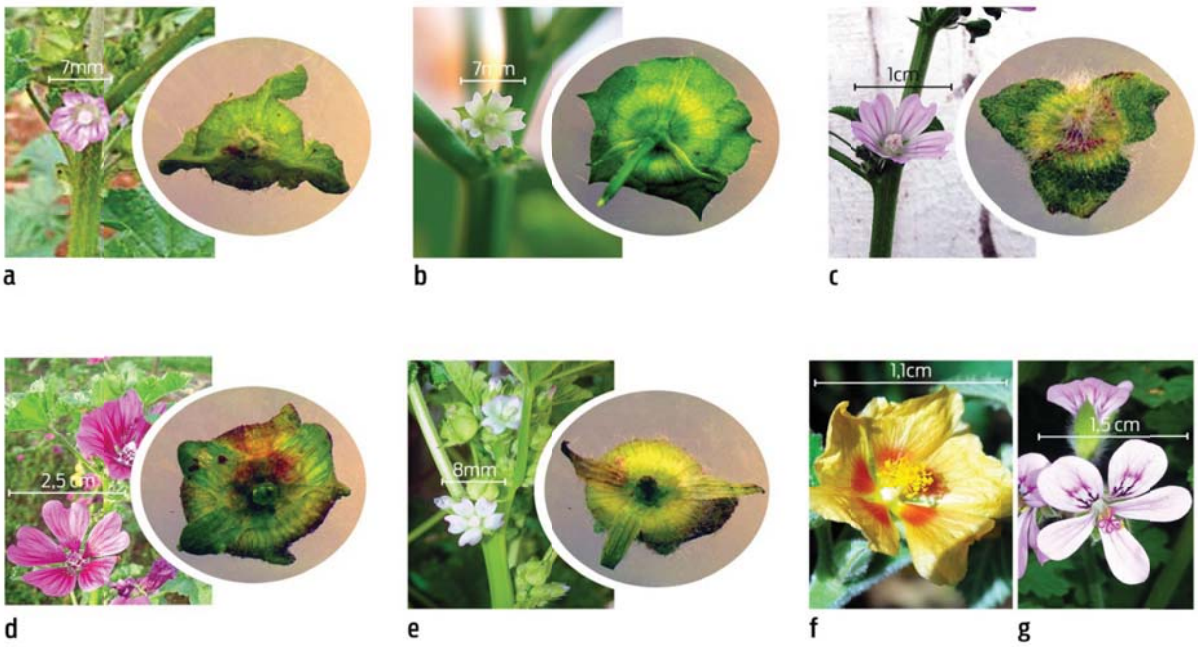

Figure 2. *Malva, Sida* and *Pelargonium flowers*

Note. a. *M. nicaeensis*, flower and detail of the epicalyx; b. *M. parviflora*, flower and detail of the epicalyx; c. *M. pseudolavatera,* flower and detail of the epicalyx; d. *M. sylvestris,* flower and detail of the epicalyx; e. *M. verticillata*, flower and detail of the epicalyx; f. *Sida cordifolia*, flower; g. *Pelargonium graveolens*, flower.

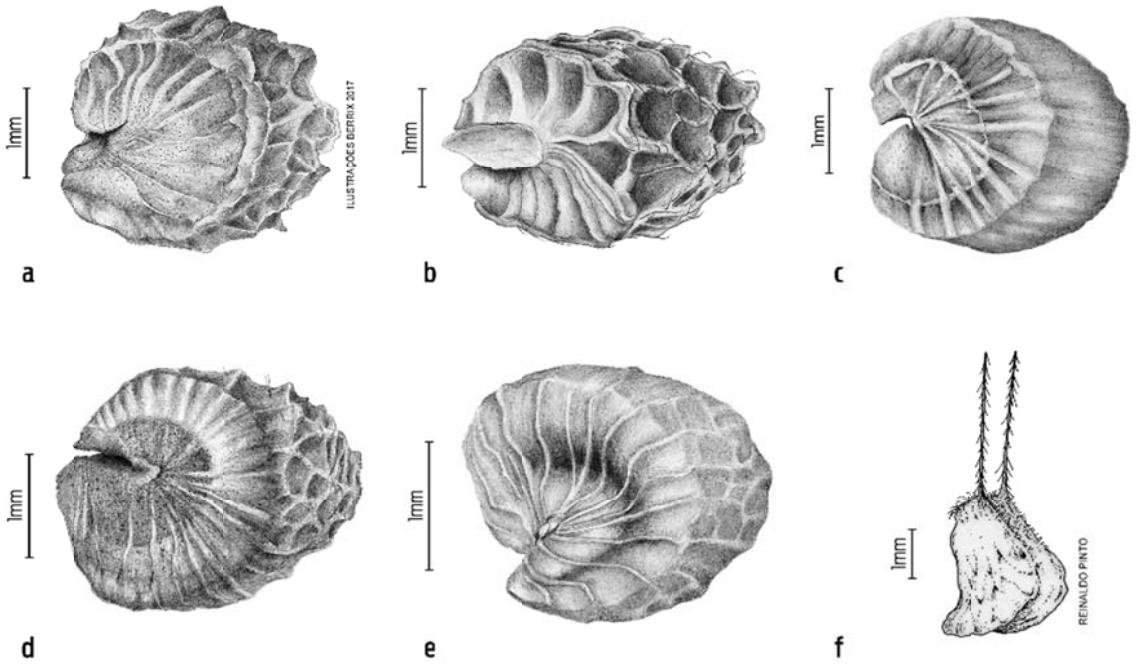

Figure 3. *Malva* and *Sida* mericarps

Note. a. *M. nicaeensis*; b. *M. parviflora*; c. *M. pseudolavatera*; d. *M. sylvestris*; e. *M. verticillata*; f. *Sida cordifolia*.

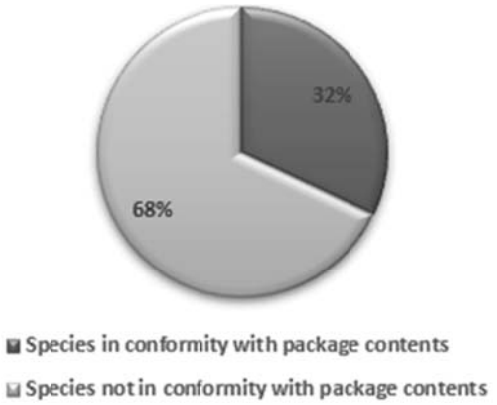

Figure 4. Representativeness of the species correctly identified, Souther Brazil, 2015-2017

4. Discussion

The most evident morphological difference between *M. parviflora* and *M. sylvestris* found in the dried samples was in the mericarp, which varies from slightly reticulate to extremely reticulate with winged projections (Figure 3). Whereas other characteristics were not of great relevance. In contrast, *S. cordifolia* (Figure 2f) is another genus of *Malvaceae* but very diverse in numbers of species, with 94 species described occurring in Brazil (Flora do Brasil 2020, n.d.). In addition to the yellow flowers with markings, morphological differences between *S. cordifolia* and *Malva* show that the mericarps in *S. cordifolia* are long awns with retrorsely trichomes, which is not observed in *Malva* (Bovini, Carvalho-Okano, & Vieira, 2001).

P. graveolens, the leaves are opposite zygomorphic flowers and schizocarps with elastic dehiscence, meaning mericarps that are released from the lower portion and are rolled up and get trapped at the apex.

It was found that 15% of the *Malva sylvestris* samples labeled under the common name "mallow" ("*malva*") were correctly identified, and 15% of those labeled as "*malva-branca*" and the scientific name of *Sida cordifolia* were correctly related to the contents of the samples (Table 2). Considering that, *M. sylvestris* is a widely-cultivated species of mallow and studied regarding its phytochemical aspects. It appears to be the target

species with the highest non-conformities. *S. cordifolia* was a species present in 80% of the samples, with 65% of them marketed as *M. sylvestris* or labeled under the common name of "mallow" ("*malva*").

After this analysis, we can point out a high degree of erroneously identified species at package labels that make up the contents of the analyzed commercial packages. Figure 4 highlights the total number of species correctly identified in relation to the contents of the packages and those that were not correctly identified.

Martins et al. (2015) analyzed samples of *M. sylvestris* or "mallow" available in dehydrated form in different Brazilian locations by using mass spectrometry and morphological analysis. They identified 50% of the samples as *S. cordifolia*. The packages labeled as *malva-cheirosa* and identified as *Malva crispa* L. (currently, *Malva verticillata*), have been incorrectly identified. They are, in the fact, *Pelargonium graveolens*, which are Geraniaceae, but very much resemble *M. sylvestris*. Romitelli and Martins (2013) and Martins et al. (2015) report that the misuse of *S. cordifolia* can be dangerous to patients with cardiovascular problems, anxiety or behavioral disorders due to the presence of ephedrine, pseudoephedrine and alkaloids.

Colet, Dal Molin, Cavinatto, Baiotto and Oliveira (2015), analyzed 44 packages of medicinal plants commercialized in pharmacies in the municipality of Ijuí/RS, based on the legilations on the subject. Among all the requirements, the results showed that all the samples had common name, while the scientific name in only 75%.

There is a high consuption of *S. cordifolia* and *P. graveolens* instead of *M. sylvestris*, which has already been recognized as medicinal due to misunderstandings in the identification of the species. Species of the *Malva* genus are difficult to recognize after processed. Nevertheless, it is likely better to recognize the species more accurately when based on different parts sampled from the commercial package. The color of the flower, the morphology of the epicalyx's bractole and the morphology of the mericarp are essential for identifying the species.

The high presence of *S. cordifolia* in the studied samples labeled as other species (65%) is probably due to the fact that the species is widely distributed in Brazil throughout all biomes, and occur on the side of paths and roads. Thus, it does not need to be cultivated, which facilitates its collection from several locations in Brazil. Furthermore, it has a slight resemblance to the *Malva* genus to the untrained eye. On the other hand, the *Malva* genus occurs under cultivation in environments with lower temperatures and it rarely occurs in the wild ecosystem.

Further studies of this nature are necessary in other states of Brazil in order for the Medicinal Plants Policy to be properly implemented in Brazil.

5. Conclusion

Five cultivated *Malva* species were found in the Western Mesoregion of Santa Catarina State-Southern Brazil: *M. nicaeensis*, *M. parviflora*, *M. pseudolavatera*, *M. sylvestris* and *M. verticillata*.

Commercial samples of dehydrated *M. sylvestris* revealed the presence of parts of plants of the family Geraniaceae. *S. cordifolia* (Malvaceae) is the main mixed species marketed in packages of medicinal plant *M. sylvestris*.

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Calcium Nitrate Priming Increases the Germination Rate of Eggplant Seeds

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Abstract

The priming may promote greater expression of the physiological potential of the seeds and contribute to the germination process under adverse environmental conditions. The objective of this study was to evaluate the physiological potential of eggplant seeds primed with different chemical agents and exposed to adverse temperatures. Seeds were subjected to priming with five chemical agents [unprimed (control); water (hydropriming); calcium nitrate (0.2%); amino acid L-phenylalanine (0.05%); L-phenylalanine (0.05%) + calcium nitrate (0.2%)] and at temperatures of 15, 25, 35 and 41 ºC, considering a completely randomized design in a factorial arrangement (5 × 4) with four replicates. The first count of radicle emission and germination, final radicle emission, seed germination, radicle emission rate index, mean radicle emission time, and seedling dry mass were measured. Germination process of eggplant seeds was completely inhibited at 41 ºC, and the optimal temperature for seed germination is 25 ºC. Calcium nitrate priming potentiates the seed germination process, resulting in higher radicle emission rate index and higher germination rate. Low temperature (15 ºC) has greater interference in the germination rate of eggplant seeds when compared to high temperature (35 ºC).

Keywords: chemical agents, physiological potential, *Solanum melongena* L., temperature

1. Introduction

Eggplant (*Solanum melongena* L.) originally from India, is an annual vegetable of the Solanaceae family. Consumption of *S. melongena* has been widely widespread due to its medicinal benefits, such as cholesterol reduction (Antonini et al., 2002). National and world seed production has increased in recent years due to the large consumption of vegetables. This higher demand for vegetable seeds requires that they have quality and vigor, and to ensure the optimal development of the seedlings, physiological priming is a technique used (Medeiros et al., 2015).

Abiotic stresses may affect seed performance either by favoring, delaying, or inhibiting their germination after sowing (Nascimento & Lima, 2008). Several techniques can be used to promote greater seed tolerance to the abiotic stresses, especially thermal and water stresses. These techniques include the physiological priming of the seeds, which promotes greater germination capacity and seedling emergence and thus ensure greater uniformity of the plant stand under adverse environmental conditions (Bisognin et al., 2016).

Seed priming is a pre-sowing treatment which leads to a physiological potential that enables the seed to germinate more efficiently. Priming reduces germination time, improves emergence uniformity and vigor of seedlings. The controlled hydration of the seeds can be accomplished by hydropriming or through osmotic solutions (Pinedo & Ferraz, 2008).

The osmopriming consists in controlling the hydration level of the seeds during the imbibition phase, which is commonly performed with a solution containing substances or chemical agents such as polyethylene glycol (PEG), calcium nitrate, potassium nitrate, among others. Osmopriming allows hydration until seed and solution water potentials reach equilibrium, and the biochemical preparatory process for germination is activated (Marcos Filho, 2015).

In eggplant seeds, the osmopriming with potassium nitrate solution improved the germination rate when exposed to low temperatures (Nascimento & Lima, 2008). The calcium nitrate solution can also be used in seed pretreatment to improve the germination process and act to overcome dormancy in some seeds (Silva et al., 2017). For example, in *Brachiaria* seeds, the dormancy is related to several factors, especially physical properties, and the priming with calcium nitrate resulted in higher tolerance of *Brachiaria brizantha* seeds to high humidity and high-temperature stress (Batista et al., 2016), thus being efficient in overcoming dormancy.

The priming of tomato seeds with $CaCl_2$ and KNO_3 solution was efficient to improve the seedling growth under salinity conditions (Ebrahimi et al., 2014). Similarly, Batista et al. (2015) reported that the priming with KNO_3 and Ca $(NO_3)_2$ resulted in greater growth of pepper (*C. frutescens*) seedlings. However, the priming of cucumber seeds with potassium nitrate showed little effect in improving the germination and growth rate of seedlings under salt stress conditions (Oliveira & Steiner, 2017).

There are few studies with the use of the amino acid phenylalanine for the priming of seeds. The beneficial effect of this amino acid application on seed germination is due to the biochemical and structural mechanisms of resistance in plants, favoring adaptation to adverse environmental conditions (Stangarlin et al., 2011). However, in the study performed by Gouveia et al. (2017), the priming of corn seeds with calcium nitrate and phenylalanine promoted greater germination rate of low vigor seeds.

The production of pepper seeds with high physiological quality has been pointed out with one of the main challenges for seed producers. Thus, the objective of this study was to evaluate the physiological potential of eggplant seeds primed with different chemical agents and exposed to adverse temperature.

2. Material and Methods

The experiment was carried out in the Laboratory of Seed Analysis and Plant Physiology of the State University of Mato Grosso do Sul (UEMS), in Cassilândia, MS, Brazil, during the year 2018. For the research, eggplant seeds (*Solanum melongena* L.) without previous treatment were used.

The treatments were arranged in a completely randomized design in a 4 × 5 factorial scheme: four stress treatment by adverse temperatures [optimal temperature at 25 °C (control), low temperature stress at 15 °C, high temperature stress at 35 °C ,and extreme temperature stress at 41 °C] and five chemical agents used in seed priming [unprimed (control); water (hydropriming); calcium nitrate ($Ca(NO_3)_2$) at 0.2%; amino acid L-phenylalanine at 0.05%; calcium nitrate ($Ca(NO_3)_2$) at 0.2% + L-phenylalanine at 0.05%], with four replicates of 50 seeds.

Seeds were submitted to priming by direct immersion in different chemical solutions for 24 h at 25 ºC, with a photoperiod of 12 °C. The solution was aerated from 2 h after the beginning of seed imbibition. After priming, seeds were removed and washed with tap water, and then put to dry at room temperature for 5 h, until the seed water content reduces to the initial value. The water content of the seeds was measured by the oven drying method at 130-133 °C, for 24 h (MAPA, 2009), obtaining initial water content (before priming) and final water content (after priming). The initial water content was 6.6%, and after the priming period, the final water content of 51.48%.

Germination test: four replicates of 50 seeds were distributed among two sheets of paper towel moistened with distilled water at the proportion of 3.0 times the mass of dry paper. The paper towel sheets were turned into rolls, and then the germination was carried out in growth chambers, under a 12 h photoperiod and at different adverse temperatures (15, 25, 35, and 41 °C).

During the seed germination process, the following characteristics were measured:

The first count of germination test (7 days) and seed germination percentage (14 days): Consisted of four subsamples of 50 seeds per treatment, and the results were expressed as a percentage of normal seedlings (MAPA, 2009);

The first count of radicle emission (7 days) and final radicle emission percentage (14 days): four subsamples of 50 seeds per treatment were used, and the results were expressed as a percentage of seeds with radicle;

Radicle emission rate index (RERI) and germination rate index (GRI): conducted in conjunction with the seed germination test, and calculated according to the equation below proposed by Maguire (1962).

$$RERI \text{ or } GRI = G1/T1 + G2/T2 + \ldots + Gi/Ti \quad (1)$$

Where, Gi = Number of radicles emitted or number of germinated seeds on a given day; Ti = Time in days from the sowing day (0).

Mean radicle emission time (MRET) or mean germination time (MGT): conducted in conjunction with the seed germination test, and calculated according to the equation below proposed by Labouriau (1983).

$$MRET \text{ or } MGT = (\Sigma\ NiTi)/(\Sigma\ Ni) \quad (2)$$

Where, Ni = Number of radicles emitted or number of germinated seeds germination on a given day; Ti = Time in days from the sowing day (0).

The total dry mass of seedlings (TDM): Four replicates of 20 seedlings per treatment were used, and the total dry mass was determined after oven drying at 65 ºC for 72 h. Values were expressed as mg seedling^{-1}.

Statistical analysis: The data were previously transformed into arcsin $(x)^{0.5}$, and then data were submitted to analysis of variance, and means were grouped by the Scott-Knott test at the 5% probability level.

3. Results and Discussion

The stress caused by the extremely high temperature (41 ºC) completely inhibited the seed germination, regardless of the priming treatments. It indicates that this temperature was very detrimental to the germination process of eggplant seeds (*Solanum melongena* L.). Therefore, since it was not possible to collect data in this extreme temperature condition, this abiotic stress treatment was not used in the statistical analyses. Thus, only the other temperature conditions (*i.e.*, 15, 25, and 35 ºC) were compared by the statistical analysis.

The seeds presented initial water content of 6.6% and after the priming reached the final water content of 51.48%. The initial water content was relatively low because the seeds were commercially purchased from packages, and after priming from the direct immersion for 24 h, there was a significant increase in the seed water content. The germination process has a direct influence on the water content of the seeds, such as the reorganization of cell membranes, biosynthesis of new enzymes, and ribonucleic acid (RNA_m), among other processes.

Analysis of variance reported that the effect of adverse temperature stress was significant on all seed germination and seedling growth traits, while the effect of seed priming methods was significant on the first count of radicle emission, final radicle emission, radicle emission rate index (RERI), and first count of germination test. Interaction between adverse temperature stress and seed priming methods showed no significant effect for any of the traits measured (Tables 1 and 2).

About the germination process, the exposure of eggplant seeds to low-temperature stress resulted in a lower percentage of seeds with radicle emission in the first count (7 days) and the final count (14 days). Low temperature also caused a lower radicle emission rate index (RERI) and higher mean radicle emission time (MRET) when compared to the other temperature stresses. The temperature of 25 °C resulted in the highest germination rate and was the optimal temperature for the eggplant germination process (Table 1).

Table 1. First count of radicle emission (7 days), final radicle emission (14 days), radicle emission rate index (RERI) and mean radicle emission time (MRET) according to the stress treatment by adverse temperature and priming with different chemical agents of eggplant seeds (*Solanum melongena* L.). Cassilândia, MS, Brazil, 2018

Stress by Adverse Temperatures	**Radicle Emission**		**RERI**	**MRET (Days)**
	Fist Count (7 Days)	**Final (14 Days)**		
15 ºC (low)	0 c	32 c	1.56 c	11.51 a
25 ºC (optimal)	93 a	96 a	9.43 a	5.15 c
35 ºC (high)	62 b	71 b	6.61 b	5.83 b
Seed Priming	**Radicle Emission**		**RERI**	**MRET (Days)**
	Fist Count (7 Days)	**Final (14 Days)**		
Unprimed seeds (control)	41 b	54 b	4.64 b	8.12 a
Water (hydropriming)	52 a	62 b	5.65 b	7.62 a
Calcium nitrate	58 a	76 a	6.68 a	6.86 a
L-phenylalanine	54 a	68 a	6.00 a	7.46 a
Calcium nitrate + L-phenylalanine	56 a	72 a	6.36 a	7.42 a
CV (%)	18.19	22.09	17.25	6.86

Note. Means followed by different letters in the column shows significant differences by the Scott-Knott test, at the 5% probability level. CV = coefficient of variation.

The osmopriming with different chemical agents improved the first count of radicle emission, final radicle emission, and radicle emission rate index; however, there was no difference between the chemical agents used (Table 1). Priming promotes the reorganization of cell membranes, especially mitochondria, which are essential for the cellular respiration process. Thus, after the reorganizations of cell membranes, the germination process of the seeds occurs faster.

The seed priming has been shown to have a beneficial effect on germination and growth rate of vegetable crops. Osmopriming of eggplant seeds provided a higher germination rate and seedling emergence rate index (Fanan & Novembre, 2007). Reis et al. (2012) also reported that KNO_3 priming resulted in higher germination rate and lower mean germination time in eggplant cv. Embu.

Similarly, to the radicle emission pattern, low-temperature stress (15 °C) was also unsuitable for the normal seedling formations (Table 2), together with high-temperature stress at 35 °C, while the temperature of 25 °C was the most suitable for the germination process.

At the first count of normal seedlings (at 7 days), the temperature at 25 °C had approximately 42% of normal seedlings, while exposure to high temperature (35 °C) was only 6.30%, and at low temperature (15 °C) still had no normal seedlings.

The germination process involves a series of metabolic activities, and these reactions have specific requirements regarding temperature, mainly because these reactions are related to enzymatic systems. In these cases, temperature changes can affect the speed, uniformity and the germination rate of the seeds, and for most cultivated species the optimum temperature is between 20 and 30 ºC (Marcos Filho, 2015).

In the first count of normal seedlings, the temperature of 15 ºC did not result in normal seedlings, because low-temperature conditions can reduce the speed of imbibition and mobilization of seed reserves, delaying the germination process and reducing seedling growth (Marcos Filho, 2015).

At the final count of germination test under 25 °C, more than 90% of the seeds formed normal seedlings, whereas the temperature of 35 °C was almost 50%, and the temperature of 15 °C had less than 2% of normal seedlings. The normal seedlings under the different temperature stress conditions did not differ about the total dry mass of the eggplant seedlings at 25 and 35 ºC. However, lower growth was observed at 15 °C (Table 2).

Table 2. First count of germination test (7 days), final seed germination (14 days), total dry mass of seedlings (TDM) according to the stress treatment by adverse temperatures and priming with different chemical agents of eggplant seeds (*Solanum melongena* L.). Cassilândia, MS, Brazil, 2018

Stress by Adverse Temperatures	**Germination**		**TDM (mg)**
	Fist Count (7 days)	**Final (14 days)**	
15 ºC (low)	0 c	2 c	8.85 b
25 ºC (optimal)	41 a	90 a	84.82 a
35 ºC (high)	6 b	48 b	73.56 a
Seed Priming	**Germination**		**TDM (mg)**
	Fist Count (7 days)	**Final (14 days)**	
Unprimed seeds (Control)	11 b	41a	50.00 a
Water (hydropriming)	17 b	49 a	54.69 a
Calcium nitrate	20 a	49 a	67.06 a
L-phenylalanine	12 b	48 a	52.79 a
Calcium nitrate + L-phenylalanine	20 a	46 a	54.17 a
CV (%)	41.10	22.38	29.80

Note. Means followed by different letters in the column shows significant differences by the Scott-Knott test, at the 5% probability level. CV = coefficient of variation.

The priming with calcium nitrate promoted a higher germination percentage in the first count. These results indicate that the seed priming with calcium nitrate solution was capable of promoting greater expression of pre-existing seed vigor, resulting in a higher germination rate at 7 days after sowing (DAS). However, at 14 DAS, there was stabilization between the seed priming treatments (Table 2). Nascimento (2005) also reported that osmopriming with KNO_3 solution resulted in higher germination percentage of eggplant seeds under

low-temperature abiotic stress conditions. The calcium nitrate solution showed to be more efficient for the germination process since the application of the antioxidant alone resulted in germination performance similar to the unprimed seeds and hydropriming. However, when the antioxidant was associated with calcium nitrate promoted a result similar to priming with calcium nitrate alone, emphasizing the efficiency of chemical agent in the osmopriming of eggplant seeds.

Nitrate stimulates the pentose phosphate pathway connected with the glycolytic pathway, responsible by the supply of free energy in the form of ATP and carbon skeleton that are used to the embryonic axis growth. In this case, the priming with the calcium nitrate favored radicle emission and promoted a higher germination percentage in the first count.

The efficiency of calcium nitrate in seed priming was also reported by Gouveia et al. (2017) when evaluating the physiological potential of maize seeds primed with different chemical agents and submitted to abiotic stress, in which the calcium nitrate priming resulted in greater germination and emergence of seedlings in sub-optimal temperature conditions when compared to the priming with water and amino acid phenylalanine.

Similarly, Batista et al. (2015) when evaluating the quality of pepper seedlings resulting from priming with water, potassium nitrate (0.2%), calcium nitrate (0.2%), GA_3 gibberellin (200 mg L^{-1}) and antioxidant riboflavin (25 mg L^{-1}), observed that the priming with KNO_3 and Ca $(NO_3)_2$ resulted in higher dry matter of *C. frutescens* seedlings.

Thus, physiological priming consists of a set of techniques that aim to express all the physiological potential of the seed lot (Marcos Filho, 2015), which was observed in the initial evaluations of seed germination. In the first count evaluations, both for radicle emission and for normal seedlings, the germination occurred more rapidly in the seeds primed with calcium nitrate. These results show that priming improved the expression of higher seed performance.

4. Conclusions

The priming of eggplant seeds with calcium nitrate solution and antioxidant L-phenylalanine either alone or in combination promotes greater expression of the physiological potential of the seeds, resulting in greater germination rate.

Low temperature (15 °C) results in a lower radicle emission rate and lower formation of normal seedlings when compared to high temperature (35 °C). The optimal temperature for the seed germination process is 25 °C, while the germination process of eggplant seeds is completely inhibited at 41 °C.

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Relationship Between Spatio-Temporal Leaf Area Index and Crop Coefficient When Monitoring Coffee Plots in Brazil Using the Sentine-2

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Abstract

Robust monitoring techniques for perennial crops have become increasingly possible due to technological advances in the area of Remote Sensing (RS), and the products are available through the European Space Agency (ESA) initiative. RS data provides valuable opportunities for detailed assessments of crop conditions at plot level using high spatial, spectral, and temporal resolution. This study addresses the monitoring of coffee at the plot level using RS, analyzing the relationship between the spatio-temporal variability of the Leaf Area Index (LAI) and the crop coefficient (Kc); the Kc being a biophysical variable that integrates the potential hydrological characteristics of an agroecosystem compared to the reference crop. Daily and one-year Kc were estimated using the relation of crop evapotranspiration and reference. ESA Sentinel-2 images were pre-analyzed and atmospherically corrected, and Top-of-the-Atmosphere (TOA) reflections converted to Top-of-the-Canopy (TOC) reflectance. The TOCs resampled at the 10m resolution, and with the angles corresponding to the directional information at the time of the acquisition, the LAI was estimated using the trained neural network available in the Sentinel Application Platform (SNAP). During 75% of the monitored days, Kc ranged between 1.2 and 1.3 and, the LAI analyzed showed high spatial and temporal variability at the plot level. Based on the relationship between the biophysical variables, the LAI variable can substitute the Kc and be used to monitor the water conditions at the production area as well as analyze spatial variability inside that area. Sentinel-2 products could be more useful in monitoring coffee in the farm production area.

Keywords: crop coefficient, leaf area index, satellite crop monitoring sentinel-2

1. Introduction

Millions of people consume 2.25 billion cups of coffee per day and represents the value in the global market of more than $19 billion USD. Coffee guarantees the livelihood of over 125 million people, and its production is key to 70 developing countries located between the Tropic of Cancer and the Tropic of Capricorn, the so-called ‘Bean Belt’ region. Brazil, Vietnam, Colombia, Ethiopia, and Indonesia are the chief producers, employing 25 million coffee farmers, mostly smallholders (Watts, 2016).

Chemura et al. (2017a) argued that the development of cost-effective, reliable and easy to implement crop condition monitoring methods are urgently required for perennial shrub crops such as coffee (*Coffea arabica*), as they are grown over large areas and represent long term and higher levels of investment. These monitoring methods are useful in identifying farm areas that experience weak crop growth, pest infestation and, disease outbreaks, in order to monitor responses to management interventions.

The coffee plantations culture has generally been developed on traditionally owned plots. The plot area is relatively small, and those can be treated as a homogeneous area to the monitoring because they are typically formed by the same variety of plants, area of the same age and receive the same amount of fertilizer and the cultural treatments.

An alternative to perennial shrub coffee monitoring is the use of remote sensing tools. Particular attention has been paid to free data sources, such as those provided by the Global Monitoring for Environment and Security (GMES), an initiative of the European Space Agency (ESA). Sentinel-2 is one of the earth observation satellites used by the ESA that provides invaluable information. This satellite overcomes the problems of Landsat satellites by incorporating three new spectral bands in the red spectral region centered at 705, 740 and 783 nm, providing an improved temporal and spatial resolution of 10m (Delegido et al., 2011; Vuolo et al., 2016) which is essential for the estimation of chlorophyll content.

The Sentinel-2 current mission consists of two identical satellites, the Sentinel-2A, launched in June 2015, and the Sentinel-2B, launched in January 2017. Although released separately, the satellites were placed in the same orbit, separated by 180°. Every five days, the satellites together cover almost the entire land surface between latitudes 84° S and 84° N, optimizing global coverage and improving temporal resolution.

Processing Sentinel-2 information is possible through the use of the Sentinel Application Platform (SNAP) software, which contains state-of-the-art tools to perform atmospheric correction processes to obtain the highest resolution of Top-of-the-Canopy (TOC) spectral reflectance. With the TOCs and a machine-learning algorithm, available in the SNAP, the monitoring of the biophysical variables of the vegetation can be realized for smallholders.

Leaf Area Index (LAI) and chlorophyll content, at canopy and plot level, are essential variables for agricultural applications because of their crucial role in photosynthesis and plant functioning (Clevers et al., 2017).

With the LAI, it is possible to define a dimensionless quantity that characterizes plant canopies. In other words, the one-sided, photosynthetically active elements of leaf area per unit ground surface area, determine the interface of the exchange of energy or mass between the canopy and atmosphere (Weiss & Baret, 2016).

At the plot level and for various ecosystems, LAI can be considered an important parameter to analyze coffee ecosystem services (Taugourdeau et al., 2014). An increase of LAI due to shade-grown coffee interfere with the microclimate (Barradas & Fanjul, 1986; Ong et al., 2000), evapotranspiration (Padovan et al., 2018), hydrological behavior (Gómez-Delgado et al., 2011), erosion control (Ataroff & Monasterio, 1997), biomass and growth (Rodríguez-López et al., 2014) production (Alves et al., 2016) and net primary productivities (Defrenet et al., 2016; Charbonnier et al., 2017).

Among the variables that affect coffee development and productivity, water availability plays an important role, but it is difficult to monitor. Water management is vital in modern agriculture, particularly for perennial shrubs such as coffee. Proper detection and monitoring of soil water conditions, therefore, became essential not only in mitigating the associated adverse impacts on crop growth and productivity but also in reducing expensive and environmentally unsustainable irrigation practices (Chemura et al., 2017b). Information on crop evapotranspiration (ETc), which represents the combined loss of water due to evaporation of the soil surface and transpiration of the crop, may facilitate better irrigation planning and, finally, water use efficiency (Akdim et al., 2014) but also improve the efficiency of cultural practices such as fertilization.

Generally, the Evapotranspiration of crops (ETc) calculations are realized by adjusting the reference evapotranspiration (ETo) by the empirical crop coefficient (Kc), *i.e.*, $ETc = Kc \times ETo$. The single values of Kc have been estimated for different cultures (Allen et al., 1998). That calculation integrates the water consumption characteristics of the crop surface under study with those of a reference crop (usually grass) that completely covers the soil surface. The Kc varies with age, size, and spacing (Allen et al., 1998; Carr, 2001).

The aim of the present study at the plot level was to reveal the monitoring of coffee shrubs with the use of information from the Sentinel-2, through the spatial-temporal analysis of the Leaf Area Index (LAI) and the Crop Coefficient (Kc).

2. Method

2.1 Characterization Site-Monitored

The study area located in the Atlantic Rainforest biome, in the eastern portion of the State of Minas Gerais, in Southeastern Brazil, monitored in the Matas de Minas region, at an altitude of around 650 m, between the municipalities of Viçosa and Paula Cândido (Figure 1).

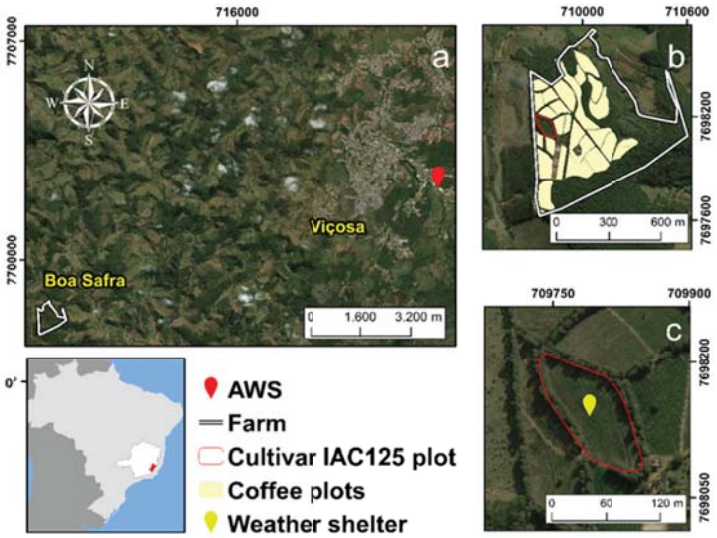

Figure 1. The Boa Safra coffee farm and the automatic weather station (AWS) in the municipality of Viçosa (a), coffee plots (b) and the plot monitored with temperature sensors in a wooden shelter (c). The images were obtained from the sensor onboard the IKONOS satellite on 2016-07-07

Arabic coffee smallholders were established in the region (Rufino et al., 2010), which has a favorable climate. The quality beverage produced there is known as "Mountain Coffee" due to its irregular topography (Ferreira et al., 2016) where the altitude ranges between 600 and 1,200 m (Zaidan et al., 2017). In 2016, the Instituto Brasileiro de Geografia e Estatística (IBGE) estimated that the 63 municipalities of the region have 259 thousand hectares in coffee production (IBGE, 2016).

The coffee plot (0.57 ha) monitored uses cultivar IAC 125. This cultivar is characterized by having low to medium size, resistance to rust, and nematodes. The coffee shrubs were planted in 2011, at the beginning of the rainy season, in a Red-Yellow Latosol of clay texture, having a density of 2.8 m × 0.7 m. It was uniform regarding soil attributes and slope (Table 1).

The cultural practices carried out in the coffee plot are the same as the coffee growers of the region apply to their crops, among which chemical fertilization, according to the analysis of soil and leaves, and the requirements related to the age of the crop, are carried out in the course of the rainy season and three additional applications. Control of natural vegetation was performed mechanically with brush cutters, four times a year. Due to slope, harvesting was done manually, avoiding defoliation.

The monitoring began with the rainy season of 2016 (October 12) and ended in the same month of the year of 2017, in total, 366 days. The biophysical variables, the Leaf Area Index (LAI) and the Crop Coefficient (Kc) at the plot level and point, respectively were analyzed.

2.2 Crop Coefficient (Kc)

The Crop Coefficient (Kc) was obtained, according to Allen et al. (1998) by the equation:

$$Kc = ETc/ETo \quad (1)$$

where, *ETo*, *ETc* are the grass-reference evapotranspiration and culture (mm d^{-1}), respectively

Daily ETo, ETc values were computed based on the equations presented by Hargreaves and Samani (1985)

$$ETo \text{ or } ETc = 0.023 \times RA \times (T°C + 17.8) \times TD^{0.5} \quad (2)$$

where, *RA* = extraterrestrial radiation; *T°C* is the daily mean temperature and *TD* = *Tmx* – *Tmi* (Maximum (*Tmx*) and Minimum (*Tmi*) daily temperatures in degrees Celsius). The values of RA were calculated for each day using equations and methodologies indicated by Bojanowski (2016).

Table 1. Coffee shrubs (cultivar IAC125) characterization and soils chemical and physical proprieties of the plot monitored

Characteristics	Attributes	Depth (cm)	IAC 125
Coffee shrub	Area in hectares (ha)		0.57
	Number of stems/plot		3.393
	Age in months[a]		69
	Shrubs height (m)		1.7
Chemical	pH (H_2O)	0-10	6.57
		10-30	6.94
	P (mg/dm^{-3})	0-10	9.8
		10-30	1.0
	K ($mg\ dm^{-3}$)	0-10	206
		10-30	206
	Ca^{2+} $cmolc\ dm^{-3}$	0-10	5.16
		10-30	2.73
	Mg^{2+} $cmolc\ dm^{-3}$	0-10	1.56
		10-30	0.59
	H+Al $cmolc\ dm^{-3}$	0-10	2.6
		10-30	1.4
	CEC (t) $cmolc\ dm^{-3}$	0-10	7.25
		10-30	3.85
	CEC (T) $cmolc\ dm^{-3}$	0-10	9.85
		10-30	5.25
Physicals	Sandy ($kg\ kg^{-1}$)	10-30	0.31
	Silt ($kg\ kg^{-1}$)	10-30	0.02
	Clay ($kg\ kg^{-1}$)	10-30	0.67
	FC[b] ($m^3\ m^{-3}$)	0-10	0.22
		10-30	0.24
	PWP[c] ($m^3\ m^{-3}$)	0-10	0.36
		10-30	0.35

Note. (a) Age of the crop at the beginning of the monitoring, (b) Moisture as field capacity, (c) Moisture at permanent wilting point.

To estimate the ETo, the temperature records of the Viçosa-86824 automatic meteorological station belonging to the meteorological network of the Instituto Nacional Meteorologia 5° INMET, located in Viçosa-Minas Gerais, were used. The station is located less than 13 km from the study area (Figure 1a).

To estimate the ETc and to reduce the interference of direct solar radiation on the temperature measurement, as well as to protect the sensor from rain and insects, a wooden meteorological shelter was installed in the middle of the row of the analyzed plot (Figures 1c and 2). Inside this structure, at the height of 1.65 m, data storage equipment with the HOBO U14 LCD logger Temperature/Relative Humidity (RH) sensors were installed.

Figure 2. Temperature/Relative Humidity sensor (a) weather shelter (b)

2.3 Satellite Data and Leaf Area Index (LAI)

For the present study, approximately 30 images provided by ESA were the pre-analyzed (https://scihub.copernicus.eu). Eight images were acquired because they had adequate processing conditions, such as cloudiness and/or low cloudiness.

The selected images were initially atmospherically corrected with the Sen2Cor plugin (version 2.05.05) and processed using the Sentinel Application Platform (SNAP) toolbox extension (Louis et al., 2016). The tool has excellent performance in the correction procedures due to the algorithm used, which it has on the APDA (Atmospheric Precorrected Differential Absorption). This algorithm calculates the ratio between the band B8A (Vegetation Red Edge) and the band B09 (Water Vapour) to convert the TOA to Bottom-of-Atmosphere (BOA), which represents the lower part of the atmosphere (Gascon et al., 2017).

With the BOA, we acquired the Top-of-the-Canopy (TOC) spectral reflectance, and next, we adjusted to a resolution of 10 m. Two components, the TOCs in high resolution and with capture angles (Table 2), were the inputs to estimate the biophysical variable Leaf Area Index (LAI). Table 2 provides an overview of the Sentinel-2 images available for the coffee plot monitoring throughout the annual productive cycle (2016-2017) and the angles used as inputs in the trained neural network to estimate the LAI.

We considered that the adopted methodology is efficient. However, because of the several sources of uncertainty associated with the inputs and the algorithm calibration, we used a tolerance margin taken for the LAI product, which oscillated from 0 to 8 (Weiss & Baret, 2016).

The LAI for each date estimated was projected in cartographic coordinates (projection UTM/WGS84). A polygon demarcated that represents the weather shelter boundaries was used to check the geometric co-registration and extraction of pixels to correlation analysis (Figure 1c).

Table 2. Specifications of Sentinel-2 overflights over the test site during the growing season of 2016-2017 in the Orbit 095.0

Date	Solar Zenith Angle (◦)	Solar Azimuth Angle (◦)	View Zenith Angle (◦)	View Azimuth Angle (◦)
2016-10-21	23.41800	68.61480	2.47777	128.61002
2016-11-21	21.03990	91.43230	2.35209	129.99600
2016-12-30	24.84480	100.3300	2.39904	129.46861
2017-01-29	29.76370	76.32980	2.47515	128.63278
2017-03-30	36.29220	50.05490	2.29089	126.31473
2017-07-03	30.32151	49.12548	2.50231	129.21562
2017-09-06	36.88810	45.60660	2.34981	130.20207
2017-12-10	22.59640	99.98620	2.45354	131.72438

2.4 Analysis of the Crop Coefficient (Kc) and the Leaf Area Index (LAI)

In order to process the data and perform the qualitative spatial and temporal analysis of the LAI of the field monitored, as well as in the correlation analysis between the Kc and LAI variables, the functions of the libraries used were “ggplot2” (Hadley Wickham, 2009) “dplyr” (Wickham et al., 2018), “nortest” and “vcd” (Meyer et al., 2017) available in the free software R Development Core Team (2008).

3. Results

3.1 Temporal Variability of the Coffee Crop Coefficient (Kc)

The Crop Coefficient (Kc-dimensionless) of cultivar IAC 125 monitored, with temperature sensors for 366 days, presented values from 0.96 to 1.49 (average range of 0.52), mean of 1.24, a variation of 0.0046, a standard deviation of 0.068 and a coefficient of variation of 5.48%. Most of the Kc values, 75%, were between 1.2 to 1.3, with a range of 0.084 (Figure 3).

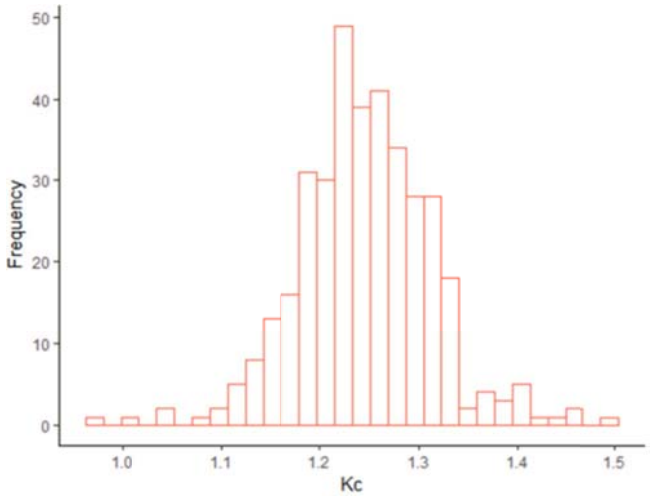

Figure 3. Crop Coefficient (Kc) estimated at the point level throughout the coffee annual productive cycle.

3.2 Variability of the Leaf Area Index at the Plot Level

In the eight selected images, composed of 83 pixels each (approximately 51 coffee plants per pixel), the atmospheric correction procedure was performed with the high-performance algorithm Sen2Cor (Louis et al., 2016). Given the presence of clouds determined the discard of other available images, therefore, it was not possible to process the series in uniform time intervals in the LAI monitoring. Considering the angles (Table 2) and eight corrected images as inputs to the neural network, these presented for the shrubs of coffee ranged from the highest LAI value of 5.22 to the lowest of 0.56, with an average value of 2.

In the first three dates evaluated, the average LAI went from 1.42 to 3.98 and reached the maximum value in the middle of the summer of 2016. Subsequently, the mean of 1.12 then increased to 2.98 in the fall of 2016. From that moment, the LAI reduced, and stabilizing in the next two dates analyzed in values of 1.98 and 1.96, arriving in the spring of 2017 with a value of 0.68 (Figure 4).

In three of the eight dates monitored (2016-11-21, 2017-01-29, 2017-03-20), the LAI pixels of the coffee plot analyzed, showed a symmetrical distribution. The other dates are asymmetric, being three (2016-10-21, 2017-07-03, 2017-09-06) with negative asymmetry and two (2016-12-30, 2017-12-10) positive. The amplitude and dispersion also vary between the monitored dates.

In the descriptive spatial analysis of LAI at the plot level (Figure 5), the total pixel population (83) per monitored date were divided into two groups: those with the lowest and highest LAI, according to the spatial variability found for each monitored period.

The lowest LAI values in all images processed were in the western part of the plot and; in seven of the eight monitored dates, the smallest LAIs located in the northwest direction; with fewer frequencies (number of pixels) in the southwest portion of the plot.

3.3 The Relationship Between the Leaf Area Index (LAI) and the Crop Coefficient (Kc)

For each monitored date, Table 2, the relationship between LAI and Kc were analyzed. The LAI pixels chosen correspond to the same location as the temperature sensor.

The Shapiro-Wilk and Shapiro-Francia tests ($p < 0.05$) indicated that the samples of the LAI variable presented a normal distribution, whereas those of Kc did not. However, the probability graph used to compare the different forms of distribution (Figure 6) indicates that the Kc values show a higher dispersion than the theoretical one (Figure 6a) when compared to the LAI (Figure 6b) and are considered acceptable.

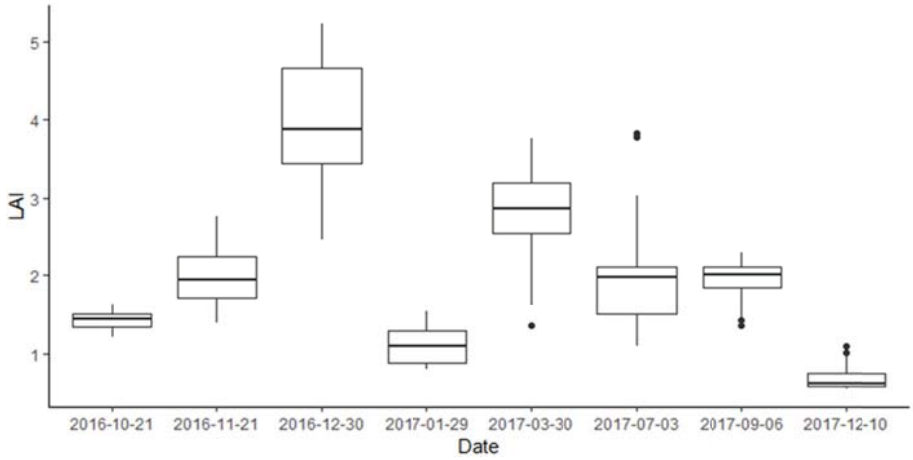

Figure 4. Leaf Area Index (LAI) monitored at the plot level throughout the annual coffee productive cycle, range and dispersion by date (yyyy-mm-dd) of LAI

Figure 5. Spatial representation of Leaf area index (LAI) monitored at the plot level throughout the coffee annual productive cycle. Each point represents the LAI of approximate 51 shrubs

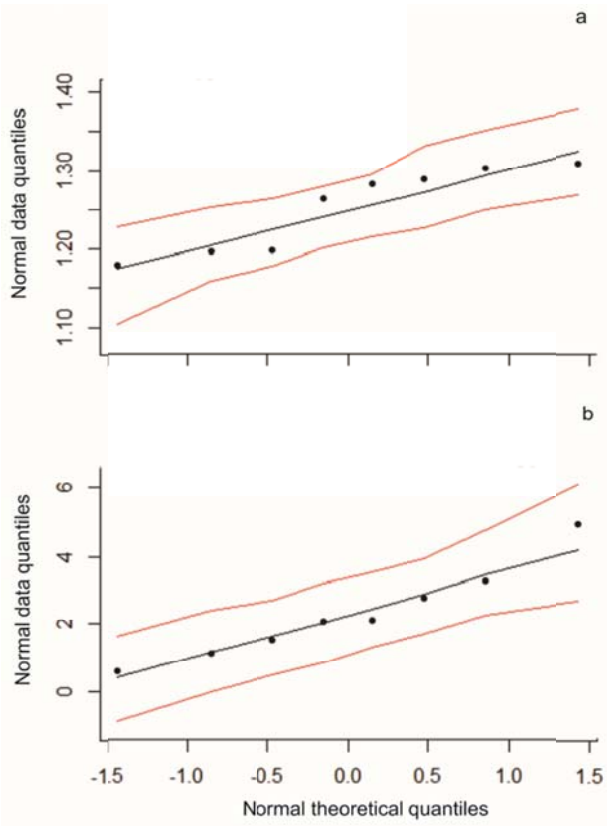

Figure 6. A normal Q-Q plot comparing randomly generated, independent standard normal data on the vertical axis: a) Crop Coefficient (Kc) and b) Leaf Area Index (LAI), to a standard normal population on the horizontal axis. The linearity of the points suggests that the data are normally distributed

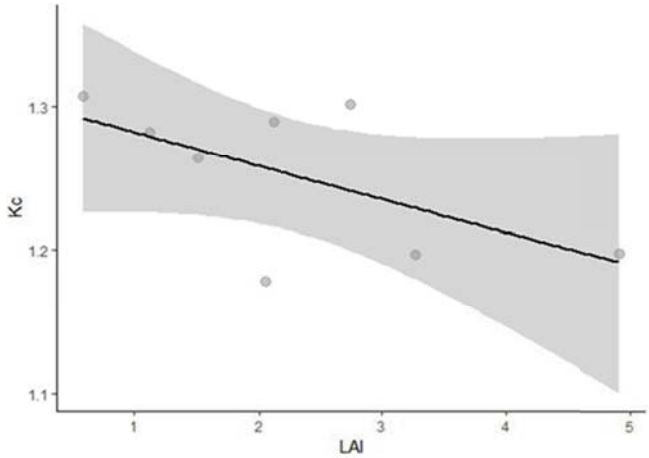

Figure 7. Scatter graph of the Leaf Area Index (LAI) and Crop Coefficient (Kc)

As the null hypothesis of normality in one of the variables rejected, the relationship between the LAI and Kc used the Spearman (rho) and Kendall (tau) rank correlation coefficient, both to determine the monotonic relationship between paired data and to verify if there is a correlation between the two variables. According to the significant tests (0.05) performed (rho = -0.45 and tau = -0.36), the negative correlation was plotted in the dispersion diagram (Figure 7).

4. Discussion

In the 2017/2018 coffee-harvest, the monitored plot showed a yield of 38.5 bags of 60 kg per hectare; these are considered higher than the average of the Mata-MG region (26.34 scs ha^{-1}) (Conab, 2018). The above indicates that the nutritional corrections of the crop and agronomic treatments were carried out efficiently.

Using a weather shelter with temperature sensors inside the plot was a practical and economical option for daily variable monitoring compared to complete automatic weather stations (EMAs). The latter are instruments permanently exposed to biological activity (bees hives, spider nets or sensor shading by Leaf growth) generating faults or errors. The shelter guaranteed the series of continuous records for the calculation of crop evapotranspiration by the Hargreaves method and then analyzed the temporal variability of the coffee crop coefficient (Kc).

The Kc values obtained in this study are consistent with those found in the literature for coffee crops (Doorenbos & Pruitt, 1977; Allen et al., 1998; Villa Nova et al., 2002; Marin et al., 2005; Oliveira et al., 2007; Sato et al., 2007; Pereira et al., 2011; Flumignan et al., 2011).

For coffee shrubs with a height of 2 m to 3 m (Allen et al., 1998), indicated the value of Kc from 0.90 to 0.95 and from 1.05 to 1.10 in the bare ground cover and with weeds, respectively. However, this estimation is applied to sub-humid climate. Besides, Doorenbos and Pruitt (1977) indicated for mature coffee grown without shade and where cultural practices involve clean cultivation with heavy cut grass mulching, Kc of around 0.9 is recommended throughout the year. If significant weed growth is allowed, coefficients close to 1.05-1.1 would be more appropriate. However, in the results of the present study, in 75% of the 366 days of monitored temperature, Kc (one year of evaluation) ranged from 1.2 to 1.3.

In Brazil, the leading coffee producing country in the world, experiments to determine the crop coefficient for the improvement of cultural practices such as irrigation, are scarce. Irrigated coffee cultivation has become an essential practice since 1984, favoring the implementation of crops in areas that have water or marginal deficits, but most of the studies conducted only verify the increase in production or test irrigation methods (Mantovani et al., 2007).

The studies published of Kc in irrigated coffee include those of Flumignan et al. (2011), Marin et al. (2005), Oliveira et al. (2007), Pereira et al. (2011) and others related with cultural practices or growth of the culture carried out and published by Villa Nova et al. (2002) and Sato et al. (2007).

In the publication of Villa Nova et al. (2002) argued that coffee crops between 12 and 36 months of age could have Kc ranging from 0.8 to 1.1. On the other hand, those older than 36 months, Kc values can range from 1.0 to 1.3. Also, the authors point out that at a density of 2,500 plants per hectare, the Kc can be 0.8, and for 6,666 plants per hectare, the Kc can be 1. According to Sato et al. (2007), after four years of pruning the coffee had Kc values ranging from 0.59 to 1.16.

Marin et al. (2005) characterized the water used by the coffee shrub in the drip irrigation system and estimated with a net radiometer (Kipp & Zonen sensors) the Kc value. The results of Kc showed a range from 0.6 to 1.9 and average 1, throughout the monitoring. Another study evaluating the irrigation management system for the 36-month-old coffee in the State of Goiás revealed Kc values ranging from 0.92 to 1.51 Oliveira et al. (2007). Also, the study by Flumignan et al. (2011), presented Kc results from 0.5 to 1.5 in three-year-old coffee shrubs grown on weighing lysimeter and different irrigation systems (spraying and dripping).

Experimental results indicate that Kc depends on several factors characteristic of the coffee shrub, including the stage of growth of vegetation, plant density, row spacing, and canopy architecture and/or cultural practices; the presence of the spontaneous plants between the rows of the crop alter the values of Kc (Allen et al., 1998; Villa Nova et al., 2002; Pereira et al., 2015).

Flumignan et al. (2011) also argued that the study presents discrepancies between Kc values of coffee according to the factors mentioned, besides the number of evaluation days and their distribution throughout the year, irrigation management or methodologies used to determine evapotranspiration (ET).

In Brazil, due to the territorial extension, wide climatic variability, diversity of production systems and coffee irrigation methods, it is necessary to quantify the water needs of the crop and to ensure the correct irrigation management through dynamic monitoring.

The size of the vegetation transpiration surface can be related to the leaf area since the leaves are the primary organs that participate in the transpiratory process, responsible for the gas exchange with the environment. On the other hand, at the plot level, the loss of water from the crop can be represented by the Leaf Area Index (LAI), which is defined as the ratio between the leaf area (one side only of the leaf) and the area of land occupied by the

crop. Thus, LAI is dimensionless and expresses the relative size of the leaf area when in competition for natural resources (water, nutrients, and light) (Pereira et al., 2008).

Leaf Area Index is a parameter that is difficult to measure but very useful. Most studies use destructive methods to quantify LAI, Coltri et al. (2015). In this study, the LAI at the plot-monitored level over eight dates during one year, the results representing the temporal and spatial variation of the variable. The trained neural network provided LAI values ranging from 0.6 to 5.6.

The study of Coffee LAI, a parameter derived from MODIS sensor (Moderate Resolution Imaging Spectroradiometer) realized by Taugourdeau et al. (2014) in the central Caribbean region, reports that the seasonal variation of the LAI ranged from 2.4 to 4.4. The authors indicate that, for perennial crops such as coffee, LAI may vary seasonally due to abiotic factors such as drought, shade, temperatures, and biological factors, such as diseases and overproduction, or even by pruning or fertilization. Brazilian studies that periodically evaluated LAI values using allometric measurements indicated results from 0.27 to 2.98 (Pereira et al., 2011) and from 2.3 to 8.7 (Flumignan et al., 2011).

Pereira et al. (2011) report that the LAI increases linearly as the coffee grows. On the other hand, Flumignan et al. (2011) reported that LAI showed large oscillations throughout the measurements in all treatments evaluated with irrigated, non-irrigated and controlled and that tendency was observed in the present study (Figure 4). To Flumignan et al. (2011) the empirical IAF for the control treatment, in coffee plants with an approximate age of 36 months, went from 2.7 to 4.7, and in the following months, it decreased and reduced to values below 2.7, and later, in the LAI again increased.

According to Carr (2001), water stress reduced the total leaf area of container-grown plants and explained that there is much evidence of the sensitivity of coffee stomatal conductance to the prevailing atmospheric conditions and in principal are high total irradiance (1000 W m^{-2}), air temperature (26-30 °C) and saturation deficit (> 1.6 kPa) These are conditions where the stomata remained closed all day.

In Brazil, coffee grows actively from the first rains of the year, which occur between September and October. During this period, vegetative growth happens at the rate of 1.1 leaves per month, peaking in the summer, with 1.6 leaves per month. Then, with the reduction of rainfall and temperature (in addition to the nutritional effect and photoperiod), the plant rests, growing at only 0.5 pairs of leaves per month (Matiello et al., 2015). This seasonal variability may also influence the LAI values.

Also sometimes some periods in a year, the locally measured coffee shrub experience higher soil evaporation (E) than leaf area transpiration (T) and Kc serves the critical purpose of representing averaged E and T process (Pereira et al., 2015b). In this work, the negative correlation between the two biophysical variables analyzed (Figure 7) may be acceptable (or reasonable) since the estimated Kc values by Allen et al. (1998); Doorenbos and Pruitt (1977); Flumignan et al. (2011); and Sato et al. (2007); and the LAI values by Taugourdeau et al. (2014); Pereira et al. (2011) were consistent with the results.

Monitoring the LAI at the plot level with Sentinel-2 products makes it possible to understand the state of the coffee as a whole. In this study, the LAI spatial variability analysis indicated that the lowest values were found at specific sites during monitoring (Figure 5), which are probably related to soil variability or deficiency of some mineral elements such as boron. Currently more monitoring time is required to confirm this trend.

The Sentinel-2 products are of quality, with an excellent spatial resolution of 10 m. They can monitor approximately 51 coffee shrubs per pixel, but they demand high computational capacity and data storage, beside technical personnel prepared for the processing and interpretation that, for coffee smallholders, is not easily accessed.

5. Conclusions

Sentinel-2 products make it possible to monitor the coffee crop at the plot level. The relationship between Leaf Area Index (LAI) and Crop Coefficient (Kc) indicates that LAI can be used to monitor crop water conditions. The use of the LAI in substitution for Kc makes it possible to identify the state of the coffee tree regarding age, spacing, natural vegetation as well as the variability of the plot, having a more reliable estimate of the available water conditions for the crop.

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Spatial-Temporal Dynamics of Vegetation Cover in a Diversity Hotspot for the Conservation of Brazilian Cerrado

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Abstract

This work investigates the spatial-temporal dynamics of land use and vegetation covers in a conservation area of Cerrado, in the county of Currais, Piauí, in which the economy depends on large agricultural projects. We used maps of a 32-year time series (1985 to 2017) of land use and cover provided by the Brazilian Annual Land Use and Land Cover Mapping Project (MapBiomas). We assessed six classes of land uses and vegetation covers: forest, savanna, grassland, agriculture/pasture, non-vegetated area, and water bodies. There was a fast increase in pressure on natural ecosystems from 1985 and 2017, primarily from 2000. The land use for agriculture and pasture increased from 0.26% (726.93 ha) in 2000 to 16% (50,772.63 ha) in 2017. During this period, the native vegetation decreased 15.90%, with savannas suffering the largest loss in hectares of vegetation (41,663.73 ha), followed by the forests (9,837.35 ha). The grassland cover, non-vegetated area, and water bodies remained unchanged. These results provide essential information for decision making and can be used to guide public policies for the conservation, monitoring, and sustainable management of remnant vegetation areas.

Keywords: Agricultural industry, deforestation, natural ecosystems, geoprocessing, time series

1. Introduction

The Cerrado biome comprises a critical area for conservation of global biological diversity (hotspot) due to its high species richness and strong anthropic pressure (Mittermeier et al., 2011; Sloan et al., 2014). Its ecosystems have a high number of endemic species, which are threatened continuously by anthropogenic processes that already modified more than 70% of original vegetation over the years (Reydon & Monteiro, 2009). As a hotspot, the Cerrado should, in practice, be regarded as a priority area for environmental conservation. However, the use and occupation of this biome for the development of agribusiness have been a leading factor altering the natural landscape, converting native vegetation areas into agricultural lands (Silva et al., 2014; Santos et al., 2017).

The incorporation of new areas for agriculture aims to meet the demand for food. However, it provokes large scale environmental impacts, putting natural resources, biodiversity, and ecosystem services at risks (Santos et al., 2017). In the state of Piauí, northeastern Brazil, the Cerrado comprises an area of 8.5 million hectares, about 70% of the state's territory. This area has been intensively exploited to agriculture since the 1970s and 1980s, with intensification in 1990 through the implementation of large projects (Aguiar & Monteiro, 2005).

The continuous occupation of Cerrado lands in Piauí occurs for several reasons, including the exhaustion of soils in other regions of Brazil, the flat topography, which favors the mechanization, the climate suitability for cultivating grain crops, lower land values, and tax incentives (Borghi et al., 2014). In this perspective, the southern region of Piauí is considered one of the last agricultural frontiers within the Cerrado biome (Botrel et al.,

2015). This area includes the Currais county, which in the last three decades has undergone an intensive process of occupation of Cerrado as a consequence of agricultural activities. Thus, the knowledge about areas occupied by agriculture and data on landscape changes over time and space become essential tools for legislators and planners of the use of natural resources.

Remote sensing and geoprocessing techniques became indispensable tools for monitoring the dynamics of land use and vegetation cover (Vaeza et al., 2010; Silva et al., 2014). In northeastern Brazil, several studies have been carried out to detect trends in changes of landscapes both at local and regional scales (Benedetti et al., 2013, Silva et al., 2014, França et al., 2018). On the other hand, little studies were carried out in southern Piauí, where large areas of relevant interest for the conservation of the Cerrado persist. Thus, the present work aimed to analyze the spatial-temporal dynamics of land use and vegetation cover to provide information for public policies of conservation, preservation, and sustainable management of natural resources in a biodiversity hotspot of Cerrado.

2. Method

2.1 Location and Characterization of Study Areas

The work was carried out in the Currais county, in center-south of the state of Piauí, under coordinates 44°18′-45º05′ W and 8°26′-9°02′ S. The county is 640 km from capital Teresina and comprises an area of 3,156.6 km², with the following bordering counties: Palmeira do Piauí and Baixa Grande do Ribeiro to the north, Bom Jesus to the South, Santa Luz and Palmeira do Piauí to the East, and Baixa Grande do Ribeiro to the West. Also, Currais lies in a region with a predominance of biodiversity hotspots of Cerrado biome, comprising priority areas for conservation according to Decree No. 5,092 (Brazil, 2004). Large agricultural projects maintain the economic activity of Currais, above all, the soybean cultivation (Figure 1).

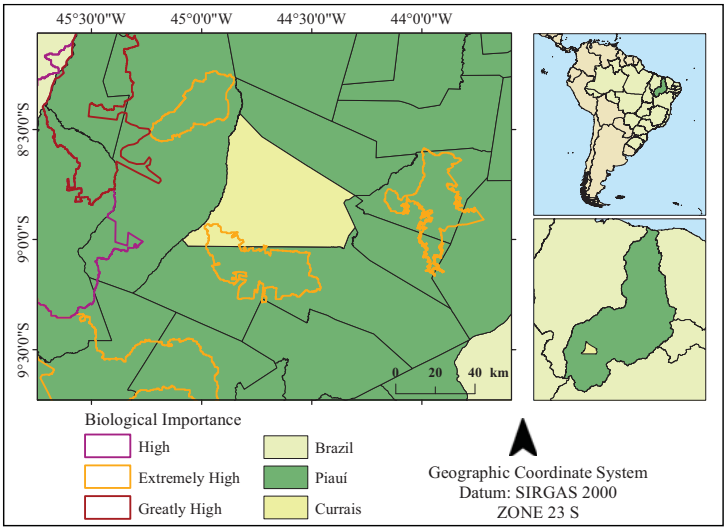

Figure 1. Geographical location of the county of Currais, Piauí, Brazil. The colored polygons on the highlighted map highlight the categories with biological importance for conservation of Cerrado biome in the region (Brasil, 2004)

According to the classification of Köppen, the regional climate is Aw' (hot and semi-humid) with temperatures above 18 °C (Alvares et al., 2013). The average annual rainfall matches the Continental Equatorial Regime, with annual isohyets around 700 to 1,200 mm and rainy season extending from November to May, with January, February, and March being the wettest quarter (IBGE, 2000; Pragana et al., 2012). The vegetation covers include formations from two biomes, Cerrado and Caatinga, and, to a lesser extent, in an ecotone area of cerrado-caatinga (Cepro, 1996; Botrel et al., 2015).

2.2 Data Acquisition and Processing

Land use and land cover maps (Collection 3) of a 32-year time series (1985 to 2017) were generated and provided free of charge by the Brazilian Annual Land Use and Land Cover Mapping Project (MapBiomas). The frequency of images had amplitude of 5 years, except from 2015 to 2017, in which amplitude of 2 years was

used. The MapBiomas project aims the mapping and quantifying of land use and coverage of the Brazilian territory in an automated and consistent way. The project brought together interdisciplinary teams to obtain the maps, including specialists in biomes and cross-cutting themes (mining, agriculture, pasture, among others).

The images used by MapBiomas come from Landsat series of satellites (5-TM, 7-ETM+, and 8-OLI), providing data from 1985 to the present year (2019). In the land use and land cover classification procedure, the project used, in collection 3, the Random Forest algorithm including a more robust sampling designed to train the classifier (MapBiomas, 2019). The classification done by the MapBiomas followed the methodological steps described in Figure 2.

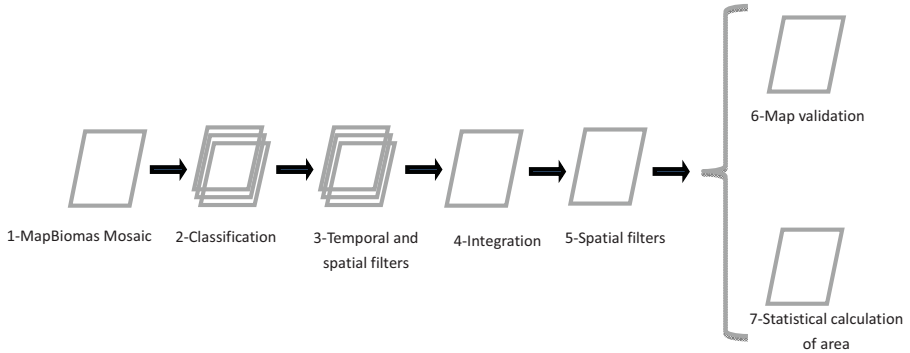

Figure 2. Methodological steps to classify and obtain land use and land cover maps in the county of Currais, Piauí, Brazil

Stage 1. Production of annual Landsat image mosaics based on specific periods to optimize the contrast between land use and land cover classes. At this stage, biomes, transition areas between biomes and transverse themes (*i.e.*, mining, agriculture, pasture, among others) were separated to obtain maps of land use and cover.

Stage 2. The setting of characteristic spectral inputs (training samples) derived from the bands of the Landsat images to carry out the classification by the Random Forest algorithm.

Stage 3. Use of spatial and temporal filters to the maps generated in the previous step with the aim of removing noise from the classification and filling the information gaps caused by clouds.

Stage 4. Integration of land use and land cover maps of each biome and cross-cutting themes, through the hierarchical overlap of each mapped class, according to specific rules of prevalence empirically defined.

Stage 5. Use of spatial filters to remove isolated classes (smaller than half a hectare) and noise resulting from an incorrect record of Landsat data.

Stage 6. Mapping validation based on two approaches: (a) spatial matching analyzes with reference maps according to their availability (b) spatial matching analyzes based on statistical techniques to define sample points based on the extent and number of classes of each biome.

Stage 7. Calculation of the zonal statistics of the classes mapped to different spatial units (biomes, states, and municipalities), including watersheds, rural settlements, and protected areas.

Based on the specificities of the county, only six classes of land use and cover were considered in this study (Table 1).

Table 1. Classes of land use and land cover used for classification of the county of Currais, Piauí, Brazil

Classes	Description
Forest Formation	**Caatinga** Types of vegetation with continuous canopy predominance-Forested Steppe-Savannah, and Decidual and Semi-Decidual Seasonal Forest.
	Cerrado Vegetation with a predominance of arboreal species, with continuous canopy formation (Riparian Forest, Gallery Forest, "Mata Seca", and "Cerradão"), as well as seasonal semi-deciduous forests.
Savanna Formation	**Caatinga** Types of vegetation with the predominance of semi-continuous canopy species - Wooded Steppe-Savannah, Wooded Savannah.
	Cerrado Savanna formations with well delimited arboreal and shrub-herbaceous strata (Cerrado in strict sense, Dense Cerrado, Typical Cerrado, Sparse Cerrado, Rocky Cerrado, and Parklands).
Grassland Formation	**Caatinga** Vegetation with the predominance of herbaceous species (Steppe-Savannah Park, Gramineous-Woody Steppe-Savannah, Savannah Park, Gramineous-Woody Savannah) + (Flooded areas with a network of interconnected ponds, and vegetation with the predominance of herbs and shrubs).
	Cerrado Grasslands with a predominance of herbaceous stratum (dirty field, clean field, and rocky field).
Non-vegetated area	**Urbanized Area** Urbanized areas with a predominance of non-vegetated surfaces, including roads and constructions.
	Exposed soil Areas with exposed soils or naturally exposed rocks without soil cover, often with partial presence of rock vegetation and high slope.
Agriculture/pasture	**Agriculture** Areas predominantly occupied with annual and perennial crops.
	Pasture Natural or planted pasture areas linked to agricultural activity.
Water bodies	Rivers, lakes, dams, reservoirs and other bodies of water.

Source: MapBiomas (2019).

After obtaining the land use and land cover maps for the time series analyzed, we calculated the statistical data of the areas of each class in the county.

3. Results and Discussion

The maps and time series showed an increasing anthropic pressure on the natural ecosystems between 1985 and 2017. The savannah vegetation cover predominated in the last year analyzed, representing 50% of all the vegetation of the county, followed by the grasslands (23.80%), and forests (10.16%). The area of agriculture/pasture corresponded to 16%. The non-vegetated area and the water bodies covered less than 1% of the county and remained unchanged over the years (Figure 3).

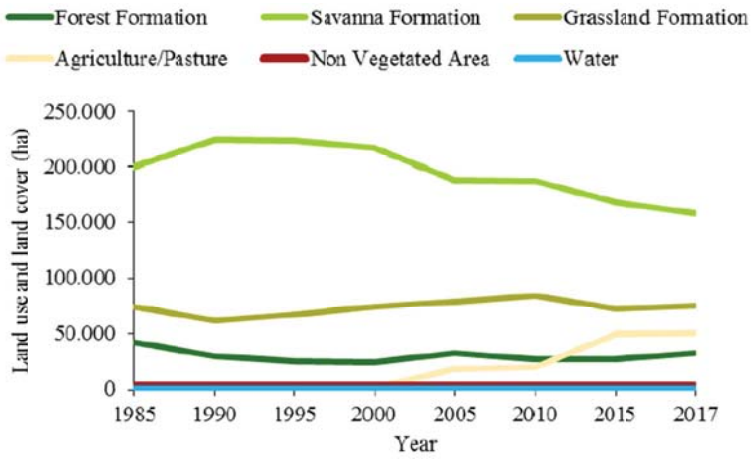

Figure 3. Spatial-temporal analysis of land use and land cover changes in Currais, Piauí, Brazil

From the year 2000, the changes in the natural landscape became more noticeable due to the beginning of use and occupation of lands mainly for agriculture. The expansion of agriculture and pasture increased dramatically, from 0.26% (726.93 ha) in 2000 to 16% (50,772.63 ha) in 2017. Due to this advance, there was a sharp decrease of 15.90% in native vegetation.

Over the years, natural ecosystems have suffered a fast process of land use and occupation, being intensively exploited for agricultural and livestock purposes. The Cerrado, formed by a complex network of plant formations, such as grasslands, savannas, and forests, is considered a biodiversity hotspot and a priority for conservation (Ribeiro & Walter, 2008; Mittermeier et al., 2011). However, it is also regarded as an ideal region for the expansion of agriculture (Borghi et al., 2014), which is often carried out intensively and disorderly. The scenario favoring agriculture lead to a significant increase in deforestation in the last 40 years (Silva et al., 2014).

The state of Piauí, with 70% of its territory covered by Cerrado vegetation, shows great potential for agricultural expansion (Silva et al., 2014), mainly due to its edaphoclimatic characteristics and the geomorphology of the area that favors the agrarian mechanization.

In the state, 24 counties stand out in agricultural production (Santos et al., 2017). Among them, Currais underwent a rapid change in the use and occupation of land in recent years, which has affected the natural landscape (Figure 4). For example, the conversion of the natural vegetation, mainly the savanna, into agricultural areas that occurred from the year 2000.

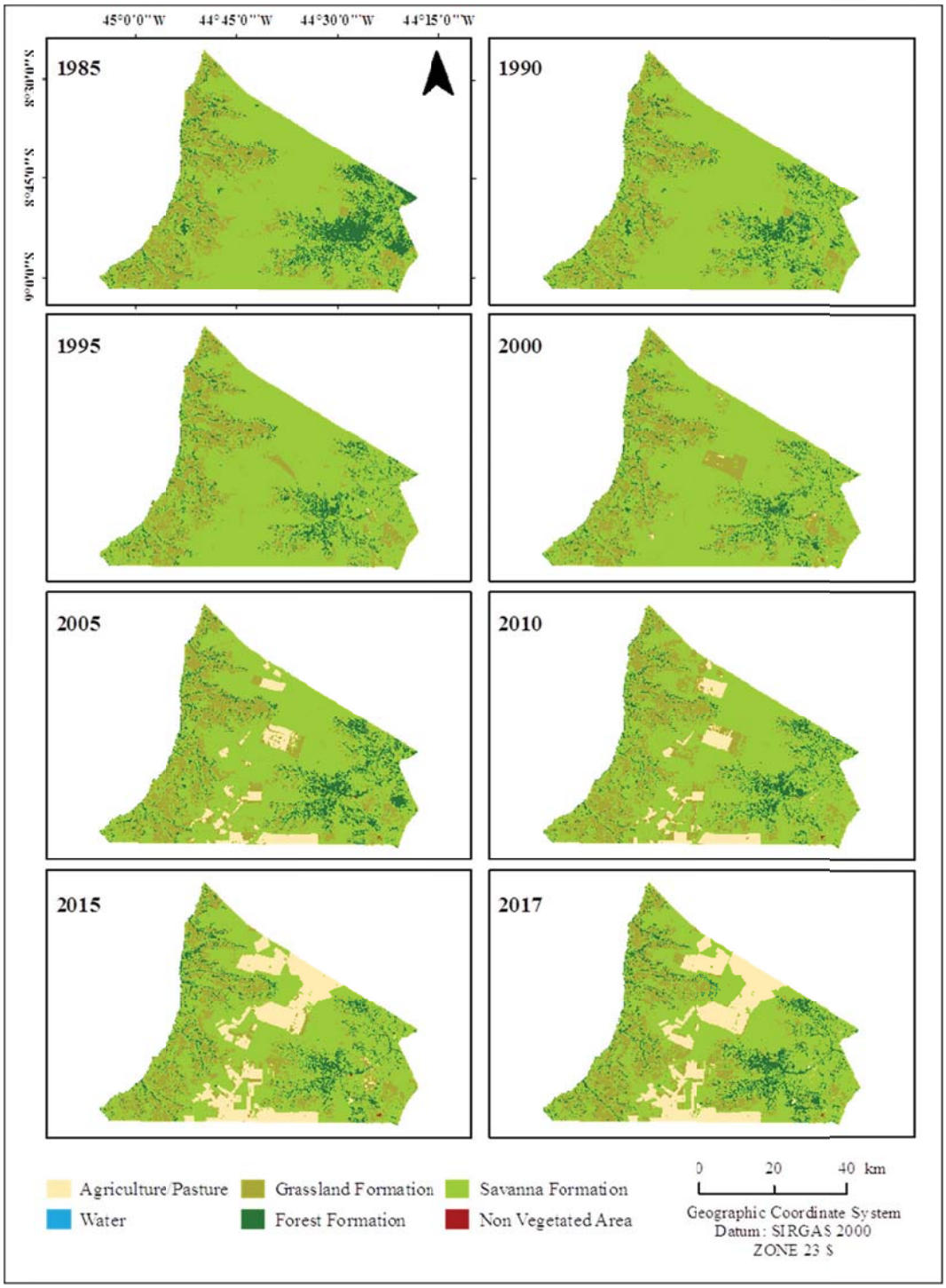

Figure 4. Spatial-temporal dynamic of land cover changes in the county of Currais, Piauí, Brazil, in a time series of 32 years (1985 to 2017)

This trend has also been observed in areas adjacent to Currais. For example, Santos et al. (2017) reported a loss of 54.81% of native vegetation and an increase of 297.98% of exposed soil between 1984 and 2015 in southwestern Piauí. França et al. (2016) pointed out an increase of 55% in the occupation of crops between 1984 and 2011 in a sub-basin of the Uruçuí-Preto river, also in southwestern Piauí.

The accelerated use and occupation of the Cerrado in Piauí from the 2000s are directly related to the decrease of lands in other regions of Brazil, the properties with low values in Piauí, the favorable soil characteristics (França

et al., 2017), and the implementation of large projects for the production of grains, mainly soybeans for exportation (Aguiar & Monteiro, 2005).

This expansion of agribusiness brought intense pressure on natural resources in the county of Currais, mainly by the conversion of areas of natural vegetation into agriculture and livestock areas. There is an urgent concern in maintaining these forests performing their ecosystem services, contributing to the maintenance of ecological functions, as well as guaranteeing the income of small rural producers and the human well-being in space and over time (Costanza et al., 2014; Mutoko et al., 2015). Some ecosystem services have already been lost and modified. Santos et al. (2017), studying the relationship between deforestation and climate change in the region, observed a reduction of 14.34 mm in the monthly average precipitation, totaling a rainfall of 172.08 mm year^{-1}, which increased the maximum temperature in 0.91 °C and decreased the relative air humidity in 7.43%. These results were corroborated by Albuquerque and Lopes (2016), who reported that changes in vegetation cover have a significant influence on the variation of temperature and relative air humidity.

The grasslands suffered little changes in vegetation-cover over the 32 years (Table 2). This fact can be associated with the type of occupation of these areas, composed mostly by small properties of family farmers located on steep slopes and scarps, which are improper for agricultural mechanization and large scale projects. On the other hand, savannas and forests had the largest losses in hectares (41,663.73 and 9,837.35 ha, respectively). The total number of hectares used to agriculture or pasture in the year 2017 was 50,772.63 ha, approaching the grassland area in the same year (75,047.37 hectares) (Table 2).

Table 2. Dynamics of the area (ha) of land use and land cover in the county of Currais, Piauí, Brazil, from 1985 to 2017

Year/Class	Forest	Savanna	Grassland	Agriculture/Pasture	Non-vegetated	Water bodies
1985	41,931.88	199,317.51	74,326.96	43.38	34.62	4.68
1990	29,528.47	224,311.80	61,727.86	59.1	31.62	0.44
1995	25,144.42	223,464.70	66,817.91	194.89	33.30	2.74
2000	23,845.11	216,576.47	74,444.61	726.93	46.99	18.29
2005	31,992.32	187,371.55	78,556.48	17,649.63	62.45	26.51
2010	26,323.97	186,468.87	83,656.46	19,118.91	69.60	20.15
2015	26,341.36	167,904.50	71,796.25	49,514.00	87.27	14.58
2017	32,094.53	157,653.78	75,047.37	50,772.63	73.14	16.53

In this aspect, the knowledge of strategies to guide the use of natural resources in a sustainable way becomes urgent and indispensable. The Brazilian law of native vegetation protection (Law No. 12,651/2012), that establishes general norms on the preservation of forests (Brazil, 2012), together with the Law No. 9,985/2000, that establishes criteria for the creation of conservation units (Brazil, 2000), are essential instruments for the maintenance, conservation, and sustainable development of ecosystems. On the other hand, the lack of supervision and cohesive application of these environmental policies, as well as the lack of study with technical-scientific bases has prevented the directing of public plans for the conservation, preservation, and sustainable management of natural resources and soil in this region.

4. Conclusion

1). The land use and vegetation cover in the county of Currais, Piauí, suffered rapid changes between 1985 and 2017, with the increasing anthropic pressure on natural resources, mainly through the conversion of savannas and forests into agriculture or livestock areas.

2). The registered changes have affected the natural landscape with loss and modification of ecosystem services that are essential for the well-being of local and regional population.

3). The results are relevant for decision making and can be safely used to guide public policies for the conservation, monitoring, preservation, and sustainable management of the vegetation remnants.

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Improving Small Weed Seeds Viability Assessment Using Tetrazolium Test

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Abstract

Tetrazolium testing in small seeds demands difficult and longstanding procedures, such as the embryo exposure by seeds section and the seeds pre-preparation. This study aims to access the viability of small seeds using the Tetrazolium test (TZ) without seeds sectioning, resulting in quicker, cheaper and precise measurements. Non-sectioned seeds of *Conyza sumatrensis*, *Bidens pilosa* and *Digitaria insularis* were put in contact with the tetrazolium solution during 0, 12, 24, 48, 72, 144, 216, 288, 360, 432 and 504 h to obtain the seeds coloring percentage in each time. When possible, the seeds coloring percentages were compared to seeds viability, obtained by the literature standard tetrazolium methodology (STZ), and with seeds germination. The proposed methodology (TZM) was cheaper and less laborious than the methodologies frequently used for weed seeds viability estimation, and provided rapid and reliable seeds viability estimations for weed species with small seeds within 24 h for *Digitaria insularis* and *Bidens pilosa,* and 48 h for *Conyza sumatrensis*.

Keywords: *Bidens pilosa*, *Conyza sumatrensis*, *Digitaria insularis*, fast test and weed science

1. Introduction

Weeds aggressiveness characteristics highly contributes to plants interference processes, impairing crops development. A part of this characteristic is related to seeds production and physiological behavior, including the high number of propagules formed, dissemination mechanisms and seed dormancy, which enable asynchronous germination (Cauwer et al., 2014). Weeds producing thousands seeds per plants are often found, resulting in increased difficulties on their control within farming areas and increasing the soil seed bank (SSB). SSB is a source of viable weed seeds, located within or on the soil body, potentially capable of infesting croplands (Hosseini et al., 2014).

As an example, *Conyza* sp. seeds exhibit increased germination levels on the beginning of autumn or spring (Buhler & Owen, 1997; Holm et al., 1997, Regehr & Bazzazn, 1979), but its germination does not stop during the year. *Digitaria* sp. and *Bidens* sp. species are spread in tropical, subtropical and temperate regions of the world (Watson & Dallwitz, 1992), and its seeds may exhibit dormancy (Souza et al., 2009). Therefore, the assessment of seeds viability on the SSB, as well as those still attached to the plant, is critical for inferring about dissemination and infestation potentials of those weeds.

The most common methods used in seeds viability assessment are top-of-paper, standard paper germination (SG), germination in sand and the agar method (Rao et al., 2006). Nevertheless, those methods may not be efficient in weed seeds viability estimation due to seeds manipulation difficulties and temperature, air humidity, light and test duration specific requirements, resulting in tests lasting days or months (Taylorson, 1987; McIvor &

Howden, 2000; Koger et al., 2004; ISTA, 2005). The SG is the most common among those, but does not provide the total seeds germination potential precisely because of seeds dormancy (Taylorson, 1970). Furthermore, seeds identified as viable by those methods cannot be used in posterior studies, since their germination makes them unfeasible.

Another problem related to the conventional germination tests used for weed seeds comes from their decreased sanitary quality. Total loss of a replication or an entire test due to fungi or bacteria attack are common. It happens because weed seeds of the SSB exhibit increased longevity (Burnside et al., 1996), which favors their contamination by pathogens or mechanical damages incidence. Tetrazolium test (TZ) avoids those problems caused by seeds contamination (Thorpe & Kaye, 2008), but has not been frequently used for small seeds testing, such as weeds. Due to its small size, weed seeds represent a challenge for TZ performing, once that this methodology requires embryo exposure to the tetrazolium salt solution with posterior evaluation of the sectioned seeds, which is a difficult task in such small seeds. Nevertheless, improvements on the TZ method for evaluation of small seeds would result in costs and time reduction, as well as increase accuracy in weed seeds viability assessment.

For *Conyza* sp. species, no TZ methodology was found to be described in literature so far. The methodology proposed by the International Seed Testing Association (Moore, 1985) for monocots using TZ is laborious, and therefore non-used. It suggests that seeds must be maintained soaked in water for 6-18 hours and sectioned in halves afterwards for posterior immersion in the tetrazolium solution for 48 h, resulting in 70 hours from the beginning to the end of the test. In addition to the duration, the sectioning of such small seeds as of *Conyza* sp. increases the difficulties of the test.

Possible contributions through improvements on small seeds viability testing are related to the identification of dormancy mechanisms or seed damages occurrence, including dead seeds. Those improvements would allow to isolate effects in studies of herbicides performance, when applied in pre-emergence. With the real physiological state of the weed seed, it would be possible to comprehend if the non-emergence of the plantlet was because of a previous physiological condition of the seed or due to the herbicide control effect. It would consequently result in less overestimations on herbicides molecules effects, as well as enable decreases on the use of low quality weed seeds in experiments. Adaptions on the TZ methodology that eliminate the need of seeds sectioning for viability evaluation may result in a simpler and more useful test. Our hypothesis is that it is possible to obtain a faster, cheaper and simpler TZ method for *C. sumatrensis*, *B. pilosa* and *D. insularis* seeds than those already described in literature. On this context, the objective of this study is to propose adaptions to the TZ test for *Digitaria insularis* and *Conyza sumatrensis* seeds that may properly and easily estimate their viability, and to compare the results obtained with the proposed method with the ones obtained with the standard and available TZ methodologies and SG.

2. Method

2.1 Seeds Collection

Seeds of *B. pilosa*, *D. insularis* and *C. sumatrensis*, growing in commercial fields at Engenheiro Coelho, São Paulo State, Brazil (22º29′18″ S; 47º12′57″ W), in September, 2017, were collected by gently rubbing the mature inflorescences of six plants each. The harvested seeds were stored during 30 days after harvest, at 25 °C and 60% air relative humidity, until the tests were conducted. The mass of a thousand seeds for *B. pilosa, D. insularis* and *C. sumatrensis* were 1.60 g, 0.80 g and 0.07 g, respectively.

2.2 Seeds Testing

All species studied were submitted to the standard germination test (SG) on germination paper, while the methodology proposed by Moore (1985) to evaluate seeds viability using TZ (STZ), was compared with the TZ methodology proposed herein (TZM) for *Bidens* spp. and *Digitaria* spp., and followed procedures described in literature for each species. No standard method was found in literature for *Conyza* spp., and therefore no STZ was performed for this species.

For the STZ, seeds were submerged in water for 12 h, and after this period were cut in two halves and kept in tetrazolium solution (0.1%) up to 48 h at 30 °C. After that, seeds with red stained embryo were considered viable.

SG tests were performed in five replications of 50 seeds each, within plastic boxes, and kept in artificially illuminated incubators (model TE-4000, Tecnal Company). These incubators are equipped with three lamp pairs in each tray level, and released 100 µmol photons/(m^2 s^{-1}) of light in each level. The gerboxes received 3 mL of distilled water each, at the beginning of the test, and the water was refilled when necessary. The seeds of *D.*

insularis were exposed to specific photoperiod (12 h under dark and 12 h under light) and stable temperature of 35 °C. *C. sumatrensis* seeds were maintained under constant 20 ºC temperature and same photoperiod used for *D. insularis*, as established in Wu et al. (2007). *B. pilosa* seeds were incubated at 25 ºC for 12 h in the dark and at 30 °C for 12 h under light (Reddy & Singh, 1992). The seeds were considered germinated when the radicle protrusion through the tegument was visible (Chivinge, 1996; Brasil, 2009). The number of germinated seeds was obtained in 0, 12, 24, 48, 72, 144, 216, 288, 360, 432 and 504 h after the test beginning.

The TZM for each species are described as follows:

2.2.1 *C. sumatrensis* and *B. pilosa*

Five replications of 50 *C. sumatrensis* and *B. pilosa* seeds were placed in individual Eppendorf tubes (20 mL) containing 10 mL of Tetrazolium solution (0.1%-w/v), covered with aluminum foil for protection against light, and exposed to 40 °C for 504 h. Every 12 h the seeds were removed from the TZ solution, in a low light environment, and examined with a microscope. Seeds that were colored in red or pink were considered viable, while the ones not colored were considered dead (Moore, 1985; Tesnier et al., 2002; Figures 1 and 2). Seeds considered viable were discarded after counting, and the ones not colored were placed in fresh TZ solution after each evaluation. *C. sumatrensis* seeds are very small, and the mean weight of a single seed is around 0.072 g. The cover tissue of *C. sumatrensis* represents around 15% of this mass and 85% corresponds to the embryo (Fenner, 1983). As the external part of the seed is translucent, it is possible to identify the tetrazolium coloring without cutting open *C. sumatrensis* seeds (Figure 1). For *B. pilosa*, seeds with reddish or pink embryo were also considered viable, while the ones not colored were considered dead, as observed in Figure 2. In this case, the embryo is visible on the cypselas cracks, uniformly present after seeds soaking. When not visible, the embryo may be exposed when removing the cypsela, which is easily opened by the touch after soaked.

Figure 1. Tetrazolium staining of *Conyza sumatrensis* seeds, with TZM. Red-stained seeds are considered viable or metabolically active, and therefore potentially capable of germination, and the uncolored seed (top-right) is considered dead

Figure 2. Tetrazolium staining of *Bidens pilosa* seeds, with TZM. Red-stained seeds (A) are considered viable or metabolically active, and therefore capable of germination, while non-colored seeds are considered dead. The arrows indicate the red-stained tissues

The evaluation of *C. sumatrensis* and *B. pilosa* seeds coloring was performed at same moments as established for the SG counting.

2.2.2 *D. insularis*

D. insularis seeds carry specific structures called palea and lemma that may interfere with translocation of the Tetrazolium salt solution into the seeds, affecting the coloring of the seed tissue. Therefore, as proposed on the international rules for seed analysis (Moore, 1985), these seeds must be cut in half for the analysis. In this study, we explore the utilization of uncut *D. insularis* seeds for the TZ test, after removal of the palea and lemma structures from each one. In this case the palea and lemma of 250 seeds were removed, and five replications of 50 seeds each were immediately incubated in 0.1% Tetrazolium salt solution, as in the other two species. Evaluations were conducted each 12 h and the seeds that presented reddish or pink embryo tissues were considered viable, and the ones not colored were considered dead; the endosperm of these grass seeds does not contain living tissue (Figure 3). The evaluation of *D. insularis* seeds coloring was performed at same moments as in SG evaluations.

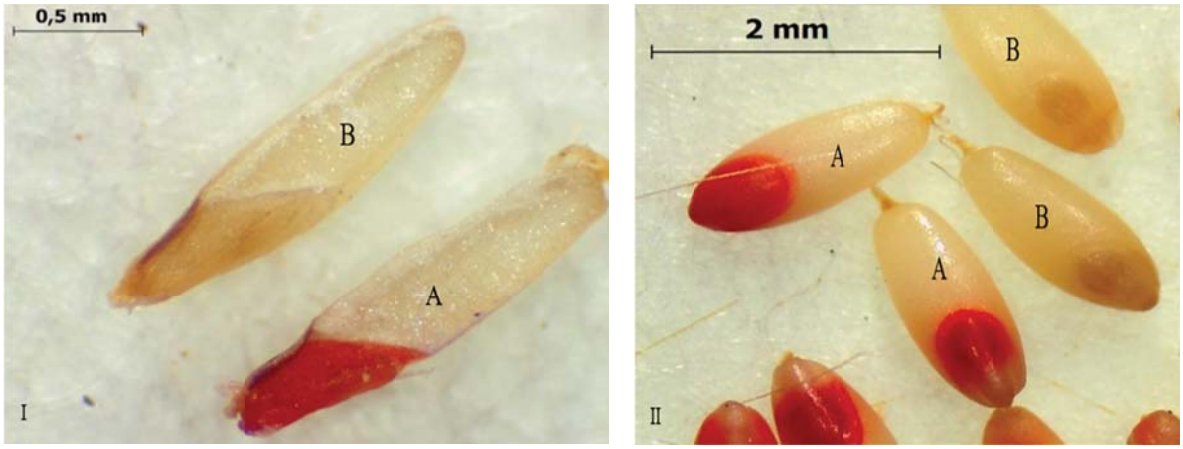

Figure 3. Tetrazolium staining of *Digitaria insularis* seeds. I: sectioned seeds (STZ) and II: uncut seeds (TZM). Red-stained (A) seeds are considered viable or metabolically active, and therefore capable of germination. Uncolored seeds (B) were considered dead

2.3 Statistical Analysis

Means were compared by the mean confidence interval ($p < 0.05$), as described in Payton et al. (2000).

3. Results and Discussion

Seeds viability of *C. sumatrensis* and *B. pilosa,* determined by TZM, were compared to the STZ method, proposed by Moore (1985), and SG of all three species studied are presented in Figures 4-6. Seeds viability and germination, obtained by the TZM and SG tests, were similar for *D. insularis* and *C. sumatrensis*, as well as STZ was similar to SG for *D. insularis*. *B. pilosa* had less germinated seeds in comparison to the number of viable seeds, obtained by SG and TZM, respectively. This difference in behavior indicates that *D. insularis* and *C. sumatrensis* seeds lacked dormancy, once their viability and germination values were statistically not different, highlighted by the plateaus obtained for percentage of colored seeds by the TZM test and percentage of seeds germinated on the SG test (Figures 4 and 6). The difference between seeds viability and germination of *B. pilosa* is probably an indication of seeds dormancy. The estimation of the overall seed viability by using the SG test may therefore be highly influenced by dormancy, and provide inaccurate results regarding weeds infestation potential. The methodology proposed herein may therefore provide reliable data regarding weed seeds viability.

Figure 4 demonstrates that, after 24 h of seeds exposure to tetrazolium salt solution, there was a stable number of viable *B. pilosa* seeds until the end of the test, while the first colored seeds were identified within 12 h after the TZM test started. The SG test on the other hand, only reached germination stability after 280 h, indicating that the TZM test was faster (11 times) to establish the viability potential than the SG test took to reach seeds germination of *B. pilosa*. As long as the number of seeds identified as viable by the TZM test reached stabilization within 24 h, we understand that this period was sufficient to be considered as a standard for future evaluations of *B. pilosa* seeds viability by the proposed TZM test. From the total amount of *B. pilosa* seeds tested, 78% were identified as viable by the TZM test (Figure 4), meaning that 22% of *B. pilosa* seeds were dead. From the total amount of viable *B. pilosa* seeds, only 23% were capable to germinate (18% of the total number of seeds), and therefore 77% of the viable *B. pilosa* seeds were dormant, as long as they were viable, but did not germinate under optimal conditions.

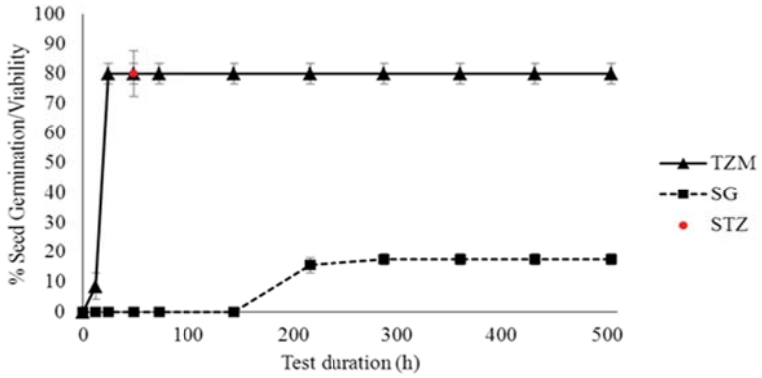

Figure 4. Comparison of two methods to estimate *Bidens pilosa* seeds viability. The ordinates axis refers to the percentage of germinated seeds in the standard germination test (SG) and percentage of seeds colored by the tetrazolium test (TZ), for *Bidens pilosa* seeds, and the abscissas axis refers to the time in which TZ and SG were taken account. The bars refer to the mean confidence interval ($p < 0.05$)

The TZ test was used to evaluate seeds overall viability, and therefore its germination potential. The SG, on the other hand, provided a realistic indication of the seedling production potential of weed seeds within 500 h after proper environmental conditions for seeds germination were reached. Seed dormancy appeared to play an important role on the differences between TZM and SG results of *B. pilosa*, causing asynchronous seeds germination and misrepresentation of the total germination capacity of the weed seeds lot by the SG test.

Figure 5 demonstrates that the stabilization of *C. sumatrensis* seeds viability, identified by the TZM test, was observed at first within 48 h, while seeds germination took 3.5 times more to reach stabilization (216 h). The TZM test identified the first viable seeds within 12 h after the test beginning, and increased the number of viable seeds identified by the method in 24 h. As no other methodology of *C. sumatrensis* seeds viability using TZ was identified by the authors in literature, it can be stated that the results expose sufficient information to establish 46 h as a standard period in future evaluations of *C. sumatrensis* seeds viability using TZM, once it provided the maximum number of viable seeds in the shortest term.

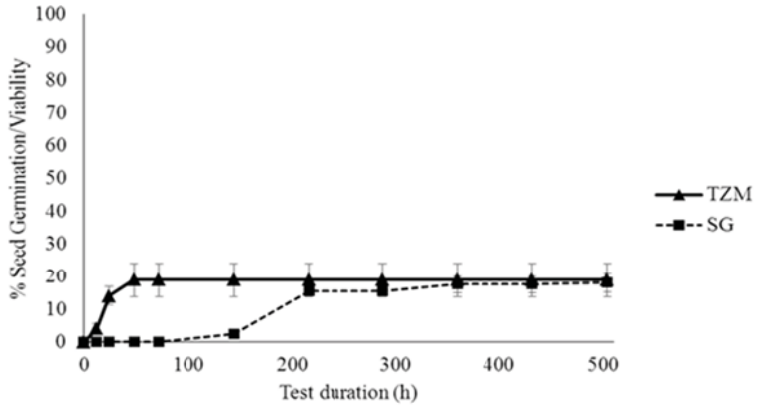

Figure 5. Comparison of two methods to estimate *Conyza sumatrensis* seeds viability. The ordinates axis refers to the percentage of germinated seeds in the standard germination test (SG) and percentage of seeds colored by the tetrazolium test (TZ), for *Conyza sumatrensis* seeds, and the abscissas axis refers to the time in which TZ and SG were taken account. The bars refer to the mean confidence interval ($p < 0.05$)

There was no difference between seeds viability and germination of *C. sumatrensis*. This is an effect of low or inexistent seeds dormancy incidence and an indication that collected seeds of this species were mature and that the germination methodology was well fitted to represent seeds viability of *C. sumatrensis* (Buhler & Hoffman, 1999). The herbicides selectivity testing of *Conyza* sp. is widely performed in weed science, once the selection of herbicide resistant populations (Weed Science, 2018) has increased complexity of its chemical control. Therefore, a quick and easy TZ test that does not impairs seeds germination may provide significant help in fast detection of viability of these populations.

Figure 6 demonstrates the SG, STZ and TZM tests results for *D. insularis*. Within 216 h, the beginning of seeds germination process was identified by the SG test, reaching a stable level at the same time. In turn, the TZM test demonstrated that 16% of the seeds tested were colored in the first 12 h, reaching a stable plateau in the first 24 h, with 90% of the seeds colored in red and identified as viable. TZM and SG results obtained after seeds viability and germination stabilization, respectively, were not significantly different from the STZ test, indicating that seeds dormancy was probably absent in this seeds lot and that SG testing of *D. insularis* is reliable to study seeds viability, in contrast with *B. pilosa*. Even though, the TZM test was eight times faster than the SG test, permitting inferences about *D. insularis* seeds viability within 24 h.

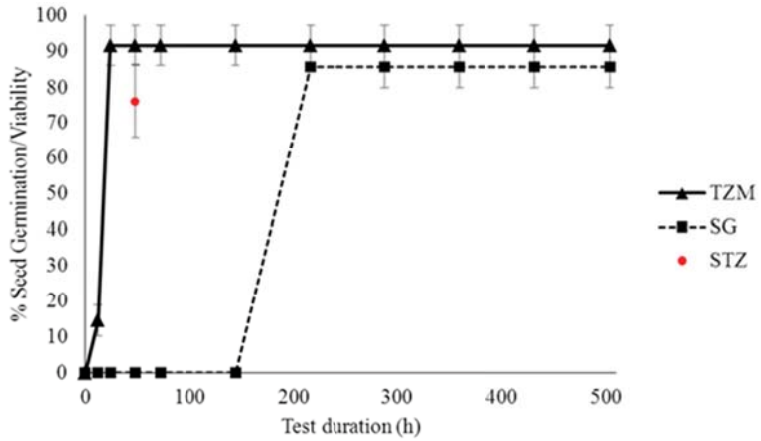

Figure 6. Comparison of two methods to estimate *Digitaria insularis* seeds viability. The ordinates axis refers to the percentage of germinated seeds in the standard germination test (SG) and percentage of seeds colored by the tetrazolium test (TZ), for *Digitaria insularis* seeds, and the abscissas axis refers to the time in which TZ and SG were taken account. The bars refer to the mean confidence interval ($p < 0.05$)

There is a limited number of studies regarding *D. insularis* seeds quality evaluation and germination tests. Considering that, methodologies were adapted to proper representation of germination levels, with different temperatures and photoperiods according to the seeds origin site. Varying temperatures from 20-30 °C, 20-35 °C and 15-35 °C with a 16-8 h of light per day are described as more suitable than constant temperature and light periods for studies involving *Digitaria* spp. (Mondo et al., 2010; Mendonça et al., 2014). Mondo et al. (2010) even question the light incidence requirements for *D. insularis* germination, while Mendonça et al. (2014) describes dependence of seeds germination in relation to the photoperiod.

Results obtained by the STZ and TZM tests for *B. pilosa* and *D. insularis*, in Figs. 4 and 6, indicate that the TZM test permitted proper inference on the seeds viability potential. The TZM methodology proposed herein aims to facilitate seeds viability determination, using a standardized methodology without cutting seeds, as seen in most weed seeds evaluation methodologies, and without any treatment to break dormancy. Furthermore, the test provided fast viability results (24-48 h) of *B. pilosa*, *C. sumatrensis* and *D. insularis* seeds. With that, we expect that studies involving weed seeds may be improved and facilitated, since herbicide resistance is expected to be an increasingly problematic issue in cropping fields (Peterson, Collavo, Ovejero, Shivrain, & Walsh, 2018).

The exposed results indicate that there is a great potential on this test application in studies involving weeds with small seeds. Studies regarding effects of pre-emergent herbicides on weed seeds may be improved by a quick and less laborious TZ test for weeds with small seeds, once the effect of the treatment may then be separated from the effect of seeds dormancy. The methodology proposed herein allows rapid and reliable results regarding seeds viability.

4. Conclusion

The proposed methodology for the tetrazolium test was rapid and reliable for small seeds viability estimation. The test provided *D. insularis* and *B. pilosa* seeds viability within 24 h, while *C. sumatrensis* seeds viability was obtained within 48 h. Besides being faster than the standard paper germination test, and simpler than the STZ test proposed for *D. insularis* and *B. pilosa*, the TZM test proposed herein did not cause damage to the seeds, enabling its later use in field trials, generating results that do not carry seeds dormancy influences.

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Morphological and Biochemical Study of *Bidens pilosa* on the Effects of Extract of *Urochloa ruziziensis*

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Abstract

Bidens pilosa is an aggressive species that competes with crops and, in addition, has a high capacity to acquire resistance or tolerance to herbicides. Thus, the objective of the present study was to investigate the allelopathic effects of aqueous extract of *Urochloa ruziziensis* on germination, development, respiration, antioxidant enzymes and cells morphology of *B. pilosa* seedlings during initial growth at laboratory.The seeds were sown with water or *U. ruziziensis* extract at concentrations of 250, 500 and 900 ppm, and after four days the percentage of germinated seeds, root and hypocotyl development, as well as respiration, peroxidase and catalase activity by seedlings, were analysed. The results were submitted to analysis of variance (ANOVA) and the means compared by the Tukey test and regression analysis. The cellular structures of the root with *U. ruziziensis* extract treatment (0, 500 and 900 ppm) were also analysed by transmission electron microscopy. The application of the extract reduced the germination of the seeds. The root growth increased, however, there was a reduction in the dry matter mass at 500 ppm. Mitochondrial respiration decreased and there was an increase in the activity of the peroxidase and catalase enzymes at 500 ppm. Morphological changes in the cells were also found, mainly with this concentration. Thus, it is possible can be concluded that allelochemicals present in extract from *U. ruziziensis* have the potential to provoke oxidative stress in *B. pilosa* seedlings in laboratory, mainly at a concentration of 500 ppm. This oxidative stress caused alterations mainly in the energetic metabolism of this plant, being this a primordial factor for its growth and survival.

Keywords: allelopathy, weed, catalase, peroxidase, respiration, electron microscopy.

1. Introduction

The *Bidens pilosa* L. weed is an annual plant originating in South America, but is widely distributed in most regions of the world (Holm et al., 1991; Xuan & Khanh, 2016). The seeds of this plant are widely dispersed by wind and animals, so a single plant can produce approximately 5000 seeds that can remain viable for years when buried in the soil (Xuan & Khanh, 2016). Due to its rapid growth, this plant is found in most parts of the world and may be in both cultivated and uncultivated fields causing problems in food crops in many countries (Holm et al., 1991; Khanh et al., 2009; Mitich, 1994; Xuan & Khanh, 2016).

In Brazil, *B. pilosa* occurs in most of the country and is considered to be one of the most important weeds in annual and perennial crops (Kissmann & Groth, 1992). It is an aggressive species that competes with crops and serves as a host for pests and diseases, causing significant reductions in productivity. In addition, this species exhibits a high capacity to acquire herbicide resistance or tolerance (Kissmann, 1997). For all these reasons, there is a growing interest in developing natural alternative methods for weed control. Thousands of secondary products are produced by plants and these can be used in the process of allelopathy among living organisms, as well as providing new molecules that can be used as natural herbicides in weed control. Allelopathy is defined as the direct or indirect inhibitory or beneficial effect of a plant on another living organism through the production

of chemical compounds that are released into the environment (Bell & Koeppe, 1972; Gressel & Holm, 1964; Muller, 1966).

Oliveira et al. (2019) studied the allelopathic potential of Brachiaria (*Brachiaria brizantha*), sunflower (*Helianthus annuus*) and sorghum extracts (*Sorghum bicolor*) on germination and initial growth of beggar ticks (*Bidens pilosa*). It was verified that Brachiaria and sorghum extracts showed no action on germination, but controlled the initial growth of beggar tick, being potential natural herbicides.

There are some studies that show the effect of allelochemicals present in plant extracts on other plants. Pacheco et al. (2016) studied the use of cover crops such as *U. ruziziensis* in no-tillage and conventional systems with soybean, corn and rice crops in the cerrado of Piauí and demonstrated significant reductions in the emergence and accumulation of weeds. Martinelli et al. (2017) studied the effect of two species of *Urochloa* on the *Citrus* and found that *Urochloa* as a cover is a good option for the sustainable and integrated management of weeds. Nepomuceno et al. (2017) studied the effect of *U. ruziziensis* when used as a vegetative cover in the cultivation of transgenic soybeans, and found that extracts collected from *U. ruziziensis* contained substances which can control the growth of weeds.

Oliveira et al. (2014) studied that the *U. ruziziensis* has shown to be specie that can be cultivated to produce straw with allelopathic potential. These effects were effective in suppressing the emergence or early growth of *E. heterophylla* and *B. pilosa.*

However, there are few studies that are concerned with modes of action of allelochemical. Mitochondrial respiratory metabolism is essential for the production of energy and precursors for biosynthesis of new cellular structures. An effect on respiratory metabolism could be a mode of action of natural compounds that suppress the germination and growth of weeds (Pergo et al., 2008). In order for these extracts to be used in the field, it is necessary to make tests that indicate mode of action first in the laboratory.

Thus, the objective of this study was to investigate the allelopathic effects of the aqueous extract of *U. ruziziensis* (brachiaria) on germination, development, respiration, antioxidant enzymes and cell morphology of *B. pilosa* seedlings during initial growth, at laboratory, indicating that the contribution of mitochondrial respiration to the energy metabolism of the seedlings was predominant.

2. Materials and Methods

2.1 Preparation of the Aqueous Extract

The aqueous extract of straw used was *U. ruziziensis* (brachiaria). This was planted, dried and prepared in 2017 at the Campus of the Umuarama, Paraná, Brazil. The brachiaria used for the production of the extract was planted on 15 February 2017 in a dystrophic red latosol and harvested on 1 May 2017. After collection, the plants were placed in an oven at 65 °C and remained there for three days to achieve a quantity of stable dry matter which was then ground. This dry material (40 g) was mixed with 1 L of water and placed in an Erlenmeyer flask. The flask was placed in an orbital shaker at 200 rpm and at a temperature of 30 °C for at least 24 h. After that time, the solution was gassed to remove larger particles, dispensed into tubes for centrifugation at 3000 g for 15 min at 4 °C. The supernatant was distributed in round-bottom flasks for lyophilization. After complete drying, the extract was removed and packed in amber flasks. Quantities extract were weighed and diluted in distilled water in order to obtain concentrations corresponding to 250, 500 and 900 ppm.

2.2 Seed Germination and Growth

The seeds of *B. pilosa* L. were purchased from a commercial supplier (Cosmos Agrícola Produtos e Serviços Rurais Ltda, Brazil). Seeds were sterilised in a 1.0% sodium hypochlorite solution. After, seeds were placed on a double sheet of germination paper in plastic germination boxes (gerbox; 110 × 110 mm), moistened with 10 mL of distilled water or 10 mL of aqueous extract of *U. ruziziensis* prepared at concentrations of 250, 500 and 900 ppm. Four replicates were used for each treatment, each replicate consisting of 50 seeds distributed in a gerbox. Experiments were repeated four times. Boxes were placed in a growth chamber with a 12/12-h light/dark photoperiod and a temperature of 30 °C.

Seeds that had germinated at four days were counted and selected for growth tests. Seedlings were removed and the primary roots and hypocotyl excised for measurement of their length and fresh matter mass. The dry matter mass of these structures were obtained after seedlings were kept in an oven with a temperature of 65 ºC until reaching a constant mass. Data were expressed as centimeters or milligrams per root or hypocotyl.

In subsequent experiments, seedlings with up to four days of incubation were used, because after this period the first leaves appear showing that photosynthesis may be contributing to the energy metabolism of these seedlings.

The experiments done in this work want to study only the contribution of mitochondrial metabolism, without the presence of photosynthetic metabolism.

2.3 Respiration of Excised Primary Roots

After four days of incubation, the primary roots of *B. pilosa* seedlings were removed to verify oxygen consumption measured by a clark electrode polarograph (Bracht & Ishii-iwamoto, 2003). For each measurement, six root samples were cut into 5 cm segments, weighed and immediately placed in the oxygen electrode vessel with 2 mL nutrient solution (pH 5.8) containing 2 mM $Ca(NO_3)_2$, 2 mM KNO_3, 0.43 mM NH_4Cl, 0.75 mM $MgSO_4$ and 20 mM NaH_2PO_4 (Larkin, 1987). To estimate the contribution of mitochondrial cytochrome oxidase (COX) and alternative mitochondrial oxidase (AOX), in addition to extramitochondrial oxidases, 270 μM potassium cyanide (KCN) was injected into the reaction. Oxygen uptake was monitored for 15 min. Absorption rates were calculated from polarographic records based on an initial dissolved oxygen concentration of 240 μM at 25 °C (Estabrook, 1967) and in relation to fresh root weight.

2.4 Peroxidase Activity

After four days of incubation, *B. pilosa* primary roots or seedlings were removed to verify the presence of peroxidase activity. Primary roots or seedlings (approximately 0.2 g fresh weight) were weighed and transferred to a mortar where they were thoroughly mixed with 2.0 mL of a cold 67 mM K-phosphate (pH 7.0) solution containing 1% PVP. Extracts were centrifuged for 15 min at 3.000 rpm and 5 °C. The reaction was measured in a medium containing 25 mM K-phosphate (pH 6.8), 10 mM H_2O_2, 2.6 mM guaiacol, and 0.1-0.4 mg protein from the enzyme extract. Tetraguaicol formation (ε, 25.5 mM^{-1} cm^{-1}) was measured at 470 nm (Pütter, 1974).

2.5 Catalase Activity

After four days of incubation, *B. pilosa* seedlings were removed to verify the presence of catalase activity. Seedlings (approximately 0.2 g fresh weight) were weighed and transferred to a mortar and thoroughly mixed with 2.0 ml of a cold 67 mM K-phosphate (pH 7.0) solution containing 1% PVP. Extracts were centrifuged for 15 min at 3.000 rpm and 5 °C. The reaction was measured in a medium containing 67 mM K-phosphate (pH 7.0), 10 mM H_2O_2, and 0.1-0.4 mg protein from the enzyme extract. The consumption of H_2O_2 was monitored at 240 nm (ε, 0.036 mM^{-1} cm^{-1}) (Aebi, 1984).

2.6 Electron Microscopy Studies

After four days of incubation, *B. pilosa* primary roots were removed to ultrastructural analysis was performed using transmission electron microscopy (TEM). The primary roots were washed in 0.01 M phosphate-buffered saline and prefixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer.

Then, the primary roots were postfixed in a solution containing 1% osmium tethoxide in 0.1 M cacodylate buffer. Afterwards, they were washed in the same buffer and dehydrated in increasing concentration acetone and embedded in EPON resin.

For the ultrafine TEM sections they were stained with 5% uranyl acetate and lead citrate and examined using a JEOL JEM 1400 transmission electron microscope.

2.7 Statistical Analyses

The experimental design adopted was completely randomized. The results of the evaluations were subjected to analysis of variance-ANOVA by the 'F' test ($P \leq 0.05$), and the means were adjusted and submitted to the Dunnett test and regression models, and the equations were chosen based on the models. ($P \leq 0.05$) with biological logic and high R^2, using the SISVAR (Ferreira, 2014).

3. Results

3.1 Germination

The germination of *B. pilosa* seeds decreased in the presence of the aqueous extract of *U. ruziziensis* with all tested concentrations, as can be observed in Figure 1. However, the concentration of 500 ppm of the extract caused the lowest germination rate, 42% lower than in the controls.

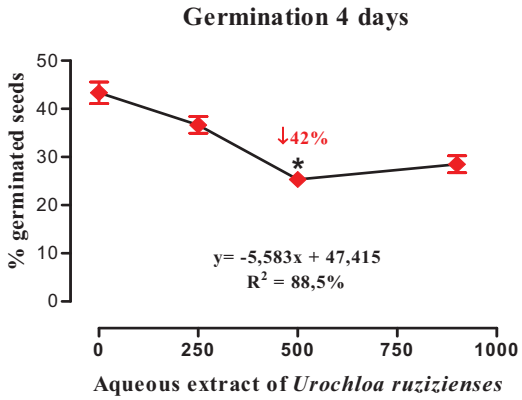

Figure 1. Germination of *Bidens pilosa*, on the effect of the aqueous extract of *Urochloa ruziziensis* in concentrations of 0, 250, 500 and 900 ppm, after four days of growth. * following by Dunnett's test

3.2 Root and Hypocotyl Development

With regard to the development of *B. pilosa* plants (Figures 2A-2F), the effect of the aqueous extract of *U. ruziziensis* was only evident for root growth and root dry matter mass. In the case of the variable root growth, as shown in Figure 2A, it is noted that the extract caused an increase in the root growth of *B. pilosa*. The increase in root growth was most accentuated with 500 ppm, which was 31% higher than the control root growth. Figure 2E shows that the dry matter mass of *B. pilosa* roots decreased by approximately 50% at all concentrations tested, despite the increase in root growth. This result can be explained by the image of the control root and roots grown in the presence of the extract in Figure 3, where rapid growth of the root caused by extract at 500 ppm resulted in thinner appearance. Thus, it is understood that the cells of the root, in an attempt to recover the damage caused by the extract, grew faster and this probably hindered the normal development of the tissue.

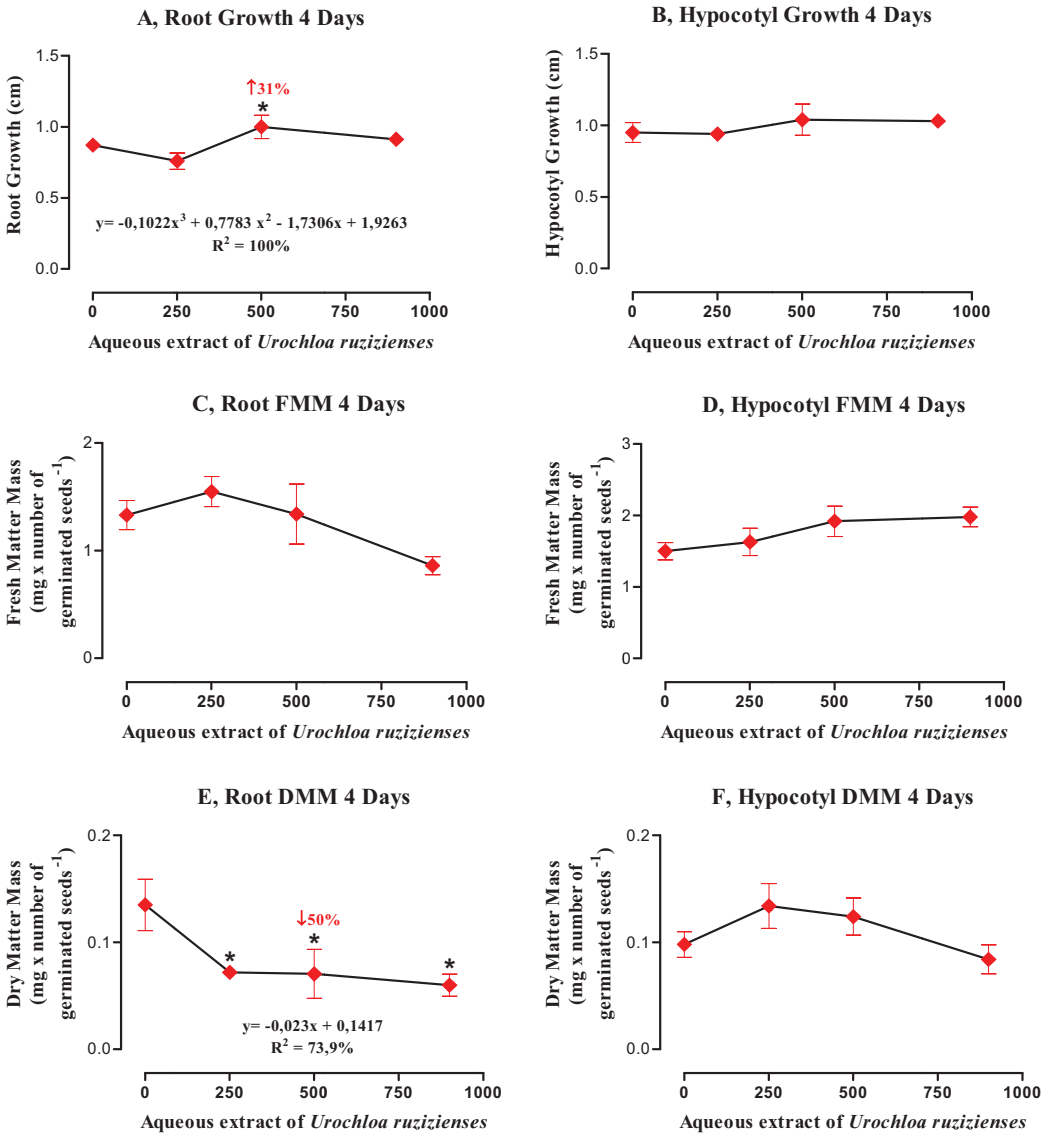

Figure 2. Primary root growth (A), hypocotyl growth (B), root fresh matter mass (C), hypocotyl fresh matter mass (D), root dry matter mass (E) and hypocotyls dry matter mass (F) of *Bidens pilosa*, on the effect of the aqueous extract of *Urochloa ruziziensis* in concentrations of 0, 250, 500 and 900 ppm, after four days of growth. * following by Dunnett's test

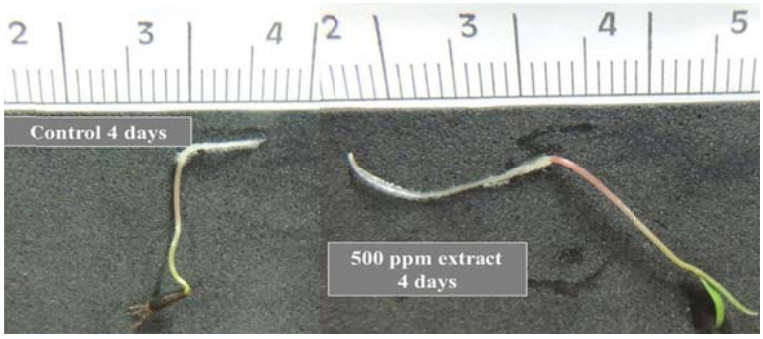

Figure 3. Images showing the morphological appearance of root of *B. pilosa* grown in the presence of water (control) and root grown in the presence of the aqueous extract of *Urochloa ruziziensis* in the concentration of 500 ppm, after four days of growth

3.3 Respiration Root

In Figure 4, it can be observed that the respiration in the roots of *B. pilosa* seedlings also suffered changes in the presence of the aqueous extract of *U. ruziziensis*. Total tissue respiration, which is the sum of respiration that is sensitive to KCN and insensitive to KCN, decreased significantly in a dose dependent manner; up to 35% and 44% inhibition occurred at concentrations of 500 and 900 ppm, respectively. The KCN-sensitive respiration, corresponding to cytochrome oxidase respiration of the mitochondria, showed the same behaviour as total respiration, except with a concentration of 500 ppm where KCN-sensitive respiration was higher than with other concentrations in relation to the control. The KCN-insensitive respiration, corresponding to the respiration involving alternative oxidase and extramitochondrial enzymes, also decreased in the presence of the extract. It was observed that at 500 ppm the lowest value for KCN-insensitive respiration occurred, reaching 68% inhibition and showing a great change in energy metabolism of *B. pilosa* seedling roots. Thus, in comparing the values for total respiration, sensitive and insensitive to KCN, we can state that the respiration that predominates in *B. pilosa* roots during this period of growth is almost exclusively mitochondrial via cytochrome oxidase, because the KCN-insensitive represented only 22% of the total respiration.

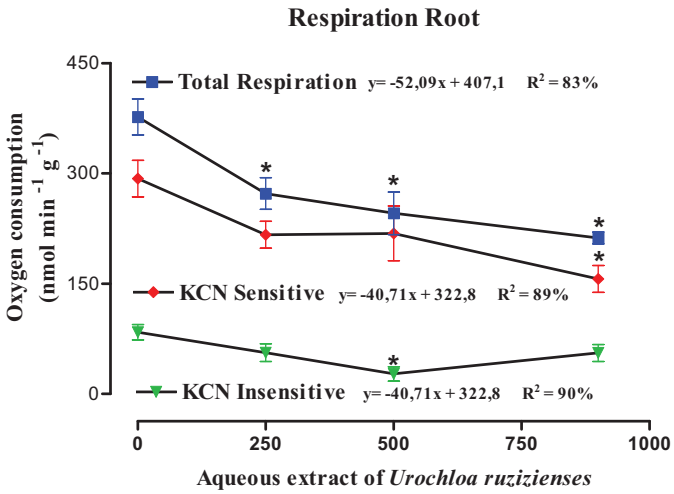

Figure 4. Respiration activity primary roots of *Bidens pilosa* on the effect of the aqueous extract of *Urochloa ruziziensis* in concentrations of 0, 250, 500 and 900 ppm, after four days of growth. Total respiration: oxygen consumption in the absence of KCN; KCN Insensitive: oxygen consumption in the presence of KCN; KCN Sensitive: difference between total respiration and KCN Insensitive. * following by Dunnett's test

3.4 Antioxidant Enzymes

Figure 5 shows effects on antioxidant enzymes of *B. pilosa* seedlings, such as peroxidase (POD) and catalase (CAT). The activity of POD (Figure 5A) in the seedlings that were grown in the presence of the aqueous extract of *U. ruziziensis* did not present a significant difference in relation to the control. Due to this, the determination of POD activity in *B. pilosa* seedling roots was performed again only with the 500 ppm concentration. Thus, it is shown in Figure 5B that extract at a concentration of 500 ppm caused a 68% increase in POD activity in relation to the control. These results are consistent with the results of CAT activity in *B. pilosa* seedlings, in that, as shown in Figure 5C the greatest effect of this enzyme also occurred at the concentration of 500 ppm, with a 36% increase in the activity of this enzyme in relation to control.

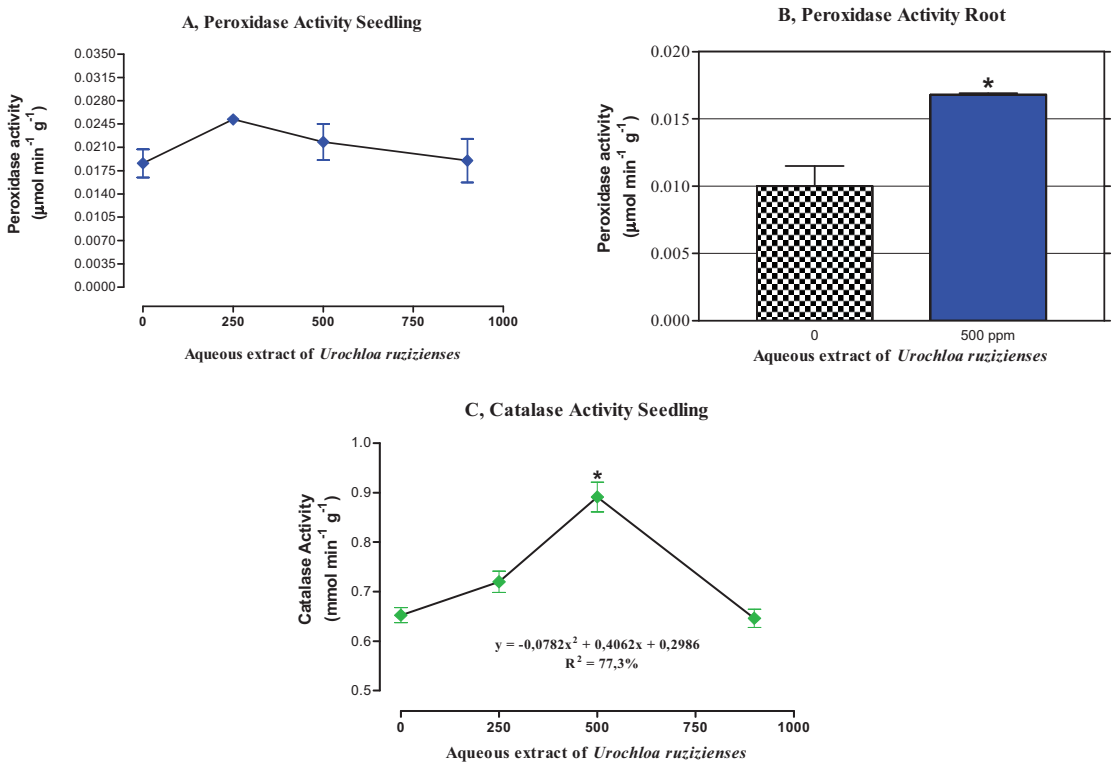

Figure 5. Peroxidase Activity seedling (A), Peroxidase Activity root (B) and Catalase activity seedling (C) of *Bidens pilosa* on the effect of the aqueous extract of *Urochloa ruziziensis* in concentrations of 0, 250, 500 and 900 ppm, after four days of growth. * significant differences according to ANOVA with Dunnett's multiple range test at 5% level significance

3.5 Images of Electron Microscopy

Figure 6a and b show the images obtained by TEM of *B. pilosa* seedlings grown in the presence of water (control). These images show normal cells during development which corresponded to four days of growth following germination. The cytoplasm contained sufficient reserves and showed vacuoles in formation, grains of amyloplasts and the presence of mitochondria. The TEM images of roots grown in the presence of the extract at a 500ppm concentration, presented differences compared to the control images (Figure 6c and d). The cells are modified in that they appear to be more elongated. The cytoplasm contained large vacuoles that push the organelles to the periphery of the cell. Also, a greater number of mitochondria is observed. In Fig. 5e and f, which shows images of *B. pilosa* roots grown in the presence of the aqueous extract at a concentration of 900 ppm, no changes were observed in relation to the control images.

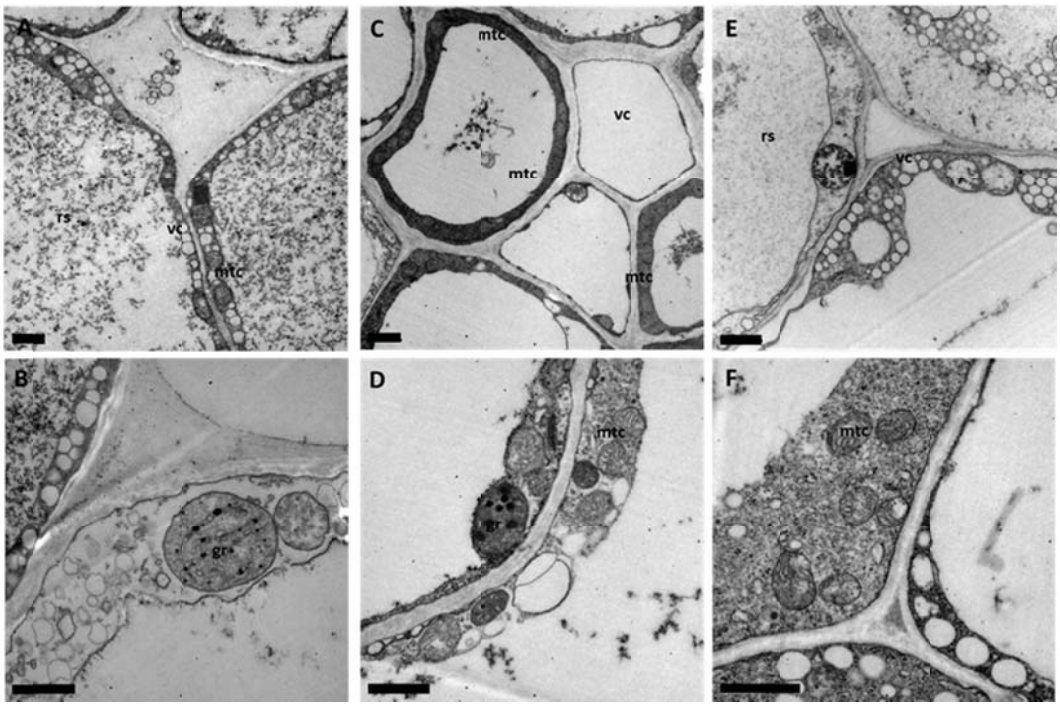

Figure 6. Electron microscopy photomicrographs of roots control (a, b) and treated at the aqueous extract of *Urochloa ruziziensis* in concentration of 500 ppm (c, d) and 900ppm (e, f) of *Bidens pilosa* on the for four days. a, c and e: transmission electron microscopy of root showing vacuoles (vc), mitochondria (mtc) and cell reservations (rs); b, d and f: transmission electron microscopy of root showing starch granules (gr) and mitochondria (mtc). Bars = 1 um

4. Discussion

In stress situations, there is an increase in the activity of antioxidant enzymes, such as peroxidase, which neutralise reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), as a mechanism to avoid further cell damage (Lima et al., 2002).

There are some studies that also study the antioxidant defense system of weeds, such as *B. pilosa*, during the early phase of growth, but no work so far has studied the defense system of this plant when submitted to the aqueous extract of *U. ruziziensis*, which makes understanding and comparison difficult.

According to Pergo and Ishii-Iwamoto (2011), and Pergo-Coelho et al. (2017), the increase in the activity of antioxidant enzymes suggests an increase in ROS generation, indicating oxidative stress caused by allelochemicals, which makes seeds and seedlings more vulnerable to dysfunction and cell death.

This stress caused by the extract was very evident in the germination that decreased, probably due to an inhibition or a delay in the germination process.

Therefore, the stress demonstrated in this study by increased POD and CAT activity with 500 ppm of the extract is probably due to activation of root growth and elongation (Figure 3). The plant probably needed more energy than normal, compromising the mitochondrial respiration, with increasing ROS and activating the defense system of antioxidant enzymes.

However, there was a cellular alteration and that this change is mainly in the energetic metabolism of the cell, so that the KCN-insensitive respiration decreased as much, about 68% in the concentration of 500 ppm of the extract, because this represents a parallel path to the consumption of oxygen by mitochondria without the production of ATP. This again shows an effect of oxidative stress since other enzymes that help neutralize ROS and are also part of the KCN-insensitive respiration have possibly been inhibited by the extract used.

According to Foletto et al., 2012, the waters soluble compounds of *U. ruziziensis* were phytotoxic to *I. triloba,* inducing perturbations in respiratory activity and lipid peroxidation. Although trans-aconitic acid exerted similar effects to the aqueous fraction, it is not the main compound responsible for the effects of aqueous fraction in *I. triloba*, because its content is very little in this fraction.

According to Nepomuceno et al. (2017) studied the extracts collected from *U. ruziziensis* contained substances such as protodioscin and triterpenoid saponins, which can control the growth of weeds.

Protodioscin (Giancotti et al., 2015) is a bidesmosidic saponin formed by a hydrophobic moiety of the furostanol type and two sugar fragments. The structural characteristics of these compounds mean that they are readily soluble in water and, therefore, easy to be absorbed by the plant root, as shown in this work, which was the tissue most affected by the aqueous extract of *U. ruziziensis*.

It is emphasized that the experimental results obtained in the laboratory or in greenhouse are difficult to be extrapolated under field conditions, since the allelochemicals derived from the secondary metabolism of plants that are released into the environment and are transformed by the action of biotic factors (soil microflora and exudates from the roots of other competitors) and abiotic (variation of soil temperature and humidity) in order to activate or inactivate them as agents of biological control (Reigosa et al., 2013; Dayan et al., 2009; Duke, 2015).

The results are laboratory experiment, the effect of the extracts need be verified further in field test with soil media. The active compounds in extracts could be absorbed, detained, transformed and degraded in soil media, thus be weaken its bioactivity, especially the 500 ppm level in laboratory bioassay.

5. Conclusion

It can be concluded that allelochemicals present in extract from *U. ruziziensis* have the potential to provoke oxidative stress in *B. pilosa* seedlings in laboratory, mainly at a concentration of 500 ppm. This oxidative stress caused alterations mainly in the energetic metabolism of this plant, being this a primordial factor for its growth and survival, mainly in this initial stage of development, where there is still no contribution of photosynthesis.

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Postharvest Quality of Yellow Pear Tomato Cultivated in Aquaponic System

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Abstract

The production of vegetables in aquaponic systems has high sustainability and conservation of natural resources, but studies that make their cultivation feasible under Brazilian conditions are still incipient. Given the influence that the cultivation system can cause on the postharvest characteristics of fruits and vegetables, this study aimed to evaluate the postharvest quality of yellow pear tomato cultivated in an aquaponic system. Tomato plants were grown in a protected environment, in the experimental area of aquaponics of the Faculty of Agrarian Sciences, belonging to the Federal University of Grande Dourados. The experimental design was completely randomized in a factorial scheme, with two factors: maturity stage at harvest and storage time. Three stages of fruit maturity (green, intermediate and ripe) and two storage times (zero and 35 days) were analyzed. Tomato samples were subjected to the analyses using the whole fruit, evaluated for mass loss, color and firmness, and the fruit pulp, evaluated for soluble solids (SS), titratable acidity (TA), SS/TA ratio, pH and lycopene content. The fruits of yellow pear tomato cultivated in aquaponic system were in satisfactory conditions with respect to the postharvest parameters evaluated, indicating great potential to be cultivated on a commercial scale under Brazilian conditions. The parameters analyzed were similar or superior to the parameters of tomatoes grown in other cropping systems.

Keywords: maturity stages, mass loss, *Solanum lycopersicum*, storage time

1. Introduction

The aquaponic system can be defined as a set of technologies that integrate the plant cultivation with fish farming in a symbiotic production system. Fish farming wastes are used as fertilizers for plant production and plants contribute to the removal of metabolic substances, which may be harmful to fish development, acting in the conditioning of water quality for fish farming (Goddek et al., 2016; Hundley et al., 2013; Roosta & Afsharipoor, 2012).

Vegetable cultivation in aquaponic system is a very widespread technique in several countries of the world, but in Brazil there are no reports about aquaponic cultivation on a commercial scale. However, studies have been conducted in an attempt to provide information that contributes to the implementation of this cultivation technique (Geisenhoff, Jordan, Santos, Oliveira, & Gomes, 2016; Jordan, Ribeiro, Oliveira, Geisenhoff, & Martins, 2018).

In order to contribute with the required information to the implementation of the aquaponic system, on a commercial scale, under Brazilian conditions, it is necessary to conduct studies on the postharvest quality of the vegetables produced in aquaponic system, since the cultivation system and postharvest handling conditions can directly influence the qualitative parameters of the fruits, especially the climacteric ones, such as tomato (Ceglie, Amodio, & Colelli, 2016; Khadka, Marasini, Rawal, Gautam, & Acedo, 2017; Rocha, Ribeiro, & Silva, 2018).

Tomato is a perishable fruit and, during the postharvest period, its qualitative and nutritional characteristics are susceptible to major alterations. Harvested fruits are subject to mass reduction (Oliveira, Coneglian, & Carmo, 2015), alterations of color (Andreuccetti, Ferreira, Moretti, & Honório, 2007), alterations in physical and chemical

composition (Ferreira et al., 2010), among others. Given the influence that the cultivation system can cause on the postharvest characteristics of tomato, this study aimed to evaluate the postharvest quality of yellow pear tomato cultivated in aquaponic system.

2. Material and Methods

Tomato plants were grown in a protected environment, in the experimental area of aquaponics of the Faculty of Agrarian Sciences (FCA), belonging to the Federal University of Grande Dourados (UFGD), located in Dourados, Mato Grosso do Sul, Brazil (22°11′45″ S, 54°55′8″ W and 446 m of altitude).

The aquaponic system used consisted of six fish-farming tanks connected in series with a plant bed. Fish farming wastewater was treated by means of decanters and biological filters. The nutrient solution used for plant cultivation consisted of a mixture of wastewater (liquid fraction from the decanter) and biofertilizer (solid fraction from the decanter) in a volumetric proportion of 100:6. Fish population density was 100 fish m^{-3}. The fish species used was tilapia (*Oreochromis niloticus*), GIFT strain. Details on the conduction of the aquaponic system can be seen in the works of Geisenhoff, Jordan, Santos, Oliveira, and Gomes (2016) and Jordan, Ribeiro, Oliveira, Geisenhoff, and Martins (2018).

Yellow pear tomato plants were cultivated on a floating raft, which was 12 m long and 1.2 m wide, at density of 6 plants m^{-2}.

The experimental design was completely randomized in a factorial scheme, with two factors: maturity stage at harvest and storage time. Three maturity stages (green, intermediate and ripe) and two storage times (zero and 35 days) were analyzed. Normality (Shapiro-Wilk test) and equality of variances (Levene's test) were analyzed at 5% significance level. For data that did not meet the assumptions of normality and equality of variance, Box-Cox transformation was used. Subsequently, the analysis of variance was carried out with F test ($p \leq 0.05$). Data of mass loss and color were evaluated using descriptive analysis, presenting the mean values obtained with their respective standard deviations. In addition, when significant, regression analysis relating the response variable as a function of the variation in storage time was performed.

After harvest, the fruits were properly sanitized with running water and dried with paper towels in the Laboratory of Physical Properties of Agricultural Products of UFGD. The tomatoes were classified into three maturity stages, according to their color in three groups: green (Stage I), intermediate (Stage II) and ripe (Stage III) (Figure 1).

Stage I Stage II Stage III

Figure 1. Classification of yellow pear tomato fruits produced in an aquaponic system and harvested at three maturity stages

Subsequently, samples of approximately 50 g were taken and placed in polyethylene terephthalate (PET) plastic packages. These samples were subjected to ambient storage conditions, simulating the shelf conditions found in the retail market.

Fruit storage began on May 24, 2017 and ended on June 26, 2017. The average air temperature ranged from 18.5 to 27.3 ºC and the relative humidity ranged from 48.7 to 91.7% (Figure 2).

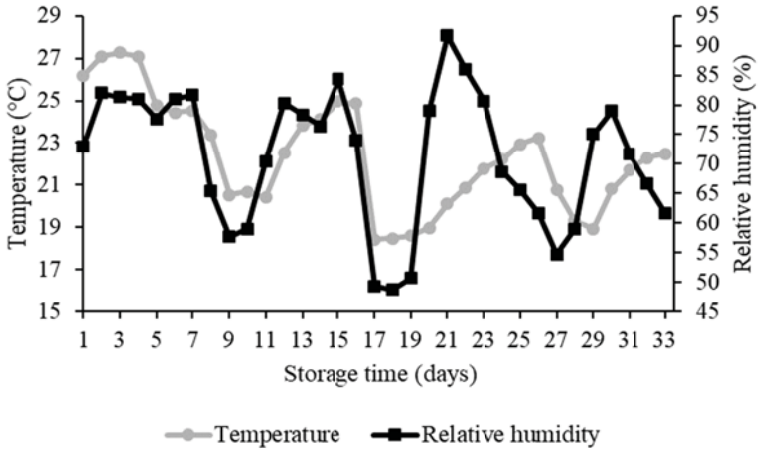

Figure 2. Average values of air temperature (ºC) and relative humidity (%) during the period of storage of yellow pear tomato fruits

Tomato samples were subjected to analyses using the whole fruit, evaluated for mass loss, color and firmness, and fruit pulp, evaluated for the content of soluble solids (SS), titratable acidity (TA), SS/TA ratio, pH and lycopene content.

2.1 Quality Analyses Using Whole Fruit

The loss of fresh mass (%) was determined based on the initial and final masses obtained daily in the measurements. Fruit color was determined by direct reading in colorimeter (Konica Minolta CR400 with CIELAB color space and illuminant D65), expressing the color variables L*, a* and b*, which correspond to the values of lightness, green-red and blue-yellow, respectively. The parameters hue angle (H*) and chroma (C*) of the fruits were determined according to Equations 1 and 2, respectively.

$$H^* = \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (1)$$

Where, H* = hue angle or chromatic tone (degrees); a* = red-green color component; and, b* = yellow-blue color component.

$$C^* = \sqrt{(a^*)^2 + (b^*)^2} \quad (2)$$

Where, C* = chroma (dimensionless); a* = red-green color component; and, b* = yellow-blue color component.

Firmness of tomato fruits was evaluated with a Texture Analyzer (TA-HDi 25 kg), individually measured in each fruit. The parameters for this analysis were pre-test, test and post-test speeds of 1.00 mm s^{-1}, 2.00 mm s^{-1} and 5.00 mm s^{-1}, respectively. Probe penetration distance was determined based on the average fruit diameter (30 mm), measured with a digital caliper, being equal to 6 mm or 20% of this value. The probe used had diameter of 45 mm according to the device's manual and is indicated for grapefruits, whose shape is the closest one to that of a yellow pear tomato.

2.2 Quality Analyses Using Fruit Pulp

Initially, the concentrated pulp of the fruits was obtained using a mixer homogenizer. After that, direct readings of soluble solids (SS), expressed in °Brix, were taken in a small sample of the pulp using a digital refractometer (MEGABRIX®) (AOAC, 2000).

Titratable acidity (TA) was obtained by volumetric titration method with indicator. 5 g of pulp were placed in a 125-mL Erlenmeyer flask, which received 2 drops of 1% alcoholic phenolphthalein. The pulp was titrated up to the turning point (pH = 8.2), under agitation, with a 1.0 mol L^{-1} NaOH solution. The results correspond to the relationship between grams of NaOH solution per 100 g of pulp (g NaOH 100 g^{-1}), expressed as % (AOAC, 2000)

Then, the SS/TA ratio was calculated as the ratio between the values of SS and TA. In addition, pH readings were also performed, using a portable digital pH meter. These readings were taken directly in the fruit pulp.

Lycopene concentration in fruits was determined by spectrophotometric analysis, using 5-g pulp samples. Each sample received 40 mL of acetone. The mixture was stirred for 1 h using an orbital shaker table at 200 rpm and filtered using filter paper, and 20 mL of acetone were added again to the filtrate for extraction. 40 mL of petroleum

ether and distilled water, together with a separation funnel, were placed in the 80 mL of solution obtained after extractions. The lower part of the filter was discarded because it contained only acetone with distilled water. The upper part of the funnel contained the ether with pigment, which were transferred to a 50-mL volumetric flask. The reading in a spectrophotometer (Micronal B495) was taken at 470 nm wavelength. Lycopene content was obtained according to Equation 3.

$$\mu g/g = \frac{AV\ 1.000.000}{A^{1\%}_{1cm}\ M\ 100} \quad (3)$$

Where, µg/g = lycopene content (µg g^{-1}); A = absorbance of the solution at 470 nm wavelength; V = final volume of the solution; $A^{1\%}_{1cm}$ = is the extinction coefficient or molar absorptivity coefficient of a pigment in a specific solvent, equal to 3450 for lycopene in petroleum ether; and, M = mass of the sample taken for analysis (g).

3. Results and Discussion

Regarding the loss of fresh mass, LFM (%), all maturity stages showed the same trend, increase of LFM as a function of the evaluation time. Stage III, after 35 days of storage, showed a higher mean reduction (3.25%), but the LFM values for the stages II and I were similar (2.96 and 3.12%, respectively) (Figure 3A).

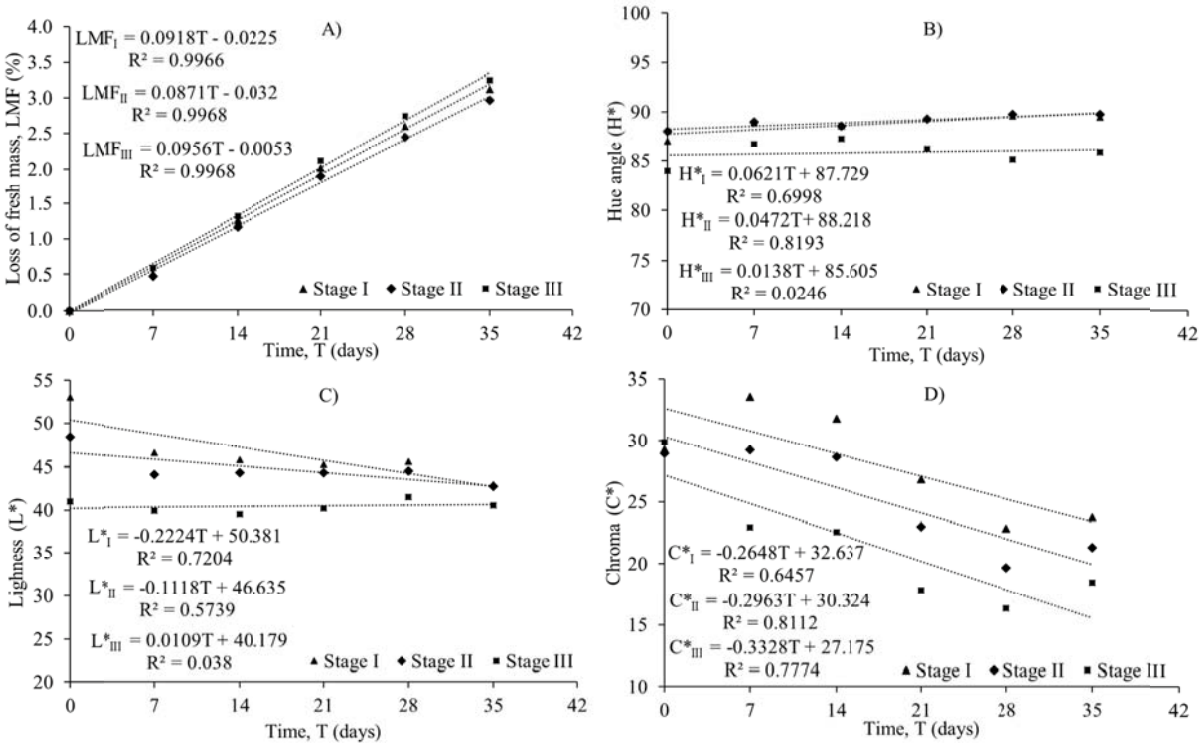

Figure 3. Loss of fresh mass, LFM (%)—(A); Hue angle (H*)—(B); Lightness (L*)—(C); and Chroma (C*)—(D) of yellow pear tomato fruits harvested at three maturity stages and stored under ambient conditions for 35 days

Regardless of the stage at which the fruits were harvested, it was observed that LFM was small in comparison to the storage time. Studies report higher losses than those found in the present study, such as Oliveira, Coneglian, and Carmo (2015), who evaluated the postharvest conservation of cherry tomatoes and observed LFM of 7% at 24 days of storage, and Modolon, Boff, Rosa, Sousa, and Miquelluti (2012), who analyzed the postharvest quality of tomatoes and observed LFM of 6% at 14 days of storage. In the present study, at 24 and 14 days of evaluation, considering maturity stage III, the LFM values were 2.29 and 1.33%, respectively. Thus, it is observed that the fruits had lower LFM compared to those reported in the literature.

Regarding the visual aspect, after 35 days of storage, under ambient conditions, the fruits remained with the characteristics desired by the consumer, with good visual appearance, firm and with no damage caused by microorganisms (Figure 4). Thus, it indicates the potential of this fruit with respect to maintaining its quality in the postharvest phase.

Figure 4. Visual appearance of yellow pear tomato fruits harvested at three maturity stages after 35 days of storage under ambient conditions

In relation to the color angle (H*), the variation of H* was higher in the first evaluation (zero storage time), demonstrating the predominance of the component a* with negative values (greener and less red) and b* with positive values (more yellow and less blue) (Figure 3B).

From the seven days of storage, the difference between the H* values decreases. All tomatoes began to show color coordinates positioned in the quadrant of positive values a* and b*, *i.e.*, predominance of yellow in the color composition. Cherry tomato maturity can be analyzed based on color (Pereira, León, Hernández, & Gonzáçez, 2012).

In general, maturation begins with fruits showing shades of green, changing to reddish and completing their maturation with yellowish color, as reported in the present study.

The coordinate L* in the CIELAB color space varies from 0 (absolute black) to 100 (absolute white). The tomatoes harvested at stages I and II showed a tendency to darkening as a function of the storage time. Tomatoes harvested at stage III maintained lightness, but were darker than tomatoes harvested at the other stages (Figure 3C).

During maturation, tomato fruits have a high synthesis of carotenoids, associated with color changes. Initially, the synthesis of the phytoene (colorless) occurs. Subsequently, the ζ-carotene (dull yellow), lycopene (red), β-carotene (orange), xanthophylls and hydroxyl carotenoids (yellow) are synthesized (Pereira et al., 2012).

Reduction in the parameter lightness as a function of storage time was also observed by Camelo & Gómez (2004). L* values, at stages I and II, indicate that the fruits were harvested before the beginning of the synthesis of red and orange pigments (lycopene). At stage III, the synthesis was already complete, so the color was maintained during the 35 days of storage.

Chroma distinguishes a strong color from a weak color, expressing the intensity of the perception of fruit color. Fruits harvested at stage I had higher values of chroma, indicating greater perception of color. The values of the component a*, as a function of time, changed from more positive values (green) to more negative values (red) (Figure 3D).

Tomatoes harvested at stages I, II and III, after 35 days of storage under ambient conditions, showed chroma reductions of 23, 40 and 61%, respectively. Thus, tomatoes harvested at stage I maintained their color intensity for longer, tending to remain yellow. Reduction of chroma as a function of storage time in cherry tomato cultivation was also observed by Pereira, León, Hernández, and Gonzáçez (2012).

This parameter can be used to assist in the selection of the best stage for fruit harvesting, especially in this case, in which there is a lack of information for aquaponic cultivation under national conditions (Jordan, Geisenhoff, Oliveira, Santos, & Martins, 2018).

In relation to the contents of soluble solids, SS (°Brix), there were significant effects of maturity stage (Figure 5A) and storage time (Figure 5B), with no interaction between them ($p \leq 0.05$). Tomatoes harvested at stage III had higher values SS (4.73 °Brix) compared to those harvested at stage I (4.38 °Brix).

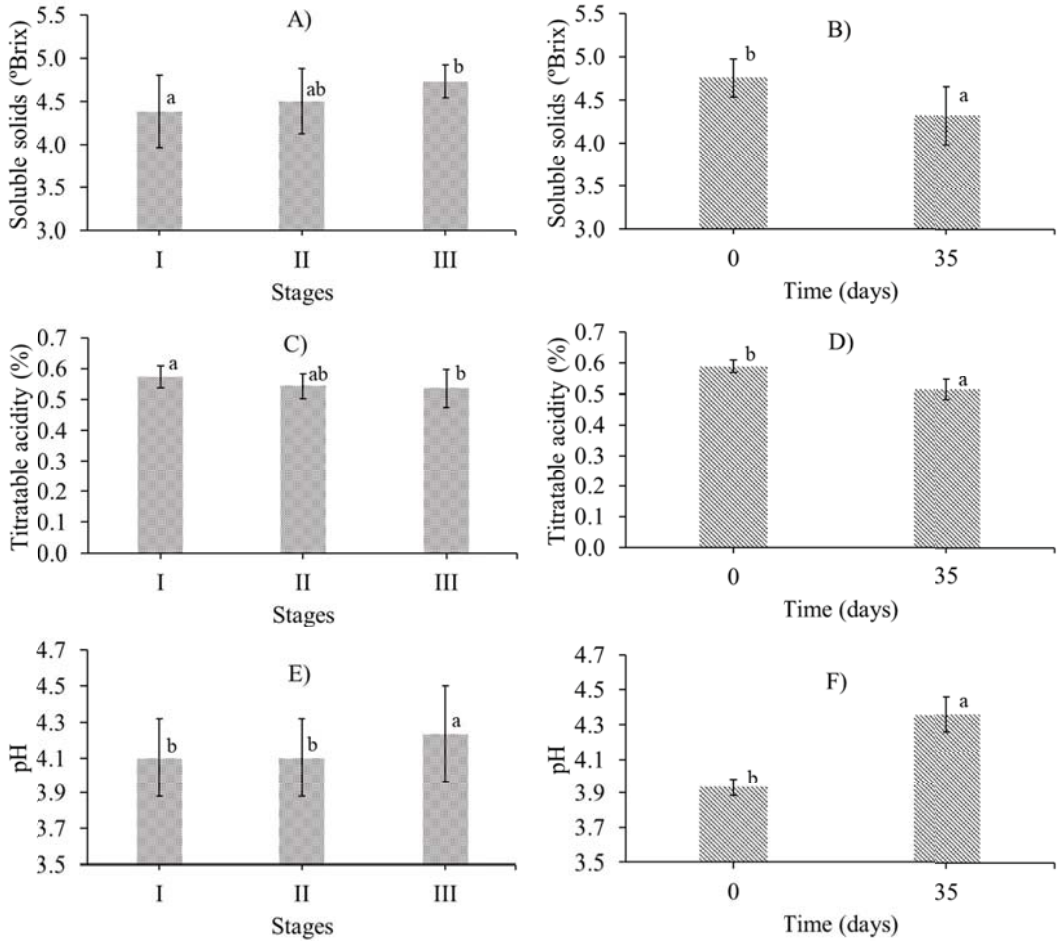

Figure 5. Mean values of soluble solids, SS (°Brix); titratable acidity, TA (%); and, pH of yellow pear tomato fruits as a function of three maturity stages at harvest and as a function of storage time under ambient conditions

Regarding storage time, there was a reduction in SS content. After 35 days of natural storage, the fruits had a 10% reduction in the SS content, from 4.76 °Brix at the beginning of the storage period to 4.32 °Brix at the end of the period.

Similar results were observed by Modolon et al. (2012), analyzing the postharvest quality of tomato fruits subjected to different dilutions, with mean value of 4.11 °Brix. The contents of soluble solids are related to the flavor of tomato fruits and, as observed in the results, the values presented in this study were similar to those obtained in tomatoes cultivated in conventional systems, demonstrating the possibility of production in an aquaponic system with no depreciation in fruit flavor.

For the parameter titratable acidity (TA), there were significant effects of maturity stage (Figure 5C) and storage time (Figure 5D), with no interaction between them ($p \leq 0.05$). There was a reduction of 6.8% in TA when the maturity stage I is compared to the stage III. TA also decreased as a function of storage time, from 0.58%, at the beginning of the evaluations, to 0.51%, after 35 days of storage under ambient conditions. Similar results were observed by Ferreira et al. (2010), evaluating the postharvest quality of table tomato in two cropping systems, conventional and organic. These authors observed reductions in titratable acidity around 0.20% for tomatoes from conventional cultivation and 0.21% for tomatoes from organic cultivation.

Reduction of TA may result from respiratory processes and/or conversion of organic acids into sugars (Ferreira et al., 2010; Wills & Ku, 2002).

Thus, it can be observed that the tomatoes from the aquaponic cultivation, even at maturity stage I, have higher contents of organic sugars compared to the contents observed in organic and conventional crops.

The factors maturity stage (Figure 5E) and storage time (Figure 5F) had significant effect on tomato pulp pH, with no interaction between them ($p \leq 0.05$).

Values of pH in tomato fruits may vary according to the cultivation system; however, in general, acidic pH values are desirable, since they reduce the proliferation of microorganisms, which can increase fruit longevity (Modolon et al., 2012; Ramos et al., 2013). In the present study, pH values ranged from 4.1 to 4.2 (in relation to the maturity stage) and 3.9 to 4.3 (in relation to storage time). Thus, with respect to the parameter pH, tomatoes from aquaponic cultivation also have potential to be marketed in a similar way to fruits from other cultivation systems.

For the SS/TA ratio, there was significant effect of maturity stage and not significant effect of storage time. The interaction between these factors was significant ($p \leq 0.05$). Table 1 shows the mean values of SS/TA ratio, lycopene content and firmness of yellow pear tomato fruits as a function of three maturity stages and of storage time under ambient conditions.

Table 1. Mean values for SS/TA ratio, lycopene content and firmness of yellow pear tomato fruits as a function of three maturity stages at harvest and of storage time under ambient conditions

Stage	Time (days)		Mean
	0	35	
SS/TA ratio			
I	7.84 ± 0.13^{aA}	7.42 ± 0.29^{bA}	7.63
II	8.35 ± 0.33^{aB}	8.43 ± 0.53^{aA}	8.39
III	8.05 ± 0.25^{aB}	9.55 ± 0.69^{aA}	8.80
Mean	8.08	8.47	-
Lycopene content (μg g^{-1})			
I	13.68 ± 0.00^{cA}	14.13 ± 0.25^{cA}	13.903
II	11.36 ± 0.00^{bB}	14.72 ± 0.41^{bA}	13.042
III	14.88 ± 0.00^{aB}	17.14 ± 0.16^{aA}	16.002
Mean	13.303	15.328	-
Firmness (N)			
I	22.46 ± 1.26^{aA}	17.94 ± 1.27^{aB}	20.204
II	18.92 ± 1.79^{bA}	16.26 ± 0.62^{aA}	17.592
III	16.70 ± 0.37^{bA}	16.61 ± 1.50^{aA}	16.657
Mean	19.364	16.938	-

Note. Mean value±standard deviation. Equal letters, uppercase in the row and lowercase in the column, correspond to equal means by Tukey test at 5% significance level.

The SS/TA ratio is responsible for the characteristic flavor of the fruits, and high values of this ratio indicate an optimal combination of sugars and acids that are correlated with the soft flavor of the fruits (Ramos et al., 2013). It is observed that tomatoes harvested at stages II and III showed significant increment after 35 days of storage under ambient conditions, with the highest increment obtained at the maturity stage III (18.6%).

Lycopene content was influenced by the factors maturity stage, storage time and the interaction between them ($p \leq 0.05$). Lycopene contents increased as a function of storage time and maturity stage. The highest value observed was 17.14 μg g^{-1}, after 35 days of storage, in fruits at maturity stage III. The lowest lycopene content was observed at the beginning of the storage period, in fruits at maturity stage II (Table 1).

The unique chemical structure of lycopene confers a remarkable antioxidant action, contributing to the prevention of degenerative diseases, cardiovascular diseases and certain types of cancer. In addition, it gives desirable appearance to the fruits, since it increases their dark reddish pigmentation. Thus, high lycopene contents in the fruits are desirable, both for fresh commercialization and for industrial processing (Carvalho, Fonseca, Silva, Boiteux, & Giordano, 2005; Marodin et al., 2016). The contents obtained in the present study were satisfactory, even at the initial maturity stages. The values obtained for lycopene content demonstrate the potential of yellow pear tomato production in aquaponic systems.

For the parameter fruit firmness, there was a significant effect of the factors maturity stage, storage time and the interaction between them ($p \leq 0.05$). Fruit firmness decreased as a function of storage time and maturity stage.

The condition with highest firmness was observed on the first day of storage, in fruits at stage I (22.46±1.26 N) (Table 1).

The results observed in the present study were higher than those reported by Bernardi et al. (2007), evaluating the production and quality of tomato fruits grown in a substrate with zeolite. These authors found values of firmness varying from 7.06 to 14.38 N. Marodin et al. (2016) analyzed the postharvest characteristics of tomato as a function of doses of silicate fertilizers and obtained firmness values from 9.5 to 10.6 N. Softening or loss of firmness in the pulp results from the solubilization of pectic substances from the cell wall through the action of pectin methylesterase (PME) and polygalacturonase (PG), whose activities are increased at the beginning of ripening and in the senescence, especially PG, which peaks at the ripe stage (Ferreira et al., 2010).

Firmness is an important attribute in the postharvest evaluations of tomato fruits, since it is related to the maintenance of other qualitative parameters during their transport and commercialization (Bernardi et al., 2007). Thus, it can be observed that fruits from the aquaponic production showed satisfactory results. These results can be attributed to the adequate nutrition provided for plants during their development, so that, in general, the higher the fertilizer dose added during plant development, the greater firmness of tomato fruits, especially fertilizers with high levels of phosphorus and silicates (Bernardi et al., 2007; Marodin et al., 2016).

4. Conclusions

This reserch aimed to contribuite with information that may enable the aquaponic production of yellow pear tomatoes in commercial scale in Brazil. For this, the post-harvest quality of fruits from the aquaponic system production was evaluated. It was observed that fruits grown in aquaponic system maintained their postharvest quality, based on the parameters evaluated, indicating great potential to be cultivated on a commercial scale under Brazilian conditions. After 35 days under ambient conditions, tomatoes harvested at different maturity stages maintained satisfactory physicochemical characteristics. The parameters analyzed were similar or superior to the parameters of tomatoes grown in other cropping systems. These results provide information to increase the technique of aquaponic production in Brazil.

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First Record of *Ectomyelois muriscis* (Dyar, 1914) (Lepidoptera: Pyralidae) on Jatoba *Hymenaea stigonocarpa* (Fabaceae) Fruits in Brazil

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Abstract

The jatoba, *Hymenaea stigonocarpa* Mart. Ex Hayne (Fabaceae), is a tree species native to the Brazilian Cerrado and is recommended for recovering degraded areas. The fruit of this species is used for food and traditional medicine purposes, and its propagation occurs occurs through seeds. This study aimed to record, for the first time in Brazil, the occurrence of an insect pest on jatoba fruits. We sampled 58 *H. stigonocarpa* fruits from September to October 2017, on a typical Brazilian Savanna area in the municipality of Monte Carmelo, Minas Gerais (18°50′26″ S and 47°19′04″ W). The emergence of nine adults belonging to the *Ectomyelois muriscis* (Dyar, 1914) (Lepidoptera: Pyralidae) species was recorded on four jatoba fruits (6.9%). The insect only occurred on injuried fruits. The larva was fed from the pulp of the fruit until reaching the pupa stage, but not consuming the seeds. This is the first record of *E. muriscis* on jatoba fruits in Brazil.

Keywords: Brazilian Cerrado, forest entomology, injury, microlepidoptera

1. Introduction

The jatoba *Hymenaea stigonocarpa* Mart. ex Hayne is a leguminous tree species belonging to the Fabaceae family, native to the Cerrado Biome, occurring naturally in Brazil, in the states of Bahia, Ceará, Goiás, Maranhão, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Pernambuco, Piauí, Rio Grande do Norte, São Paulo and the Federal District. Its propagation occurs through seeds, and the dispersion of fruits and seeds is essentially zoocorical, by avifauna. The species is recommended for recovering degraded areas, and its fruit is used for food and traditional medicines (Carvalho, 2007).

In this sense, studies were developed to evaluate the dormancy of *H. stigonocarpa*, as a strategy for forest restoration of tropical pastures (Pereira, Laura, & Souza, 2013), to test the survival of seedlings in the recovery of former mining areas (Silva & Corrêa, 2008), as well as in the restoration of disturbed area in an urban environment (Oliveira, 2006).

A flour with a high content of total dietary fiber is obtained from the fruit of the jatobá used as a replacement for wheat flour, in the manufacturing of biscuits, breads and porridge (D. B. Silva, J. A. Silva, Junqueira, & Andrade, 2001). The jatobá plant has medicinal qualities and is often used as a depurative, anti inflammatory, appetite stimulant and iron rich fortifier (Souza & Felfili, 2006).

Surveys carried out in different Brazilian biomes with leguminous tree species highlight the predation of seeds and fruits by bean weevils/seed beetles (Coleoptera: Chrysomelidae) (Santos, J. A. S. Costa, C. B. N. Costa, & Calado, 2015; Mojena & Barreto, 2016) *Cryptophlebia carpophagoides* Clarke, 1951 (Lepidoptera: Olethreutidae) (Wink, Guedes, Murari, & Pelentir, 2007), microlepidoptera (Lepidoptera: Tortricidae, Olethreutinae) (Boscardin, Costa, Garlet, & Oliveira, 2012), *Agathodes designalis* (Guenée, 1854), *Liopasia ochracealis* (Walker, 1865) and *Terastia meticulosalis* (Guenée, 1854) (Lepidoptera: Crambidae) (Pereira & Silva, 2013; Pedron et al., 2015).

In this sense, planting legumes such as *H. stigonocarpa* can be compromised by insect attacks on its fruits and seeds, which compromises its propagation. Thus, the present work aimed to record, for the first time in Brazil, the occurrence of an insect pest on jatoba fruits in an area of the Cerrado Biome.

2. Method

For this purpose, *H. stigonocarpa* fruits were harvested on a typical Cerrado biome vegetation, located in the Gonçalves community in the municipality of Monte Carmelo (18°50′26″ S and 47°19′04″ W). The municipality belongs to the mesoregion of the Alto Paranaíba, Minas Gerais, and is at about 890 m altitude.

The area is located on the Paranaíba River Basin, with a predominance of Red Latosol. The region is characterized by a seasonal Aw climate type according to Köppen classification, with two well-defined seasons; one hot and rainy summer, and the other with cold and dry winter. The average temperature is 20.7 °C and annual average rainfall of 1569.1 mm (Prado Júnior et al., 2012).

The fruits were randomly sampled from four matrix trees. The fruits were collected in the four cardinal directions of the canopy tree (north, south, east and west). Pruning was employed for the fruit sampling when necessary. A total o 58 fruit were collected on Septempber and October 15th, 2017.

After sampling, the fruits were properly packed in plastic bags, identified and taken to the laboratory where the diameter and length variables of the fruits were measured. These fruits were then stored into plastic containers insulated with "voile" type fabric, and kept in natural environment. The daily emergence of adult insects was verified.

Adult insects were identified by Dr. Vitor Osmar Becker, from the Uiraçu Institute, Camacan, Bahia, Brazil. Voucher specimens were deposited in the Vitor O. Becker Colection. The jatobá species was identified by Forest engineer, Msc. Kelen Pureza Soares.

3. Results

The insect species found on the jatoba was identified as a microlepidoptera species, *Ectomyelois muriscis* (Dyar, 1914) (Lepidoptera, Pyralidae, Phycitinae) (Figure 1). The presence of this insect was observed from orifices with the presence of pupae. Endocarp consumption was also observed, but only on the fruits that presented some type of previous injury (Figure 2).

Figure 1. *Ectomyelois muriscis* adult

Nine adult insects emerged from the 58 sampled fruits, from September to October, 2017. The pupae were verified on 6.9% of the fruits. Non-occurrence of the insects attacking the jatoba seeds is noteworthy, as well as the absence of associated insects. The sampled jatoba fruits had an average length of 12.91 ± σ = 1.81 cm, largest diameter thickness of 4.48 ± σ = 0.57 cm, while the diameter of the lowest thickness was 3.24 ± σ = 0.51 cm.

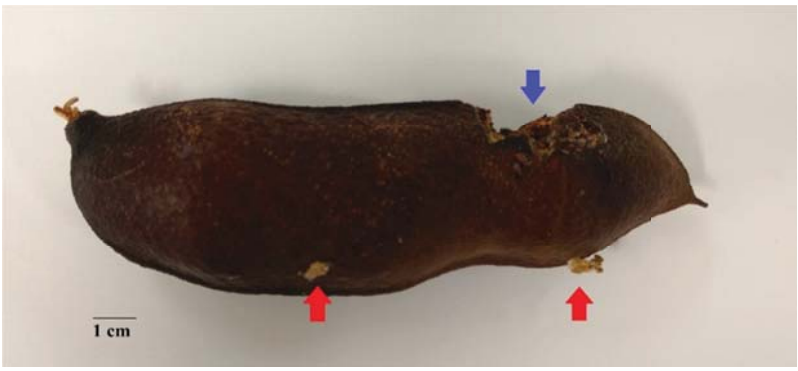

Figure 2. Injury of jatoba *Hymenaea stigonocarpa* (arrow in blue), and presence of pupae (arrow in red) of *Ectomyelois muriscis*

4. Discussion

A study performed by Ribeiro, Sales, Miranda, Soares, and Oliveira (2007) indicates that insect predation negatively influences the vigor of healthy seeds of the Cerrado legume tree species such as *Dalbergia miscolobium* Benth., *Enterolobium gummiferum* (Mart.) J. F. Macbr., *Plathymenia reticulata* Benth. and *Stryphnodendron adstringens* (Mart.) Coville.

The microlepidoptera *E. muriscis* is widely distributed in the Neotropical region (Neunzig, 2003). In this sense, the species is reported to feed on fruits, using host plants *Mammea americana* L. (Calophyllaceae) and *Theobroma cacao* L. (Malvaceae) in Latin American countries (Heinrich, 1956), and occurring in Costa Rica in 80% of the fruits of *Hymenaea courbaril* L. (Fabaceae) (Janzen, 1983) and *Theobroma simiarum* Donn. Sm. (Malvaceae) (Young, 1986). Also, a natural infestation by *E. muriscis* larvae on fruits and branches of *Jatropha curcas* L. (Euphorbiaceae) was reported in Chiapas, México (Gómez-Ruiz, López-Guillén, Barrera, Solis, & Zamarripa-Colmenero, 2015).

In Costa Rica, the oviposition of 20 *E. muriscis* eggs of was verified in the epicarp of *H. courbaril*. After hatching, the cream-to-yellow-colored month larvae enter the fruit and spend about a month feeding on the flesh of the fruit. There were 7 to 20 larvae per fruit of *H. courbaril*, and these did not consume the seeds. About two weeks after pupation the adult emerges. The newly emerged adults disperse in the habitat and do not oviposit in *H. courbaril* fruits until the next harvest, approximately 10 months later (Janzen, 1983).

In a field study of *E. muriscis* attacking *J. curcas* it was verified that the first instar larvae penetrate the fruit through its petiole, developing into a seed. The larvae leave the infested fruit after 18-20 days, then the emerged larva perforates the stem of the plant where it remains for approximately 10 months before pupation. After 15 days in the pupae stage, adults emerge from the stem. The total life cycle from oviposition to adult emergence in *J. curcas* is 11 months. Overlapping generation were not observed (Gómez-Ruiz, López-Guillén, Barrera, Solis, & Zamarripa-Colmenero, 2015).

This is the first report of *E. muriscis* attacking *H. stigonocarpa* fruits in Brazil, constituting a potential pest since they may compromise the propagation of this Fabaceae species, which is economically and ecologically important for the Brazilian Cerrado region.

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Research on Advance of Rice False Smut *Ustilaginoidea virens* (Cooke) Takah Worldwide: *Part I. Research Status of Rice False Smut*

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Abstract

Since hybrid rice was planted, rice false smut (RFS) caused by *Ustilaginoidea virens* (Cooke) Takah has risen from a sporadic secondary disease to a major devastating and common disease, due to the changes in climatic conditions, cultivation system, fertilization and water management and cultivar replacement, and has become one of the new three major rice diseases in China. In addition to cause rice yield decrease and economic losses, RFS also causes toxic effects on humans and animals, due to the fact that the pathogen has color, produces toxins, affects rice appearance, and reduces rice quality. Therefore, RFS has attracted great attention from various governmental agencies, research institutions and scientists. More than 300 papers related to RFS composed over the past 100 years were reviewed. In this part, the occurrence, epidemiology of RFS, the relationship between occurrence sverity of RFS and yield loss, field distribution pattern and sampling method of RFS and disease severity classification were discussed.

Keywords: rice false smut (RFS), occurrence and harm, *Ustilaginoidea virens* (Cooke) Takah, research progress

1. Introduction

1.1 Importance of Rice and the Occurrence Status of RFS

Rice is a staple food of more than half of the world's population, and more than 60% of the population of China. Increasing rice yield is thus crucial for solving problems of food shortage, ensuring food security, and reducing poverty (Yuan, 2014). Given that rice is a staple food of half the world's population, rice false smut (RFS) (*Ustilaginoidea virens* (Cooke) Takah) presents a constant threat to global food supplies. The development of durable and environment friendly strategies for the control of RFS depend on a better understanding of the developmental process of this pathogen, and the establishment of the disease (Hu et al., 2014).

RFS is a type of global rice heading stage fungal disease induced by Deuteromycotina Ustilaginoidea (*U. virens* (Cooke) Takah) (Tanaka et al., 2008a; Ashizawa et al., 2010; Kepler et al., 2012), and its main typical symptom is the formation of yellow to deep green RFS on the spikes of rice (false smut balls), commonly known as black ball disease, dustbrand or false smut (Suwa, 1915; Nakamura et al., 1992). RFS was recognized as a symbol of a bumper harvest and was categorized as a minor disease due to its sporadic occurrence (Ladhalakshmi, et al., 2012). Now, it is a serious disease of rice worldwide (Rush et al., 2000; Tsuda et al., 2006; Zhou et al., 2008; Brooks et al., 2009; Ladhalakshmi et al., 2012). It was called "bumper disease" or "good harvest fruit" in China. The pathogen of *U. virens* produces both sexual ascospores and asexual chlamydospores in its life cycle. In

recent years, RFS has already become an important disease in rice producing areas in Asia, America, Aferica and Europe (Yaegashi et al., 1989; Brooks et al., 2009; Singh et al., 2010). False smut has become the main and serious rice disease since 2001 in India. The infected tillers were found to vary between 2%-85%, the number of infected grains reached even more than 100 per panicle in severe cases. Due to the heavy false smut, the air above the infected field gave a black smoky appearance from a distance as a result of the release of spore mass in the atmosphere (Dodan, et al., 1996; Mandhare et al., 2008). In recent years, it has emerged as the most devastating grain disease in the majority of the rice-growing areas of the world including China (Wang et al., 2004), India (Ladhalakshmi et al., 2012), the United States (Brooks et al., 2009, 2010) and Japan (Tsuda et al., 2006; Ashizawa et al., 2011).

In China, RFS has a long history. *Bencao Gangmu*, written in the Ming Dynasty (1368-1644 AD), includes a description of "mildew grain of hard rice and slave rice" (*i.e.*, false smut). However, RFS only occurred sporadically throughout a very long period of time, the damage was not severe, and the loss was small, thus it has been regarded as a minor disease of rice. Internationally, RFS, caused by the fungus *U. virens* (teleomorph: *Villosiclava virens*), was historically a minor rice disease (Padwick, 1950; Tanaka et al., 2008a). High-yield hybrid rice has been planted in large areas since the late 1970s, especially high yield varieties of Indica × japonica hybrid rice, and too much nitrogen fertilizer is topdressed at the tillering stage and booting stage. Especially in recent years, climate change and change of the farming system have led to increasingly serious cases of RFS in China, the occurrence of the disease displaying the characteristics of wide area, high frequency, and cuased severe yield losses (Pan et al., 1993; Wang et al., 2004; Zhou et al., 2008; Hou et al., 2009). At present, RFS is one of the three major diseases of rice in China, Table 1.

Table 1. Occurrence and control areas of main rice diseases between 2013 and 2015 in China (Thousand hectare, thousand ton)

Diseases	Year	Ocurred area	Controlled area	Reduced yield loss	Actual yield loss	
Total of diseases	2013	26807.49	52392.90	12468.30	1647.30	If establish national level post system expert
	2014	29935.80	59787.67	16082.00	2066.70	
	2015	29439.40	59802.27	14904.60	1943.90	
Rice sheath blight	2013	17448.97	25454.53	8279.70	1057.50	Yes
	2014	17878.67	28234.33	8728.00	1116.30	
	2015	17934.13	27656.07	8152.40	1075.50	
Rice blast	2013	3679.18	12702.68	2247.90	318.7	Yes
	2014	5136.20	16129.00	4632.10	558.30	
	2015	5112.67	17150.67	4738.20	565.90	
Rice false smut	2013	1995.30	5388.75	565.20	78.70	No
	2014	3154.73	7608.60	1011.80	189.10	
	2015	3023.40	7779.20	919.20	161.10	
Rice virus diseases	2013	573.26	2917.01	428.80	35.30	Yes
	2014	350.67	1357.47	247.10	19.40	
	2015	267.87	1003.07	165.80	18.40	

1.2 Research Status of RFS

"Rice and *Ustilaginoidea*", "Rice and *Claviceps*" and "Rice and *Villosiclava*" were used as the keywords to search in the "Web of Science", and 278, 64 and 23 records were respectively obtained (including patents), among which 182, 23 and 17 papers were published by Chinese scholars, accounting for 65.47%, 35.94% and 73.91%, respectively, and an average of 58.44%. From the quantity and quality of the published papers, the countries with greater numbers of and more in-depth studies on RFS, in order, are China, followed by Japan, the USA, India, and the International Rice Research Institute (IRRI, Philippines). "Rice and *Ustilaginoidea*" was used as the keyword in the "Web of Science" to search the contents between January 2013 and December 2014, including relevant papers and patents, and there was a total of 65 records, of which Chinese scholars published 56 papers (86.15%), including 24 Chinese papers, 23 periodical English papers, and 9 patents; a total of 9 papers were published by other countries. Whether in terms of quantity or quality, the published papers on RFS by China occupy an absolute advantage.

Jiang et al. (2012) used the full text database of Agricultural Science and Technology Periodical Literature in Chinese National Knowledge Infrastructure (CNKI) as the data source, using the bibliometric method, they counted and analyzed the published research papers on RFS by China for a period of more than 50 years (1957-2011) (Table 2).

Table 2. Published papers of RFS in China between 1957 and 2011

Years	Before 1980	Between 1980-1989	Between 1990-1999	Between 2000-2009	Between 2001-2011
Number of Published papers	4	155	398	1081	1242
Average (papers/year)	?	15.5	39.8	108.1	112.9

The Chinese keywords of "rice and RFS" were employed for retrieval (fuzzy matching) (time: January 1, 2012 to December 31, 2014), and a total of 305 records were selected.

The related literatures of RFS in China were as early as in 1957 published in "Agricultural Science Communication" (which is limited to the retrieval system, and there may be earlier references). Before 1980, China's agricultural science and technology periodicals published a total of four papers related to RFS; between 1980 and 1989, 155 papers were published; between 1990 and 1999, there were 398 papers published, the document number of which is 2.6 times that of 1980-1989; between 2000 and 2009, a total of 1081 papers were published, 7.0 times of that of 1980-1989, and 2.7 times of that of 1990-1999. These related papers mainly included the contents such as disease investigation and chemical control. Currently, the selection and breeding of rice varieties resistance to RFS, genetic mechanism of disease resistance, and identification of resistance genes are relatively underdeveloped.

The present paper focuses on the analysis of a number of papers related to RFS and funding-supported project information over the period of 11 years (2001-2011) in China. There were 1242 papers related to Chinese RFS in 2001-2011. Before 2004, the papers related to RFS were published in 40 journals, and the number of published papers was less than 100. In 2005-2011, the annual average number of published papers related to RFS was greater than 100, and the papers were distributed in 40 journals. In 2001-2005, the number of published papers supported by projects was 27, among which 5 papers were supported by state-funded projects and 22 were supported by local projects. In 2006-2011, the number of published papers funded by projects was 77, of which 48 papers were supported by state-funded projects, and 29 by local projects. These data indicated that research on RFS in China carried out earlier, however, early research is relatively rare. After 2000, the number of papers related to RFS published in China increased rapidly, which may be related to the fact that the occurrence and damage of RFS have increased year by year, and more attention has been given to the research of RFS, funded by the relevant departments and strengthening of national project support.

2. Occurrence and Damage and Economic Losses of RFS

When the incidence rate of RFS is low, it has little effect on the yield of rice. The damage mainly includes the pathogenic fungus with color, toxic production, pollution of rice grains, reduction of rice quality, and toxicity to humans and animals. If occurrence of RFS is severe, it not only causes reduction of yield, pollutes rice grains and decreases the quality of rice, it also causes damage to consumers (Huang et al., 2003); Aside from the loss of production, false smut is also a threat to food safety as the chlamydospores of *U. virens* produce mycotoxins (Koiso et al., 1992; Miyazaki et al., 2009; Shan et al., 2012). The disease also causes economic losses to farmers due to a lower market price for their produce owing to the presence of black chlamydospores masses on healthy rice grains (Ladhalakshmi et al., 2012).

RFS is an intermittent occurrence disease in the past, nowadays, it has increased to be a main and common rice disease, especially in the single season rice and late rice. The infected panicle rate can reach as high as 30%, and the incidence rate of grain reach 0.8% in the seriously diseased fields, which will result in a decreased maturing rate of seed, reduced 1000-grain weight, and yield loss rate of diseased panicle reach 11.5-54.5% (Dong et al., 2003).

Figure 1. Seriously infected by *U. virens* of Yongyou 12, an indica/japonica hybrid rice combination

2.1 Occurrence of RFS

RFS was first reported by Cooke from Tirunelveli district of Tamil Nadu State of India, and named *Ustilago virens* (Cooke, 1878). In 1918, RFS occurred in large areas in the Philippines, occurred in Burma in 1945 and then was widespread in 1980. In 1975, the disease occurred in large parts of West Africa, and serious occurred in parts of India in 1992 (Wang et al., 2003). Thereafter, RFS has gradually spread, and now it is widely distributed throughout the world, including Asia, Africa, South and North America and Europe, to nearly 40 major rice production countries, such as China, India, Japan, the Philippines, Burma, Bangladesh, Australia, Brazil, Italy, the USA and Egypt, of which the spreading of RFS was quite serious in China, India, the Philippines, Japan and other Asian countries (Ou, 1985; Dodan et al., 1996； Atia, 2004; Xia et al., 2009; Ladhalakshmi et al., 2012). RFS was first reported in the 1930s and 1940s in some rice fields of the southern and the southwestern China, south of the Yangtze River and northeast China. In the 1950s and 1960s, RFS occurred sporadically in some parts of China. In the late 1970s, the disease showed a rising trend year by year, generally with large areas of planting of high-yield hybrid rice and increasing application of fertilizer level, but there were annual fluctuations. The occurrence and damage of RFS in China have become the third major disease, ranking only second to sheath blight (*Rhizoctonia solani*) and rice blast (*Pyricularia oryzae*).

2.2 Damage Caused by RFS

The damage of RFS has been investigated in a large area in various parts of China, while in other countries was not comprehensively investigated and reported. RFS was listed as the officially investigated rice disease by Ministry of Agriculture of P.R. China since 2009. In 2013-2014, the area of occurrence of RFS was 1995.3-3154.73 ha, and the yield loss of rice reached 643.90-1200.90 kilotons if without controlled (Table 1).

RFS is one of the important diseases currently constraining high quality and high yield of rice in China (Fu, 1994). In 1980, the occurrence area of RFS in Yunnan Province was only 6000 hm^2, by 1991, the occurrence area of the disease was more than 60,000 hm^2, in 1993, the areas reached 130,000 hm^2. The average rate of diseased panicle was 3-5%, while the infected rate of panicle was as high as 40% in severely infected rice fields (Liao et al., 1994). The occurrence area of RFS was more than 600,000 hm^2 in Jiangxi province in 1982, and it caused 30 million kg yield loss of rice (Ji et al., 1995). In 1980-1982, there were more than 340,000 kg of rice grain loss because of RFS in a town in Fenghua county of Zhejiang province. In 1984-1986, the infected rice area by RFS in Liaoning province increased from 200,000 hm^2 to 330,000 hm^2, showing a rising trend year by year. In 1986, the occurrence area of RFS in Funing, Luannan and Tanghai counties of Hebei province was only 212,000 hm^2, while in 1987 and 1988, the infected area increased to 360,000 hm^2 and more than 500,000 hm^2. Even more serious, nearly more than 70% of the paddy fields were infected by RFS in varying degrees in 1989. In 1990, the diseased area of RFS in Mengzi county of Yunnan province reached more than 65.8% (Li et al., 2006). In 1994, RFS occurred in 70,000 hm^2 of rice fields in eastern Hebei province, and caused 37.2 million kg loss of rice grain (Pan et al., 1997a, 1997b). In 1984, the occurrence area of RFS in Liaoning province was about 200,000 hm^2, accounting for 43% of all the planting area of rice in the province. The rate of diseased panicle was generally 5-10%, and more than 30% in serious plots. In 1996, the disease occurrence area has increased to

330,000 hm^2 (Ji, 2002). RFS was first discovered in Heilongjiang province in 1986, at present, the disease has become one of the main rice diseases in the local (Gao et al., 2001).

In 1982, the occurrence of RFS in Hunan province was serious, the infected rice area was about 670,000 hm^2, and there were 75.4% areas were seriously infected among the diseased fileds (Ji et al., 1995). In 2003, an area of more than 233,000 hm^2 had infected by RFS, and the loss was nearly 100 million kg. In 2004, there was false smut disease with different degrees in various counties, states and cities throughout Hunan province. The infected area was 633,000 hm^2, the loss of rice was 137 million kg, and the direct economic loss was 200 million RMB yuan (Wang et al., 2005). The area with a diseased panicle rate of less than 10% accounted for 46.75%; that with a diseased panicle rate of 10%-20% accounted for 24.5%; that with a diseased panicle rate of 20%-40% accounted for 15.5%; that with a diseased panicle rate of more than 40% accounted for 3.75%; the diseased panicle rate reached 80% in the most seriously infected field, and there was only 9.5% of rice areas not be infected. In general, the number of diseased grains was 5-10 grains per panicle, however, at the seriously infected panicle, there were about 15-20 diseased grains per panicle (Zhang et al., 2009). In 2005, the RFS occurrence area throughout the province was nearly 266,700 hm^2, and the loss of rice was 100 million kg. According to incomplete statistics, the area with occurrence of RFS of middle and late rice in Hunan province in 2003-2005 was more than one million hm^2, and the direct economic loss was 200 million RMB yuan. RFS mainly harms double-cropping late rice, followed by one season of middle and late rice in Hunan province, and the early rice generally has a lower incidence. The panicle maturation rate, brown rice rate, polished rice rate and head rice rate of the panicle which infected with RFS all decreased, while the rate of green rice and death rice rate increased significantly (Hou et al., 2009).

In 2004, RFS universally occurred in one-season middle rice in Changde city of Hunan province, covering an area of 40,000 hm^2, and accounting for 31.9% of the total sown area of 125,300 hm^2, which is rare in the past 10 years. The rice yield loss caused by RFS was 12.63 million kg, of which there was 1.09 hm^2 of Honglianyou No. 6 and 1.68 hm^2 Yueyou 938, with a yield reduction of 70%; there was 1.41 hm^2 of II You 084, with a reduction of 50%; and there was 1.41 hm^2 of Liangyoupeijiu (LYPJ), with a reduction of 40% (Liu et al., 2006). In 2005, RFS generally occurred in the middle and late rice of Zhenba county in Shaanxi province, and was especially serious in the late rice. There were 4433 hm^2 rice fields were moderate to seriously infected by RFS throughout all of Zhenba county, the average yield loss was 407.4 kg per hectare, and the direct loss of rice was 1.81 million kg, of which the area with a diseased panicle rate of 8-43% was 3500 hm^2, with an average yield loss of 662.4 kg per hectare, and the area with a diseased panicle rate of less than 8% was 933 hm^2, with average yield loss of 197.4 kg per hectare. The direct economic loss was 1.26 million RMB yuan (Lu et al., 2006). In 2008, the area of RFS in Anhui province was more than 500,000 hm^2 (Lv et al., 2008).

In India, it was reported that RFS caused the resistant and susceptible rice varieties' yield loss from 2.0%-12.9%; grain filling decrease from 3.28%-19.27%; 1000-grain weight decrease from 4.93-21.84%. Germination percentage of seeds were reduction of most rice cultivars，19.43% reduction in seedling length and 22.79% decrease in vigour index (Srivastava et al., 2014).

3. Field Distribution and Disease Classification of RFS

3.1 Field Distribution Pattern and Sampling Method of RFS

It is generally believed that the distribution type of RFS in the field is the aggregated distribution, but there are differences among the varieties and degree of incidence of diseases. Tang et al. (1998) believed that the diseased rice panicle of RFS and the diseased grain exhibited aggregated distribution in the field, and the reason for aggregation was induced by environmental factors. Therefore, the investigation of RFS in fields should use the parallel sampling method, which is the most suitable. Li (1995) employed different varieties of rice for experiments, and found that the field distribution type of RFS conformed to the negative binomial distribution, but it conformed to both the negative binomial distribution and Neyman distribution in mild cases. The sampling accuracy of the two-parallel linear jump methods in the field investigation is the highest, followed by the diagonal line, and the suitable sampling number in each region is 200 hills. Pan et al. (1998) researched and believed that both the distributions of the diseased panicle of RFS and diseased grain are aggregated (negative binomial) distributions, and the basic component of the distribution was the loose individual group, and the distribution in individual group was random. The individual population ranges of the diseased panicle and diseased grain were 4-8 and 2-4 clusters, respectively. Sun et al. (2006) believed that the distribution of the diseased grain was the aggregation distribution, but the distribution types of the different varieties were different. The diseased panicle distributions of "Yueyou 938" and "Wuyunjing" were the uniform type, and the diseased panicle distributions of "W9707" and "Taihu rice" were the aggregation types. Xiao et al. (2007) conducted a

large area of investigations in the maturity period of the late rice variety, and concluded that the field distribution of RFS belonged to the Neyman distribution type of the aggregation distribution through theoretical frequency calculation and tests of goodness of fit of various distribution types, and the parallel jumping sampling method was the field survey method most suitable for the disease. For the highly susceptible cultivar, RFS spatial distribution in the fields conform to nonrandomness aggregation. The best way for sampling survey of RFS in the field was parallel and saccad sampling (Xie et al., 2019).

3.2 Relationship Between Occurrence Degree of RFS and Yield Loss

In addition to the lesions of grains, the loss of rice yield caused by RFS mainly leads to the decrease of the actual grain number of the whole rice grains, 1000-grain weight and single grain weight. Research has shown that there is an extremely significant negative correlation between the number of the diseased grain per panicle and elements of rice yield. There was a significant positive correlation between the percentage of empty grain and the number of diseased grains per panicle, with a correlation coefficient of r = 0.9886. One diseased grain per rice panicle is increased, and the empty grain rate is increased by 3.5%, while the single panicle weight is decreased by 0.14 g. The correlation coefficient between single panicle weight and diseased grain number per rice panicle was r = -0.9913. One diseased grain is increased per panicle, and the loss rate of average per panicle weight is increased by 6.1%, of which one diseased grain caused weight loss of per panicle was 9.3%, and 9 diseased grain caused the weight loss of per panicle was 48.2%, with an average value of 30.3%. The effect of the number of diseased grains per panicle on 1000-grain weight is not significant, at r = 0.5958. The increased diseased rice grain of RFS and degree of the decreased grain weight of single panicle was different between varieties (Huang et al., 1988). Zou et al. (1994) analyzed the relationship between RFS and the yield loss by using the grey correlation degree method, and they pointed out that the decline of the setting percentage was the main reason for yield loss of rice. There was a significant negative correlation between the number of the diseased grains of RFS per panicle and 1000-grain weight and grain number per panicle and panicle weight (the correlation coefficients were -0.9663, -0.9036 and -0.9723), and it was significant positively correlated with the immature grain rate (correlation coefficient: +0.8965) (Shi et al., 2003). Gao et al. (2001) believed that there was a significant linear correlation among the number of diseased grains per panicle of RFS and empty grain rate, 1000-grain weight and yield loss rate. According to the control and treatment cost and price of rice and other factors, the economic threshold of RFS was obtained. Japonica rice “9516” had 1-10 diseased grains per panicle, and the percentage of empty grain was in the range of 14.26-50.19%, and the 1000-grain weight was 26.3-21.8 g, and rate of yield loss was 2.79-54.88%. Indica hybrid rice “R405” had 1-10 diseased grains per panicle, the percentage of empty grain was in the range of 29.12-44.22%, the 1000-grain weight was 25.0-22.0 g, and the rate of yield loss was 7.64-36.22%. The effects of increasing one diseased grain per panicle on the percentage of empty grain, 1000-grain weight and yield loss rate on Japonica rice “9516” were greater than those of indica hybrid rice “R405”. Jiang et al. (2009) and Yang et al. (2013) also believed that the presence of diseased grain strongly influenced the 1000-grain weight of health grain which close to the spindle panicle, and the empty grain rate of the diseased panicle was increased, and the 1000-grain weight was decreased, ultimately affecting the yield of rice. Ding et al. (1997) established the Weibull model of rice of the number of diseased grains per panicle, reduction rate of rice yield and the rate of decline of milled rice. Wang et al. (2007) used the statistical software of SAS to analyze the field data, and they pointed out that the main factors affecting the rice yield were the rate of diseased panicle of RFS, followed by the rate of diseased grain, and the rate of infected hill was the secondary factor.

3.3 Disease Severity Classifications of RFS

The relationship between the degree of the occurrence of RFS and yield loss of rice is significantly different because of different varieties, and the results of different researchers. According to the differences of degree of RFS, disease index or damage loss index, the degrees of occurrence of RFS were divided into multiple levels, but yet there is no unified classification standard. Li (1996) divided the diseases into 10 grades according to the percentage of the diseased grain number in the total number of grains of each panicle, which are as follows: scales 0, no obvious symptoms (none diseased grain of RFS in the panicle); scales 1-9, the percentages of the diseased grain in the total grain number per panicle were 0.1-2%, 2.1-5.0%, 5.1-10.0%, 10.1-15.0%, 15.1-20.0%, 20.1-30.0%, 30.1-50.0%, 50.1-75.0%, and more than 75.1%, respectively. Tang et al. (2000) employed Q type systematic cluster analysis, and they used five indicators of aspect ratio of false smut ball (FSB), weight of 100-grain FSB, filled grain weight per panicle, seed setting rate, and loss rate for the classification of the number of the RFS diseases, and the RFS was divided into six levels according to the numbers of FSB in a panicle: Level 0, no diseased grain; Level 1, 1 grain of false smut ball; Level 2, 2 grains of false smut ball (chlamydospore); Level 3, 3-5 grains of false smut ball; Level 4, 6-9 grains of false smut ball; and Level 5, more than 10 false smut

ball. Shi et al. (2003) used the loss rate of panicle weight as the index, and they divided the RFS disease into six grades: scale 0, panicle weight loss rate 0; Grade 1, panicle weight loss rate ≤ 5%; Grade 2, panicle weight loss rate ≤ 10%; scale 3, panicle weight loss rate ≤ 20%; scale 4, panicle weight loss rate ≤ 50%; and scale 5, panicle weight loss rate more than 50%. Li et al. (2011) studied and concluded that the regression equation of yield loss rate caused by RFS and the average grain number per panicle was y = 2.8624x + 0.5427 (P = 0.0001), and they divided the severity of RFS into six scale with a more accurate average grain number per panicle and yield loss rate. Some of the above classification standards were complex to a certain degree, and some standards were too fine to be determined rapidly, thus it was difficult to implement in the field investigation operation.

Zhang et al. (2006) reference grading standard of rice neck blast (*Pyricularia oryzae*) incidence to differentiate the resistance or susceptible of rice varieties to RFS. The grading standards are as follows: Level 0: diseased panicle rate less than or equal to 1% (HR); Level 1: 1% < diseased panicle rate ≤ 5% (R); Level 3: 5% < diseased panicle rate ≤ 10% (MR); Level 5: 10% < diseased panicle rate ≤ 25% (MS); Level 7: 25.0% < diseased panicle rate ≤ 50% (S); and Level 9: diseased panicle rate > 50.1% (HS). Deng (1989) divided the RFS disease severity into six levels according to the number of false smut ball in a single panicle: scale 0, no diseased grain; scale 1, 1 false smut ball; scale 2, 2-5 false smut balls; scale 3, 6-10 false smut balls; Grade 4, 11-15 false smut balls; and scale 5, more than 16 false smut balls. The above two grade standards are too rough for practical purposes, and thus cannot reflect the incidence of RFS and actual output loss.

Disease resistance levels are usually conducted on domestic RFS according to the infected hill rate, diseased panicle rate, number of diseased grains per panicle, diseased grain rate of a panicle, and panicle weight loss rate, then the disease index is calculated. Shiwen Huang (professional standard, 2019) set the classification standard by combining the standards of different researchers, taking into account the current rice varieties and occurrence of RFS, harm and toxins and other factors, and he personally believe that this grading standard is quite scientific and reasonable. Table 3 shows the grading standard of RFS currently used in China and give the commentary of each grading standard.

Table 3. Disease index grading standard of RFS and their merit and demerit

	Disease scale	0	1	3	5	7	9	Commentary
	Resistance	HR	R	MR	MS	S	HS	
Chen Jiafu et al. (1992)	Infected panicle rate (%)	0	0.1-5.0	5.1-10.0	10.1-25.0	25.1-50.0	≥50.1	Easy to operate, but low accuracy
Zhang Yushu et al. (1992)	Infected grains per panicle	0	1	2	3-6	7-10	≥11	The former is easy to operate and basically reasonable; the later is difficult to operate
	Weight loss rate per panicle (%)	0	0.1-5.0	5.1-10.0	10.1-20.0	20.1-50.0	≥50.1	
Li Xian (1996)	infected grains per panicle	0	1-2	3	4-5	6-7	8-9	The former is easy to operate and basically reasonable; the later is difficult to operate
	Infected grain rate (%)	0	0.1-5.0	5.1-10.0	10.1-20.0	20.1-50,	≥50.1	
Liu Yongfeng et al. (2000)	Infected grains per panicle	0	1	2-4	5-7	8-10	>11	Easy to operate, Basically reasonable
Shi Chenzhi et al. (2003)	Infected grains per panicle	0	1	2-3	4-7	8-15	≥16	The former basically reasonable and easy to operate; the later is difficult to operate
	Weight loss rate per panicle (%)	0	0.1-5.0	5.1-10.0	10.1-20.0	20.1-50.0	≥50.1	
Zhang Juncheng et al. (2004)	Infected grain rate per panicle (%)	0	0<X≤2	2<X≤5	5<X≤10	10<X≤20	20<X	Heavy workload for investigation and evaluation
Zhang Shu et al. (2006)	Infected panicle rate (%)	<1	1.0-5.0	5.1-10.0	10.1-25.0	25.1-50.0	≥50.1	Easy to operate, but low accuracy
Huang Shiwen (professional standard of China, 2019)	Infected grains per panicle	0	1	2-4	5-8	9-15	≥16	The former is scientific and reasonable and easy to operate; the later is difficult to operate
	Weight loss rate per panicle (%)	0	0.1-5.0	5.1-10.0	10.1-20.0	20.1-50.0	≥50.1	

Note. Disease index = [Σ(number of diseased plants × representative value of disease grade)/total number of investigated plants × highest representative value of disease grade] × 100%; diseased panicle rate (%) = (diseased panicle number/total surveyed panicle number) × 100%; panicle weight loss (%) = (diseased panicle weight/panicle weight without diseases) × 100%.

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Research on Advance of Rice False Smut *Ustilaginoidea virens* (Cooke) Takah Worldwide:
Part II. Studies Progress on the Pathogen and Its Toxin of U. virens

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Abstract

In this part, the history of the study on RFS pathogen *U. virens* was reviewed, including the pathogen naming and the change process, morphological characteristics of *U. virens* and culture characters both of asexual and sexual stages, and mycelium, chlamydospore, conidiophore and sclerotium germination. Genetic diversity, pathogenicity, the strain-host interaction, host range of *U. virens* and it's early detection were also discussed. The research of Ustiloxins of RFS, including biological activity, toxicity to plants and animal, the potential possibility utilization of Ustiloxins, for example use as screening agent for rice varieties resistance to RFS, and anticancer drugs.

Keywords: *U. virens*, pathogen classification, characteristics, ustiloxins

1. Introduction

It possesses great significance for realizing the occurrence, epidemiology, harm and the management of the diseases to study the biological characters and morphological characteristics of the pathogens which caused the diseases. Rice false smut was first found in 1878 and the caused pathogen was named *Ustilago virens*. The pathogen was finally named *Ustilaginoidea virens* (anamorph) after more than once alteration, and the sexual generation was *Villosiclava virens* (teleomorph). The biological characters and morphological characteristics of *U. virens* was discussed in this paper. The *U. virens* has genetic diversity, and the pathogenicity shows obvious difference of different source pathogen. There was strong interaction between the *U. virens* and the host rice varieties. The pathogen of RFS could infect various plants. The abilities of different *U. virens* producing toxin there are differences. The toxin produced by *U. virens* shows obvious poison and negative effect on both plants and animals, on the other hand, the toxins of *U. virens* potentially develop the beneficial preparation products.

2. Research Progress of RFS Pathogen

The Basidiomycota smut fungi have been intensively studied over the last century because of their threat to the yield and quality of major crop plants (Kronstad, 1996).

2.1 Naming and Classification of the RFS Pathogen

RFS was first recognized by Cooke from infected rice samples from India, and at the time was named by Cooke as *Ustilago virens*, as a species of *Ustilago* (Cooke, 1878). Then, Patouillard carried out independent study on RFS samples from Japan, and named it *Tilletia oryzae* Pat., a species of rice *Tilletia*. In 1895, Brefeld held that

the development and sporulation pattern of *Tilletia oryzae* Pat. was similar to an asexual stage of *Ustilaginoidea*, namely the sac fungi of *Ustilaginoidea*. Therefore, *U. virens* was transferred to the genus of *U. virens*, and its name was changed by Brefeld to *U. oryzae* (Pat.) (Padwick, 1950; Ou, 1985; Tanaka et al., 2008b). In 1934, Sakurai found that the sexual spores of the pathogen, the sporangia sclerotium produced by sclerotium germination and the fungus was attributed to the Ergot fungi genera of sac fungi known as *Claviceps virens* (che). However, it did not obtain a valid name for various reasons. The teleomorph of *U. virens* had been named *Claviceps virens* (Sakurai ex Nakata) and *Claviceps oryaze-sativae* (Hashioka) because its characteristics of teleomorph are similar to those of *Claviceps* (Hashioka, 1971). Due to the fact that the characteristics of the spores in the conidiospore stage of *Claviceps* and *U. virens* differed, the name has not been admitted in academy (Dodan et al., 1996). In 1988, Ahuja and Payak proposed that the genetic difference discrimination of family should not be based on the same sex in most cases, instead it should be based on the characteristics of conidial stage. They also suggested that *U. virens* (che) was the valid name of the pathogen *U. virens*, and *Claviceps oryzae sativae* was its alias (Ahuja et al., 1988). This name has gradually been accepted in academic circles, thus RFS was officially named as *U. virens*. Until 2008, RFS has been independent of Claviceps by Tanaka based on comparative research, and the sexual state was named as *Villosiclava virens* (Tanaka et al., 2008a, 2008b).

However, the molecular phylogenetic analysis, which based on both large subunit of the rRNA gene and acetaldehyde dehydrogenase gene sequences, revealed that members of *Ustilaginoideae* are distinct from teleomorph genera of *Clavicipitaceae* and should be recognized as amonophyletic group within *Hypocreales* (Bischoff et al., 2004; Tanaka et al., 2008b). As a result, it was suggested *Villosiclava virens* as the new name for the teleomorph of *U. virens* (Tanaka et al., 2008a), which was accepted and used in recent reports (Ashizawa et al., 2012; Fu et al., 2012; Tang et al., 2012).

A new type of *U. virens* strain was isolated from white false smut balls. The pathogenicity test and the analysis results of isoenzymes and RAPD demonstrated that the taxonomy of albino strains was independent of *U. virens*. Whether or not it can establish its new position as a species still requires further research (Wang et al., 1998, 2008b; Jecmen et al., 2015).

2.2 Morphological Characteristics of Pathogens

The *U. virens* forms smut ball on the rice panicle, the symptoms (smut ball) produced by *U. virens* are visible after flowering only (Biswas, 2001), its color changes from cream white, yellow to dark green or dark brown with the passing of time, and this is the conidia pedestal. The spore pedestal section is divided into three layers: the outer layer of yellow green is mature chlamydospores, the middle orange layer is hypha and spore, and the inner white or pale yellow layer is radial hyphae and spores that are in the process of being formed (Ou, 1985; Lee et al., 1992; Biswas, 2001). The morphological characteristics of *U. virens* include the asexual stage and sexual stage.

2.2.1 Morphological Characters in Asexual Stages

The vegetative state of *U. virens* includes mycelia, chlamydospores and conidia. Chlamydospores are conidiospores with thick walls, round or ovular in shape, with a size of 4.5-7.8 × 4.5-7.0 μm, and yellow to dark brown in color. The cell wall is thick, and on the surface there is a large amount of verruca (Zhang, 1988). The chlamydospore on the white false smut of RFS balls is spherical, colorless and transparent, and the outer wall is smooth (Verma et al., 1988; Wang et al., 1997). Under appropriate conditions, chlamydospore germinates and produces a germ tube, and the germ tube forms dissepiment and differentiates into conidiophores. The tips of the conidiophores produce secondary conidium (Zhang, 1988). Conidiospores are thin-walled spores, ovoid or oblong in shape, with a size of 2.6-8.0×2.0-5.0 μm, have single cells, colorless and transparent, and have a smooth appearance (Mulder et al., 1971; Zhang et al., 2003a, 2003b, 2003c).

2.2.2 Morphological Characters in Sexual Stages

The sexual stage of RFS mainly includes the formation of stroma by sclerotium germination, ascus and ascospore. The fungus can form sclerotium on rice diseased grain. Sclerotium is black, hard, falls off easily, fusiform, horseshoe shape and various shapes, and has irregular sizes (length of 2-20 mm). The newly grown stroma is usually yellow in color, and the color turns black green after reaching maturity. The monolayer in stroma has many perithecia, the perithecium is ovular or pear-shaped, with a front opening, and the size is 357.5 × 247.0 μm, containing about 300 asci. The asci have a long cylindrical shape, colorless and transparent, and have a smooth surface, with a size of 130-234 × 3.12-5.2 μm and 8 ascospores within. The ascospore is colorless unit cells, linear, easily broken, with a size of 52-176.8 × 0.52-1.04 μm (Zhang et al., 2003a, 2003b).

2.3 Culture Characteristics of U. virens

2.3.1 Isolation of Pathogenes

The pure isolate of RFS pathogen *U. virens* was acquired for the first time in 1895 by Brefeld. In 1975, Sharma & Joshi isolated conidiospores from a fresh sclerotium on a yeast PDA medium (Wang et al., 1990; Zhou et al., 1999; Ji, 2001; Chen, 2004). Thereafter, the isolation technology of *U. virens* has been further developed and improved. At present, the main methods of separation of *U. virens* include the tissue, sclerotia, RFS ball isolation method and chlamydospore suspension method.

2.3.2 Mycelium Culture

The mycelium growth rate is related to environmental conditions and medium types. The temperature range of mycelial growth is 10-37 °C, and the optimum temperature range is 26-28 °C. The pH range is 3-10, and the most suitable pH is 5-7. Light demonstrates no significant effect on mycelia growth. The growth rates of *U. virens* in different media are different. The optimum carbon source of mycelial growth is sucrose, followed in order by maltose, glucose and starch; the optimum nitrogen source is L-asparagine; inorganic salt is a mixture of disodium hydrogen phosphate and magnesium sulfate (Lu et al., 1996; Zhang et al., 2003a; Pan et al., 2007a; Wang et al., 2012a). The optimum nitrogen and carbon source for different virulent strains different (Wang et al., 2013). The growth speed of different growing stage of *U. virens* is different at various media. The growth of *U. virens* in PDA and PSA media is slow, while large amounts of sclerotia may be produced in the PDYP medium (Zhou et al., 1999).

The sporulation ability and pigmentation of *U. virens* are positively correlated with pathogenicity, and the strain growth rate is negatively correlated with pathogenicity (Wang et al., 2013). In the same kind of solid medium, the colony morphology and color of *U. virens* strain in the initial stage are similar, but different characteristics appear after one month of culturing (Zhou et al., 1999).

2.3.3 Chlamydospore Culture

The production of chlamydospores of *U. virens* is correlated with sporulation ability and medium type. Some *U. virens* strains have sporulation ability, and some strains cannot produce spores (Verma et al., 1988; Cheng et al., 1996). An oatmeal liquid medium is more conducive to *U. virens* produced chlamydospores than in the liquid media of PS and PD (Zhou et al., 1999).

The life span of chlamydospore is quite long, as it can survive for more than 19 months under dry conditions (Lv et al., 1994). The optimum temperature for chlamydospore germination is 28 °C, and the optimum pH value is 5.8-6.3 (Lu et al., 1996). Some nutrients could improve the germination rate, proper order is 2% sucrose > 2% maltose > rice washing water > 2% millet sprout liquid. The rice tissue liquid in different parts is also conducive to spore germination, and the effect of pollen was the best. Under suitable temperature, the germination rates of yellow chlamydospore in water and rice pollen exceed 80% and 90% after culturing for 5-6 h, and the dark green chlamydospores were only 10% and 35%, respectively (Liu et al., 1989). pH value showed a significant effect on the germination and sporulation of chlamydospore, Neutral partial acid was conducive to germination and sporulation of chlamydospores, but peracid or parlkaline (pH 3.0 and pH 10.0) obviously inhibited sporulation and spore germination (Wang et al., 1998). Regarding the effects of light on the germination of chlamydospores, there are several different views. Wang (1988) and Liu et al. (1989) held that sunlight, fluorescent lamp, UV lamp irradiation had no significant effects on chlamydospore germination, but they could inhibit the formation of microspores (Wang, 1988; Liu et al., 1989). However, Lu et al. (1996) demonstrated that light had a stimulating effect on the germination of spores.

2.3.4 Conidiophore Culture

Conidia production and germination of *U. virens* are closely related to strains and culture conditions. Some strains can produce conidia, while some cannot. The same *U. virens* was cultured in four types of media for 144 h, and the medium with the most sporulation quantity was PS, followed in order by PD, YPPD and PW (Wang et al., 1998). *U. virens* was cultured in a liquid medium for 7-9 d at a temperature of 26 °C, and the cultured mycelium was placed into the plate medium for culturing for 3-5 d in the dark, after which a large number of spores was produced (Fujita et al., 1989). A large number of conidia were also produced by shaking the culture of mycelia in a PS medium for more than 7 d (Lu et al., 1996).

Potato, glucose and rice juice solid medium are the most suitable media for mycelium growth, while potato and dextrose broth are the most suitable for mycelial growth and sporulation (Lv et al., 2009). *U. virens* was cultured in PSB medium at altered temperatures of 22-29 °C and a constant temperature of 28 °C under natural lighting conditions for shaking culture for 12 d, and the lowest sporulation quantity reached 6.3×10^7/mL, followed by

PDB medium, at 1.1 × 10^6/mL (He et al., 2011). The conidia germination temperature was 22-31 °C, and the optimum temperature was 28 °C, the optimal pH was 6-7. The PSA media was most suitable for germination (Zhang et al., 2003a, 2003b). *U. virens* was cultured in a PSB for 9 d, and the conidia concentration reached 7.2 × 10^7/mL (He et al., 2011). More than 12 months can survive if *U. virens* was periodically transferred in paraffin oil storage, which was known as suitable method for the storage of *U. virens*.

2.4 Sclerotium Germination

The temperature, humidity and illumination could affect the germination of sclerotium. The germination of *U. virens* sclerotia must undergo a period of dormancy. After winter dormancy, the sclerotium is more conducive to producing sporophores and ascospores. Whether the collected sclerotia germinate and produce sporophores, 12 h of light is needed (Dong et al., 1989). Sclerotia do not germinate after wintering under dry conditions, while at moist conditions and the temperature is 26-28 °C it could germinate. The dormant period of sclerotia can reach up to more than 6-7 months when the average temperature is below 20 °C, while the average temperature is above 27 °C, the dormant period is 3-6 weeks (Liao, 1994).

2.5 Genetic Diversity of U. virens

Information about the genetic diversity and population structure of *U. Virens* is essential for rice breeding and efficient control of the RFS (Sun et al., 2013).

Strains isolated from different regions or different rice varieties are distinguishable in genetic diversity and in virulence to rice. 110 isolates of *U. virens* isolated from Liaoning and Beijing of north China were analyzed by using amplified fragment length polymorphism (AFLP) markers. The isolates can be divided into three groups according to the genetic distance and the isolates from the same region can be placed into one group (Zhou et al., 2008). The coefficient of strains from Liaoning and Beijing was 0.92 and 0.55, respectively. There was no specific DNA pattern for the isolates from the same rice varieties，and there was no co-relation between the clusters based on genetic similarity coefficient and variety origin of isolates (Pan et al., 2007b). 59 isolates of *U. virens* what isolated from three rice varieties of hybrid in Sichuan province of west China could be classified into six groups based on their virulence to rice varieties (Lu et al., 2009).

The rDNA-ITS fragment of *U. virens* was amplified by using ITS4 and ITS5, and the electrophoresis band of PCR. The sequencing analysis showed that the rDNA-ITS sequences of 35 strains came from different parts of China were completely consistent, i.e. the homology was 100%. The sequence alignment results showed that the ITS homology of 35 strains and the strains collected from Zhejiang, Liaoning, Yunnan provinces, and Japanese (AB116645 and AB105954) were all 100%, indicating that the ITS sequences of *U. virens* from different geographical origins or ecological zones were highly homologous or completely consistent (Zhou et al., 2003). RAPD technology was used to analyze the population genetic structure of 55 strains from nine regions of eight provinces of China and one strain in Japan. The results exhibited that for the strains from different geographical origins was difficult to divide their geographical lineages, and the degree of differentiation of diversity of *U. virens* was relatively low (Zhou et al., 2004; Pan et al., 2006; Wang et al., 2009).

However, some studies suggested that *U. virens* exhibited rich DNA polymorphism and genetic diversity. The strains from different years and different regions had significant genetic differences, and the genetic grouping of RFS in different located was related to geographic origin (Zhou et al., 2008; Zhang et al., 2009). Yang et al. (2011) illustrated that *U. virens* in Fujian province had a rich genetic diversity, and the change range of genetic distance was between 0.02 and 0.67. The genetic diversity level of the strains isolated from western Fujian province was the highest (*PPB* = 76.43, *H* = 0.2212, *I* = 0.3383), and the genetic diversity of the isolate group from late rice (*PPB* = 91.08, *H* = 0.2402, *I* = 0.3655) was higher than that of early rice populations (*PPB* = 63.06, *H* = 0.1892, *I* = 0.2870). It was deemed to the geographic origin of isolates, rice varieties and their growing season are the main factors affecting the genetic diversity of *U. virens* in Fujian province, which may play an important role in the genetic variation and occurrence and prevalence of RFS.

The biological method and RAPD-PCR technology were used to analyze the mycelial growth rate, conidia production, spore germination rate and genetic diversity of 84 strains from 11 provinces (municipalities) in China. Based on the mycelium growth rate, the strains can be divided into two types of fast and slow, accounting for 58.33% and 41.67%, respectively. According to the sporulation ability and conidia germination ability, the isolates can be divided into three types of strong, medium and weak. Isolates both from the same and different regions showed different variations, and the variation degree of the strain groups of inland areas was significantly higher than that in the coastal areas (Wang et al., 2012b).

The DNA genetic diversities of 60 *U. virens* strains from six *indica* rice area in Sichuan province were investigated by means of ERIC-PCR fingerprint technology with UPGMA cluster analysis and similarity analysis. At the similarity level of 0.75, the tested strains were divided into 11 genetic types. The genetic similarity of *U. virens* from the same area is higher, while from different regions showed different degrees of variation. The correlation between the varieties and genetic differences of *U. virens* was low (Zhang et al., 2009).

2.6 Pathogenicity of U. Virens

Forty-six single spore of *U. virens* isolates were employed to inoculate three rice varieties of "Yue 938", "Huai 9508" and "Wuyunjing 3", which show different resistance level to RFS, to study the differentiation of the pathogenicity of *U. virens*. The response of different resistance rice varieties showed different on the same strain; similarly, the pathogenicity of different isolates to the same variety also showed significant differences, suggesting that the pathogenic differentiation of the strains of *U. virens* is significant (Chen et al., 2009; Pan et al., 2012; Yin et al., 2014).

2.7 Strain-Host Interaction

There were different viewpoints regarding whether there is interaction between the *U. virens* strains and rice varieties among different researchers. Zhang et al. (2003b) and Lu (2013) held that there were specific and significant interaction phenomena between rice varieties and strains of *U. virens*, the reasons are: (1) The pathogenicity differences of different *U. virens* strains on the same rice variety can generally be divided into three strain types of weak, moderate and strong virulence; (2) different rice varieties had different resistance to the same strain, which can be divided into the four types of moderate resistance (MR), moderate susceptible (MS), susceptible (S) and high susceptible (HS).

Jiang (2014) held that different rice varieties, showed significant difference in resistance to RFS, and there were significant pathogenicity difference among 25 *U. virens* strains. The relationship of *U. virens* strains and rice varieties could be divided into weak interaction and strong interaction, of which the weak interaction accounted for 91.3%, and the strong interaction was 8.7% (Yin et al. 2014). For example, the variety of Hui 9 was immune to strain GD1001 of *U. virens*, and the variety Jinyou 207 was susceptible to GD1001; in addition, the variety Hui 9 was susceptible to strain GZ1001 of *U. virens*, while the variety Jinyou 207 was immune to strain GZ1001. It indicates that there is strong interaction of rice varieties and *U. virens* strains of RFS (Pan et al., 2012). However, according to the Zhou et al. (2004) and Pan et al. (2006, 2007b) preliminarily concluded that there was no specific interaction between rice varieties and *U. virens*.

2.8 Host Range of U. virens

There has been no report on the host range of *U. virens* by artificial study, but the survey found that *U. virens* not only infected rice, it also infected corn and some weeds in fields, such as *Digitaria marginata*, *Panicum trypheron* and wild rice (Shetty et al., 1987; Abbas et al., 2000). It was found that there was a similar RFS pathogen on dry grass, and the two pathogens cross inoculations could lead to pathopoiesis of each other from rice and dry grass (Shetty et al., 1987). Atia (2004) reported that the weeds of barnyard grass (*Echinochloa crusgalli*) and cogongrass (*Imperata cylindrica*) in Egypt could be infected by *U. virens*. It has been reported in China that there were similar cases of RFS in *Sporobolus fertilis* (Steud.) (Li et al., 1986), and weeds with similar symptoms also discovered in paddy field weeds in many other locations (Hu et al., 2012).

2.9 Molecular Detection of U. virens

The advent of genetic transformation and several techniques have opened the possibilities for studying the interactions between plant and pathogen, including agrobacterium mediated transformation (Zhang et al., 2006) and electroporation (Tanaka et al., 2011) have been developed for the transformation of *U. virens*. A recent study utilized a transgenic strain expressing green fluorescent protein gene (GFP) (Ashizawa et al., 2012) to observe the initial infection of rice panicles before heading.

Zhou et al. (2003) designed specific primers and established a method for detection of *U. virens* with nested PCR, by using the sequences intraspecific conservative characteristics of the rDNA-ITS of ribosomal internal transcribed spacer of *U. virens*. It was found that there was attachment or infection of *U. virens* on the auricle of flag leaves at early reproductive growth stage of rice; at the same time, the *U. virens* could also be detected in the flag leaf ear of rice early reproduction growth and duckweed in the field (Zhou et al., 2006). Ashizawa et al. (2005) detected *U. virens* in the inoculated and non-inoculated rice at the booting stage, indicating that the *U. virens* spores could naturally intrude into the spike bud outer rice husk, and could attach to or infect young glume, thus suggesting that early and late booting stages of rice was an period of vadility for *U. virens* conidia

infection (Chen et al., 2013). The establishment and application of these technologies laid a solid foundation for the in-depth study the regularity of *U. virens* infection, as well as rapid and accurate detection and prediction of RFS (Zhou, 2004).

A series method of high sensitivity to detect the pathogen of RFS in rice plants and soil have been developed recently, known as PCR-based (Zhou et al., 2003), nested PCR (Zhou et al., 2006) and the "real-time PCR" method (Ashizawa et al., 2010). We can use these methods to detect less than 50 fg DNA of *U. virens*, the equivalent of eight chlamydospores in a gram of soil. The real-time PCR assay for the soil samples was at least 100-fold more sensitive than the conventional and nested-PCR assays tested. It may be a useful tool for optimization of disease control strategies (Ashizawa et al., 2010).

3. Ustiloxins

RFS not only caused a reduction of rice yield, increased empty grains and broken rice, decreased milled rice rate and quality of rice, but also had harm to plants and animals due to the toxins produced by *U virens*. The *U. virens* could produces large amounts of mycotoxins, the ustilotoxins (more than 100 mg kg^{-1} false smut balls) which inhibit cell division in animals and plants and thus frequently cause animal poisoning (Koiso et al., 1998; Nakamura et al., 1994; Li et al., 1995). The toxicity produced by different *U. virens* strains was quite different, the toxicity of toxin produced by white strain of *U. virens* was stronger than that of ordinary (black) strain (Bai et al., 1997). The *U. virens* of RFS is poisonous when the incidence exceeds certain degree and the grain should not be fed to animal. The chlamydospores and conidia also contaminate the rice grains and straws with their antimitotic cyclic peptides (known as ustiloxin), which are poisonous to both humans and animals (Koiso et al., 1994).

3.1 Research of Ustiloxins

In the early 20th century, it was found that *U. virens* extract was toxic to rabbits and other animals. In 1933-1937, Yabuta isolated a pigment from the ether extract of *U. virens* for the first time, called Ustilaginoidin. The structure of Ustilaginoidin and its homologues were ascertained (Shibata et al., 1963; Tsuchita et al., 1987), and found that the mechanism of the action of *U. virens* was different from the mechanism of plant toxins. In the 1950s, Chinese pathologist pointed out that *U. virens* contained toxic pigment $C_9H_6O_7$. Further study found that the toxin was a kind of alkaloid compound (Deng, 1989; Ma et al., 2001). Japanese scholars found that the Ustiloxins of *U. virens* was a cyclic peptide, a kind of anti-eukaryotic cell mitosis, including a 13-ring, in which there is an ether bond (Koiso et al., 1992).

Up to now, it has been found that there are two kinds of secondary metabolites of *U. virens*, one is colored fat soluble substance called "Ustilaginoidins", belongs to naphtho-pyrones; the other one is a water-soluble colorless substance called "Ustiloxins", also known as ustilazin , which is a cyclic peptide. It believed that the RFS toxin was produced by the chlamydospores of *U. virens* and the false smut (Jiang et al., 2010). There were six kinds of toxins had been isolated from *U. virens* till now, namely Ustiloxin A, B, C, D, F and E, and their molecular formulas were $C_{28}H_{43}N_5O_{12}S$, $C_{26}H_{39}N_5O_{12}S$, $C_{23}H_{34}N_4O_{10}S$, $C_{23}H_{34}N_4O_8$ and $C_{21}H_{30}N_4O_8$, respectively. Due to the fact that the isolated quantity of Ustiloxin E was too less to conduct an experiment, its structure and molecular formula were not clear (Kosio et al., 1994, 1998).

3.2 Biological Activity of the Toxins of U. virens

3.2.1 Toxicity to Plants

Crude toxin of *U. virens* had strong inhibition effects on the germination of rice, wheat and maize seeds, as well as the growth of radicles and plumules. The inhibitory effect on the radicle growth is stronger than that of embryo growth and seed germination (Bai et al., 1997; Tian et al., 2000; Gao et al., 2013). Rice seeds were treated with the toxins of *U. virens*, the seeds germination of resistant varieties could be inhibited, on the contrary, the seeds germination of susceptible varieties were promoted. This suggested that there is a correlation between the inhibition ability of ustiloxins on rice seed germination and the resistance level of rice varieties (Gao et al., 2013). Ustiloxins could inhibit the mitosis of garlic root tip cells, but it did not inhibit cell elongation (Chen et al., 2004). Abbas et al. (2014) demonstrated that the extract of *U. virens* from Arkansas, USA, had almost no effect on rice seed germination, but it did exhibit toxicity to duckweed.

3.2.2 Toxicity to Animal

Ustiloxins of *U. virens* is a kind of cyclic peptide that resistant to mitosis of eukaryotic cells (Koiso et al., 1994), and it has a wide range of biological activity on animal cells. The active mechanism of Ustiloxins is the inhibition of mitosis of animal and plant cells (Nakamura et al., 1992; Ludueña et al., 1994; Li et al., 1995). The liver cells and renal tubular cells of mice *in vitro* were rapid necrosis after one-time injection with Ustiloxins

(Koiso et al., 1994). It also suppressed the cell mitosis or caused abnormal mitosis, which was similar to the symptoms expressed with colchicine. Ustiloxins A and B could inhibit the mitosis of a variety of human tumor cells, it is stable to heat, and the toxicity is not destroyed by heating at 100 °C for 30 min (Chen et al., 2004).

Crude toxins of *U. virens* can caused acute, occasional necrosis of hepatocytes and renal tubular cells, followed by increased number of mitotic figures with occasional multinuclear giant cells. Erosions and ulceration of the forestomach and atrophy of the thymus were observed a week later (Nakamura et al., 1994). Feeding rabbits, chickens, mice and other animals with rice grains mixed with RFS can cause lesions of the liver, kidney and other internal organs (Shang et al., 1985; Nakamura et al., 1993; Bai et al., 1997). The pathological change of the animals' organs and/or death were caused after feeding rice grains contaminated by RFS for 35-84 d. The mortality rate of the rock roosters was 37.5%, and the lethal dose was 0.14-0.17 g RFS grains daily consumption of per kg of animal body weight, which could lead to an inability in the laying hens to lay eggs, as well as ovarian atrophy (Leng, 1984; Gao, 1992).

Feeding pigs with the feedstuff mix with 0.5% infected rice grains of RFS, it could slowed down the growth of the pigs, decreased the pigs' weight gain rate, and pathological changes of multiple organs, such as liver, kidney and spleen and other diseases were caused. It also affected sow's reproductive performance, such as ovarian hyperemia, hemorrhage; decreased the litter size, the weaning litter weight and the survival rate of piglets(Shang et al., 1985). At the same time, the phenomena of stillbirth and/or mummification of fetal and fetal malformation were also present (Huang et al., 2002). Ducks fed with rice containing 5% of RFS grains could cause hepatomegaly (Huang et al., 2002; Wang et al., 2008a). A typical example was from Shexue Township of Guizhou Province, from 1999 to 2002, 1914 livestock and poultry appeared a kind of disease with the main symptoms of diarrhea, fever, salivation, vomiting, central nervous excitement or paralysis, shortness of breath, and rapid heartbeat. The animals often died of severe dehydration and exhaustion, and the death rate reached 71.12%. It was diagnosed as feeding infected rice grains of RFS and resulting in toxin poisoning (Wu, 2004).

3.3 Utilization of Ustiloxins

3.3.1 Use as Resistance Screening Agent

Rice seeds of resistance varieties were treated with toxin of *U. virens*, the seeds germination could be inhibited; on the contrary, the seeds germination rate of susceptible varieties were promoted. It provided a simple and efficient method for the identification the resistance of rice varieties to false smut (Ma et al., 2007; Gao et al., 2013). The crude toxins of RFS were employed as selection pressure to screen rice resistance mutants to RFS, and the resistance of various rice varieties at the cellular level was consistent with that of the rice in fields. It suggested that it is feasible to select disease resistant mutants with the crude toxins of *U. virens* as the selection pressure.

3.3.2 Anticancer Drugs

Due to the Ustiloxin A and B can inhibit the mitosis of a variety of human tumor cells (Kosio et al., 1994), it is possible to develop the fungal toxin of false smut into cancer targeted therapy drug by using modern molecular biological technique and gene engineering technology.

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Research on Advance of Rice False Smut *Ustilaginoidea virens* (Cooke) Takah Worldwide: *III. Infection Cycle and Invasion Mechanism of U. virens and Rice Resistance to RFS*

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Abstract

In this part, the infection cycle and invasion mechanism of RFS, including the primary and secondary source of RFS infection. The factors affecting the disease occurrence and epidemiology, including the infection time and pathway, different conditions affecting the incidence of RFS, for example, the type of rice varieties and morphological characteristic, meteorological factors, environmental conditions, cultivation management, and amount of *U. virens* in the field. The mechanism of rice varieties' resistance to RFS were also discussed, including morphological characteristics, and biochemical mechanism, resistance genes of rice.

Keywords: *U. virens*, Infection cycle and mechanism, affect factors, rice resistance and mechanism

1. Introduction

To understand the invasion mechanism and cycle of *U virens*, the resistance and the resistant mechanism of rice varieties to RFS, there are significance for further study of *U. virens* and effective prevention and control of RFS, as well as rice genetic breeding for disease resistance. There are different viewpoints on the source of infection in the early, but a more consistent view was that the overwintering sclerotia and chlamydospore are the main original resources of infection. There are many factors affect the occurrence and prevalence, for example, the climate conditions, temperature, humidity, hours of illumination, especially the "key growth stage of rice" (KGSR, *i.e.*, the late booting stage and begin heading to flowering period are the susceptible period of rice to RFS) encounter the local climatic conditions which suitable for the occurrence of RFS; The resistance of rice to RFS, fertilizer and water management, and amount of original *U. virens*. The mechanism of rice varieties' resistance to RFS was determined by itself genetic, for example, the morphological characteristics of rice variety, the content and activity of biochemical substances. However, the most essential factors are the resistance genes of rice varieties.

2. Disease Cycle of RFS and Factors Affecting the Disease Occurrence

Knowledge of the pathogen's life history and infection process in nature is critical for disease control (Tang et al., 2012b).

2.1 Primary Infection Source of RFS

U. virens can overwintering in the form of mycelium, chlamydospore, sclerotium and RFS balls. The overwintering sclerotia germinated and produced ascospores, which caused rice false smut in the coming year (Liao, 1994). Inoculation with chlamydospore, ascospores and thin-wall conidia can successfully induce RFS (Chen et al., 1995; Huang et al., 2002; Yao et al., 2012). Zuo et al. (1996) captured chlamydospores over the rice fields, suggesting that chlamydospores have the ability of air flow spreading. Therefore, it is deduced that there is a pathogen source base or intermediate host suitable for the dormancy of chlamydospore outside rice fields. However, there is a totally different point of view on this point. Chen et al. (1994c) believed that chlamydospores in soil germinated and produced conidia, and the conidia spreading by wind and rain and caused the primary infection; However, the sclerotia were not found in some areas of some provinces in China, but the rice was infected by *U. virens* every year, so it was really questionable that sclerotium was the major primary infection source (Wang, 1992; Chen et al., 1995). On the other hands, Liu et al. (2009b) demonstrated that the primary infection source of RFS mainly was the seeds with pathogen, followed by the overwintering pathogen in soil.

Most scholars abroad were consistent with the views that the pathogens overwintering in the form of sclerotium and chlamydospore were the major primary infection sources, and the chlamydospores played a decisive role in the secondary infection (Ikegami, 1963; Ou, 1985).

2.2 Secondary Source of RFS Infection

A more consistent view on the secondary infection source of false smut is chlamydospores. The chlamydospores in soil germinated and produced conidia, and the conidia caused primary infection by spreading through wind and rain. Many studies have reported that there was a severe incidence of RFS in late maturing varieties, and that the secondary infection may be the major factor (Chen et al., 1994c). A large number of chlamydospores could be captured over the rice field, and the number of spores was relatively increased with the arrival of the flowering period. Evidently, the secondary infection source of RFS is mainly chlamydospores and conidia by air spread (Ou, 1985).

2.3 Infection Time and Pathway

The infection periods and pathways of *U. virens* are not very clear. There are main views of the seedling stage system infection, or late booting to early heading stage infection (late stage infection), or systemic infection and late growing stage infection. However, more advanced inoculation and detection technology have been employed recently, indicating that RFS infection is mainly in the late stages.

2.3.1 Systematic Infection in Seedling Stage

The experimental results demonstrated that the infection of *U. virens* may be the seedling stage systematic infection. When rice seeds with pathogen germination, the pathogenic spores can successfully infect a large number of radicles and coleoptiles, and extend along the outer surface of the sieve tube of the phloem until reaching the middle and late stages of tillering (Ikegami, 1962, 1963; Schroud et al., 2005). It was subsequently found by histological observation and molecular techniques detected that the pathogen can attack the root at the seedling stage and lead to symptomless colonization of the entire plant (Ditmore et al., 2006; TeBeest, 2010). Dai et al. (2005) detected and found that the conidia of *U. virens* formed mycelium on the surface of the glume and extended into the inner hull, which provides evidence for the direct infection of conidia on rice grains.

A large number of comparative field studies regarding to whether the rice seeds with pathogen can lead to occurrence of RFS have conducted. Some results indicated that seeds with pathogens can cause RFS (Chen et al., 1994b; Liu et al., 2009b). Overwintered chlamydospores could infect seeds, seedling coleoptiles, leaves and roots of the early rice, and cause RFS during heading stage (Chen et al., 1995; Gao et al., 2011). Rice seeds contaminated by *U. virens* and treated with biocidal (pathogens-free) were sown and planted under sterile soil and isolated conditions, the rice seeds carrying pathogens could induce RFS, while the sterilized seeds could not induce diseases, indicating that the seeds could carry pathogens and caused RFS (Liao, 1993). By using spray or injection inoculation with chlamydospores at the rice seeds germination, seedling or booting stages, it could successful caused RFS (Ikegami, 1962, 1963; Miao, 1992).

2.3.2 Late Growth Stage Infection

Most researchers believed that the primary infection site was the floral organ of rice, and the infection period was between the middle and late booting stages to the early heading period (Xu et al., 2001; Wang et al., 2008; Chen et al., 2013). Liu et al. (2007) believed that the main infection period of *U. virens* was between the big belly stage to begin heading period, but not the seed germination stages. Strong evidences support that the infection occurred in flowering stage was that artificial inoculation in the late booting stage could increase the

diseased panicle rate substantially (Cai et al., 2009; Gao et al., 2011). At present, it was believed that 1-2 weeks before heading are the main invasion period of RFS (Li et al., 1986; Wang, 1992; Guo et al., 2000). Du et al. (1990) illustrated that seed treated with biocidal there was no effect of prevention and cure RFS, and inoculation with *U. virens* after germination of rice seeds also did not incur the disease, proving that *U. virens* is not systemic infection.

An experiment of bagging protection was conducted, the results have shown that there was no occurrence of RFS when rice plants were bagged in elongation stage and early booting stage, while RFS occurred in all of the treatments of bagging after the late booting stage (pollen mother cells (PMC) filling stage), indicating that the infection of RFS began early booting and late booting stage (Deng et al., 1990) (Table 1).

Table 1. Studies on the infection stage of RFS by bagging

Treatment	Rate of infected panicle%	Rate of diseased grain‰	Treatment	Rate of infected panicle%	Rate of diseased grain‰
Bagging at elongation stage	0	0	Inoculation at elongation stage	0	0
Bagging at early booting stage	0	0	Inoculation at early booting stage	0	0
Bagging at late booting stage	15.0	5.68	Inoculation at late booting stage	38.6	17.8
Bagging at begin heading stage	9.5	3.86	Inoculation at begin heading stage	18.5	8.24
Bagging at full heading stage	5.2	1.12	Inoculation at full heading stage	5.2	3.36

From the incidences of RFS of different period inoculation with *U. virens*, there was no RFS occurred during the elongation stage and the meiosis stage of PMC, while most serious occurred of RFS with inoculation during late booting stage and begin heading stage. Inoculation at begin heading and full heading stages of rice, there were a small amount of RFS balls appeared, suggesting that the infection of RFS mainly occurred after late booting stage (Hu et al., 2010; Liu et al., 2009b). The inoculation was performed at 6 to 9 d before heading, then the incidence were the highest either for resistant or susceptible rice varieties; in addition, the incidence was very low or there was no occurrence of RFS for the inoculation at 10 to 13 d before heading (Zhang et al., 2004).

Field observations and the results of inoculation experiments combined with histological studies, now suggest that the most likely route of *U. virens* infection is when rice plants are at the booting stage (Sonoda et al., 1997; Ashizawa et al., 2012). Furthermore, experiments using a mixture of conidia and hyphae of *U. virens* cultured demonstrated that serious cases of RFS could arise when the inoculation was made by injecting the panicles at the booting stage (Zhang et al., 2004; Sonoda et al., 1997; Ashizawa et al., 2011).

2.3.3 Systematic Infection + Late Stage Infection

There were also experiments supporting that RFS not only could infect systematically, but also infect at late stage (Liao, 1992). Artificial inoculation with chlamydospores at early and middle seedling stages of rice, it could induce RFS at adult periods. After three years and nine experiment replicates, it was proven that the *U. virens* could infect rice most easily from the formation of young panicle to the middle of the booting stage, and the rice was basically not be infected after begin heading (Chen et al., 1994a).

2.3.4 Infection Site and Infection Mechanism of *U. virens*

A recent cytological study indicated that the pathogen of *U. virens* infected the filaments intercellular and extended intercellular along the filament base (Tang et al., 2012b). It was found that the hyphae of *U. virens* are able to invade the spikelet apices, via a small gap between the lemma and palea.

Infection sites: Examination of serial semi-thin and ultrathin sections of infected spikelets showed that the primary infection sites of *U. virens* was upper parts of the three stamen filaments located between the ovary and the lodicules. The pathogen did not penetrate host cell walls directly and did not form typical appressoria structures and haustorium. The ovary remained alive in the RFS ball and was never infected (Tang et al., 2012b). *U. virens* did not kill the host cells, so it belongs to living nutrition type fungus (Hu et al., 2012a; Tang et al., 2012b).

In the booting stage, *U. virens* specifically infects the stamen filaments of rice, and thus it grows and develops into chlamydospores and finally formed smut ball. *U. virens* could not infect the ovary and anther, however, the secondary hyphae can occasionally infect the stigma and outer cells of lodicule (Dai et al., 2005). However,

Dodan et al. (1996) and Mandhare et al. (2008) found that conidiophore of *U. virens* could infect the ovary and single spikelet and then transformed into chlamydospores and false smut ball.

A recent study has indicated that *U. virens* follows a specific route, with the hyphae colonizing the outer surface of the spikelet, and then entering the inside of spikelet from the apex (Ashizawa et al., 2012). Consistent with a previous report (Tang et al., 2012b), *U. virens* initially attaches itself to the surface of the filaments, and then formed several discrete structures, including mycelial stroma and infection hyphae.

Mechanism of infection: The route of *U. virens* penetrates rice panicles has long been a question of debate. A recent study utilized a transgenic strain expressing green fluorescent protein gene (GFP) (Ashizawa et al., 2012) to observe the *U. virens*' initial infection of rice panicles before heading. The detection method of nested PCR to detect the *U. virens*, and found that there were attachment and infection in the early reproduction stage of rice (Zhou et al., 2003, 2006; Wang et al., 2005a). The GFP-labeled conidia of *U. virens* were injected into rice sheaths at booting stage, there were a lot of conidia present on spikelet surfaces 48 hour post-inoculation (hpi), hyphae had invaded spikelets through the apices, via the small gap between the lemma and palea and had already reached all floral organs 144 hpi (Ashizawa et al., 2012).

The primary site of *U. virens* colonization was at the base of the filaments with the inner spikelets becoming infected by hyphae at 24 hpi. The accumulation of hyphae reached its highest level at 168 hpi, before rice heading stage, as the infection extended upward from basal filaments to the anther apex, and then enclosed all the floral organs to produce a velvety smut ball (Hu et al., 2014).

2.4 Conditions Affecting the Incidence of RFS

The occurrence and epidemic of RFS was affected by many factors, for example, the amount of *U. virens* and its pathogenicity, resistance of rice varieties, climate factors, and cultivation management. The susceptible varieties and continuous rainfall for more than 5 d during rice booting and heading period are the two key factors to cause outbreak and epidemiology of RFS (Hu et al., 2010). Rice plants at late booting stage, and flowering to filling stage encounter a relatively low temperature (*i.e.*, 22-28 °C), rainy and humid climate or rainy weather exceed consecutive 5 d days, the degree of RFS incidence will be serious (Wang et al., 2005b; Liu et al., 2000).

Figure 1. Late maturing variety (left) was seriously infect by RFS but early maturing (right) no any infection

2.4.1 Resistance of Rice Varieties

The resistance difference of different rice varieties to RFS varies greatly (Chen et al., 1992, 1994c). Observation in the paddy fields combined with artificial inoculation in greenhouse and field natural infection have both proven that there was real resistance to RFS of different rice varieties (Ashizawa et al., 2011; Tang et al，2012a). Under natural conditions, the infected panicle rate and the disease index of different varieties vary greatly, and the values were in the ranges of 0.43-33.04% and 0.05-16.14%, respectively (Jin et al., 2005; Lu et al., 2008). The resistances of rice varieties were affected by the internal genetic mechanism (Xu et al., 2002; Fang et al., 2008) and external morphological characteristics.

Rice varieties types: The resistance difference of rice varieties has close relationships to types of rice varieties, plant types, and growth characteristics. In general, the waxy type varieties are more susceptible to RFS than that of the japonica varieties, and the *japonica* is more susceptible than *indica* varieties. Erect dense panicle varieties are more susceptible to RFS than that of general varieties, the incidence of RFS in hybrid rice was much more severe than that of the conventional rice varieties (Wang et al., 2004; Lv et al., 2007). The short-stalked, large panicle, wide leaf but small angle varieties are fertilizer tolerant and lodging-resistant, and they are suitable for high density planting, which are in favor of the occurrence of RFS. Varieties with long duration of tillering, booting and flowering stage, the incidence of RFS is also more serious. The RFS in the two-line hybrid rice was more serious than that of the three-line hybrid rice (Liu et al., 2009a; Tang et al., 2012a; Gan et al., 2013).

Rice plants morphology: The effects of rice plant types on the occurrence of RFS vary greatly (Hu et al., 2012b). The occurrence of RFS has a close relationship to the panicle traits, and the correlations are as follows: grain number per panicle > secondary branch grain number > secondary branch number > the seed density. The number of grains per panicle, especially the number of grains in the secondary branch, is the main causes of the high incidence of RFS (Wang et al., 2004).

2.4.2 Meteorological Factors: Temperature, Rainy Days and Rainfall, Humidity, Light, etc.

It was demonstrated with many years observation in the fields, the RFS incidence of the same rice variety in different years are quite different. If the resistant varieties of rice encounter rainy days during the booting period and begin heading stage, then the incidence of RFS will be aggravate; on the contrary, if the susceptible varieties encounter dryness and high temperature weather during these stages, then the incidence of RFS will decrease or even no be infected (Wang et al., 2004).

The climate factor that rice plants encounter in vulnerable period or KGSR is one of the key factors determining the degree of RFS incidence. If KGSR encounter more rainy days, abundant rainfall, a short sunshine duration, the relative humidity (RH) was high (above 85%), the temperature was suitable (22-28 °C), and a small temperature difference between day and night, then the degree of RFS incidence was severe. If these factors were contrary, then the degree of RFS incidence was low (Yashoda et al., 2000; Ye et al., 2005; Yang, 2007; Fei et al., 2010). It was found that the severity of RFS was closely related to the local accumulated sunshine hours and total rainfall in KGSR. If the number of sunshine hours was reduced and the rainfall was increased, then the morbidity of RFS was aggravated (Pan et al., 1997b).

The rain days and rainfall during the KGSR were positively correlated with the infected panicle rate, the correlation coefficient was $r = 0.8342^*$ and $r = 0.8826^*$, and the related equation was $Y = -6.7985 + 6.0538x$ and $Y = -2.6963 + 0.3652x$, respectively. RFS is negatively correlated with the daily mean temperature but positive correlated with humidity at the begin heading stage. When the daily mean temperature was 23-24 °C and RH 82%-87% during the begin heading period are conducive to the occurrence of RFS (Lv et al., 2007).

Shading and RFS: Rice plants were treated with two or three layers of gauze for shading, the number of infected hills, panicles and grains by RFS are 1.08-1.37, 1.19-1.66 and 2.53-3.94 times of those no-shading controls, respectively (Qian et al., 1993). The experimental results verified that cloudy and rainy weather was conducive to the occurrence of RFS (Table 1).

2.4.3 Environmental Conditions

The RFS incidences of the same rice varieties in the same year which planted in different areas are different. Occurrences of RFS are associated with altitude and ecological environment, and the incidence of high altitude was more severe than that of the low altitude; the incidence of RFS in temperate and highlands is low, while in tropical lowlands is high (Zhang et al., 2006a). Even in the same paddy field, the rice plants incidence of RFS on the edge was severe, while in the middle of the field was low (Jin et al., 2005; Chen et al., 2005).

2.4.4 Cultivation Management

The occurrence of RFS is also associated with rice cultivation and management, especially the management of fertilizer and water.

Fertilization: Different fertilizer, dosage of application and application time of fertilizer significantly affect the occurrence and severity of RFS. The incidence of RFS was higher if more nitrogen fertilizer was applied and the application time was late (Bhardwaj, 1990; Pan et al., 1993; Ye et al., 2005; Yang, 2007). Increasing the amount of nitrogen fertilizer significantly increased the rate of infected rice plants.

Fertilization habit of partial nitrogen, excess dosage of fertilizer and late fertilization will reduce the rice resistance ability to RFS (Zhao, 2008). The applied total quantity of nitrogen (X_1), amount of panicle fertilizer

(X_2) and application time of panicle fertilizer (X_3) were strongly affect the incidence of RFS (Y), the regression equation was $Y = -93.053 + 3.393X_1 + 9.265X_2 + 3.711X_3$, and the correlation coefficient $R = 0.8922^{**}$. Among all of these factors, the direct effect of the application amount of ammonia fertilizer on the RFS incidence was the highest, $P_{0.1} = 0.393$ (Pan et al., 1997a). If 600 kg/hectare of urea was used as panicle fertilizer, then the infected panicle rate was 17.5%, and increased by 34.6% and 48.9% compared with that of the 300 kg/hectare and no application of the panicle fertilizer (Chen et al., 2009a).

When 165.0, 225.0, 232.5, 240.0 and 300.0 kg of pure N was used as the topdressing fertilizer (urea) per hectare at heading period, the infected rice panicle rates of RFS were 1.33%, 1.97%, 2.13%, 2.33% and 3.13% (Chen et al., 2000), respectively. Reasonable amounts ratio of nitrogen, phosphorus and potassium fertilizer were beneficial to increasing rice yield and reducing the occurrence and harm degree of RFS (Wang et al., 2010; Hong et al., 2013; Qing et al., 2014).

Transplanting method and water management: The planting density and water management also affect the incidence of RFS. Close planting and long-term deep water irrigation can increase the incidence of RFS. Waterlogging paddy fields, especially long-term waterlogging in late stage of rice growing will lead to high humidity and result in serious occurrence of RFS (Zhang et al., 1997; Zhang et al., 2005; Yang, 2007; Wang et al., 2010).

Sowing and transplanting period. Generally speaking, the incidence of RFS of early transplanting rice groups were significantly lower than those of the late transplanting for each rice variety in the same area (Wang et al., 2010). The infected rate of the late maturing group and early maturing group varieties were investigated in 2005 and 2006, the infected rate of hill, panicle and grain were 76.3%, 26.6%, 4.6% and 16.8%, 5.4%, 4.7% for the same rice variety, respectively (Lu et al., 2006).

The sowing and transplanting time of rice will determine the time of KGSR, and affects the KGSR if encounter the local climatic conditions which suitable for the occurrence of RFS. It was reported that the reason for the severe incidence of RFS was that the heading stage met the most appropriate of the local meteorological conditions for the occurrence of RFS (Singh et al., 1981). The incidence rate of RFS of the early sowing was only 0-3.1%, while for the late sowing rice it was 48.5-56.1% (Ahonsi et al., 2000).

2.4.5 Amount of *U. virens* in the Field

The occurrence of RFS was positively correlated with the accumulation degree of *U. virens* in the fields. In general, the occurrence of RFS in the old disease areas and/or used to be serious occur areas then the RFS is high, due to the large number of *U. virens* left behind previous year was significantly higher than that of the field with little pathogen, and vice versa (Liao, 1994; Zhou et al., 2010).

3. Rice Resistance to RFS and Their Mechanism

Most researches did not find any rice varieties (materials) with immunity or high resistance to RFS (Guo et al., 2010; Jiang et al., 2010a, 2010b; Zheng et al., 2013), but there were some studies which found highly resistant and immune varieties (Huang et al., 2010). It was verified by natural infection or artificial inoculation that there were significant differences in resistance to RFS among rice varieties (Yang et al., 2008). Many research results and long-term field observation have both shown that the resistance of rice varieties to RFS was real subsistent. 198 rice varieties were identified by using artificial injection inoculation method of the conidia suspension liquid, there were 44 rice varieties with complete immunity to RFS, 34 varieties with high resistance (Chen et al., 2009b). The identification results of Pan et al. (2012) showed that the resistance of rice varieties to RFS were actual existence, for example, the most serious infected panicle rate reached up to 80%-90% of the susceptible rice variety, while for the resistant one, there was low infected panicle rate (Singh et al., 1987; Phatak et al., 1991; Lore et al., 2013). The incidence of 160 rice varieties were investigated under natural conditions, and demonstrated that there were 16 varieties with resistance (Urmila et al., 1999).

3.1 Morphological Characteristics and Rice Resistance to RFS

There are also differences in the resistances to RFS of rice varieties with different morphological characteristics (Hu et al., 2012b). RFS incidence was closely related to panicle traits, the following order affect the RFS severity from large to small, secondary branch number > secondary branch grain number > grain number per panicle > grain density. Grain density was the main factor affecting the incidence of RFS (Xu et al., 1987). The incidence was significantly and positively correlated with the flag leaf width, and extremely significantly negatively correlated with the number of panicles per unit area, flag leaf angle and plant height. The more number of grain per panicle was, the higher incidence of RFS. Large-panicle type, more grain numbers and high grain density of rice cultivars were more easily to be infected by RFS (Chen et al., 2011).

3.2 Biochemical Mechanism and Rice Resistance to RFS

The glume of resistance varieties had large amounts of lignin, while the susceptible varieties had less lignin; and the red fluorescent substances in the glume of the resistant cultivars were higher than those of the susceptible one. The endosperm cells of the resistance varieties had more polyphenols, but there was no polyphenols in the susceptible varieties (Dai et al., 2005). Rice plants were inoculated with conidia suspension of *U. virens*, the content of MDA was significantly increased in the susceptible varieties, and the content changes of resistant cultivars were smaller; the CAT activity of the resistant varieties was decreased, and that of the susceptible varieties was increased. The activities of POD, PAL and PPO of the resistant varieties increased, and the susceptible cultivars did not show significant changes when rice plants were inoculated with conidia suspension of *U. virens*; but the differences of SOD activity between the resistant and susceptible varieties were not significant (Jiang, 2010; Li et al., 2010). The activity of the CAT of the susceptible varieties decreased, while the PAL activity increased after inoculation (Lu, 2013). Gan et al. (2013) believed that there were no inevitable correlations between rice resistance and the activities of the defense enzymes POD and SOD. Brassinosteroids in rice plant may have more important roles than that of salicylic acid and ethylene in response to *U. virens* infection, the disturbance of other hormones such as auxin, gibberellins and jasmonates may also affect *U. virens* infection (Yang et al., 2014).

3.3 Resistance Genes of Rice

Hereditary resistance to RFS of rice was not only controlled by major gene, but also affected by multiple genes, which was consistent with the E-1-3 genetic model, namely two pairs of major genes plus the polygene mixed genetic model. The heritability of the major gene was 76.67%, and the polygene was 22.86% (Li et al., 2008). The resistance of rice varieties to RFS was affected by additive and non-additive effects, and the effect of the female parent had a critical influence (Wang et al., 2013). The dynamic expressions of RFS progression- related protein genes including *OsPR10a*, *OsPRla* and *OsPR1b* were analyzed by using RT-PCR. The expressions intensity of PRPG *OsPR10a*, *OsPR1a* and *OsPR1b* in incompatible interaction process were shown to be higher than those of the compatible interaction (Lu, 2013).

3.4 The Genes Location of Rice Resistance to RFS and Their Distribution

There were 146 molecular markers were selected from the recombinant inbred lines (RILs) of Lemont/Teqing. The total genetic distance of 146 markers was 2227.6 cM, covering 95.6% of the donor genomes, and the average adjacent marker distance was 15.2 cM. There were two anti-RFS of rice QTL (*QFsr10* and *QFsr12*) were located on the 10^{th} and 12^{th} chromosomes, the enhanced resistance alleles were from the parent Lemont, and the respective additive effects were 3.38 and 3.34 (Xu et al., 2002). Quantitative resistance loci (QRL) was identified using 213 introgression lines (ILs) from a cross between Teqing (recipient) and Lemont (donor). Ten QRL affecting percentages of infected hills, panicles and spikelets were detected and mapped to rice chromosomes 2, 3, 4, 6, 8, 10, 11 and 12. Four QRL of qFSR-6-7, qFSR-10-5, qFSR-10-2 and qFSR-11-2 had relatively larger and consistent effects (Zhou et al., 2014).

Seven QTLs of *qFsr1, qFsr2, qFsr4, qFsr8, qFsr10, qFsr11* and *qFsr12* were detected (Li et al., 2011), which were respectively located on the 1^{st}, 2^{nd}, 4^{th}, 8^{th}, 10^{th}, 11^{th} and 12^{th} chromosomes, and the contribution rate was 9.8%-22.5%. The explanation rate of the trait of *qFsr10* and *qFsr11* was between 18.0%-19.3% and the disease index of RFS was decreased by 8.0-16.3%.

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Research on Advance of Rice False Smut *Ustilaginoidea virens* (Cooke) Takah Worldwide: *IV. Identification of Rice Resistance to RFS, Management and Prospection of RFS*

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Abstract

Technological issues in identification of rice resistance were discussed in this part, including inoculum and its preparation, inoculation concentration of inoculum, inoculation time and method. It is very difficult to control RFS, RFS can only prevent but not cure, *i.e.*, to control the RFS only spray fungicides before symptomatic appearance (rice smut balls appeared) can achieve better control efficiency, while once the smut ball appeared it could not be controlled even the concentration of fungicides increase several times, or spraying multiple times. Therefore, in order to achieve better control efficiency to RFS, integratd control measures need to be adopted. The integratd control measures including agricultural control, rice resistant varieties application, cultivation techniques, fertilization and water management, and fungicides application is the last approach and emergency measure for controlling RFS.

Keywords: RFS, resistance identification, identification technologies, accurate control techniques, prospection

1. Introduction

To obtain rice materials with true resistance to RFS is the basis and the key of success or failure for rice genetic resistance breeding to RFS. It is necessary to carry out the identification of rice varieties' (lines, materials) resistance to RFS. Scientific, reliable and standardized technologies of identification is the basis for screening out true resistance materials of rice. To establish the unified and standardized identification technology system for rice resistance to RFS is necessary. Technological issues in identification of rice resistance to RFS were discussed. Due to the difficulty to control RFS, and it only can prevent but not cure. Therefore, in order to achieve better control efficiency to RFS, integratd control measures need to be adopted. The integratd control measures including agricultural control, resistant varieties application, cultivation techniques, fertilization and water management, and fungicides are the last approach and emergency measures for controlling RFS. Precise timing of the optimum fungicide application time by "physical signs" of rice was presented, "one soaking and two spraying, timing by phyllula distance". "One soaking" means rice seeds soaked with fungicides for sterilization, "two spraying" means first spraying fungicides at 5-12 d before begin heading and begin heading, or 1-3 cm negative phyllula distance, or 1/3-1/2 rice plants in the field were at zero phyllula distance, the second spraying was at begin heading or 5-12 d after first spraying. The technology shows the characteristic of simple and convenient, easy to master, precision and high control efficiency. The authors' views of problems existing in research of RFS, as well as the research direction in the future, are put forward in this paper.

2. Identification of Rice Resistance to RFS

Field testing is the closest to the natural conditions, and it is the main method to evaluate the resistance of rice varieties (Sonoda et al., 1992; Kurauchi et al., 2006). However, due to the fact that the field experiment consumed large amounts of time and man power; at same time, it was restricted by many factors, and the accuracy of the evaluation was affected, the repeatability of the test results was poor. Under controlled greenhouse conditions, artificial inoculation identification was employed, the efficiency was high and it could be repeated several times in a year. The results were accurate and the repeatability was good (Ashizawa et al., 2011). However, for the artificial inoculation under either natural conditions in the field (Zhang et al., 2003, 2004) or greenhouse of controlled conditions (Yang et al., 2011; Ashizawa et al., 2011; Hu et al., 2014), the identification of rice varieties' resistance to RFS was successful.

2.1 Inoculum and the Preparation

The chlamydospores, conidia, or conidia and mycelium fragments were used as the inocula for inoculation, which all could successfully induce RFS, and the effects of conidia and mycelium fragments were the best (Zhang et al., 2004; Yang et al., 2011; Ashizawa et al., 2011; Hu et al., 2014). At same time, the strains with strong pathogenicity, large sporulation and high germination rate of spores should be selected as inocula.

The inoculation effect of *U.virens* + potato sucrose broth (PSB) media as inocula was the best. The inoculation effect of the pathogen cultured in PSB for 5-7 d was good, but the inoculation effect was significantly decreased with the prolonging of the culture time. If the conidia concentration of the inocula was high, then the infected panicle rate and the diseased grain number was high. The PSB culture medium with a conidial concentration of 4×10^6 spores /mL was used for injection inoculation at middle and late booting stages of the susceptible variety, the incidence rate of panicle was 100%, and the average number of the diseased grain was 35.1, with the highest number of 87 (Yang et al., 2011). Rice plants were inoculated with conidia and chlamydospores of *U. virens*, the disease index of inoculation with conidia was higher than that of chlamydospore in both the field and greenhouse (Zhang et al., 2004). There was also a report showing that the inoculation effect with conidia cultured for 3-4 days was good (Ashizawa et al., 2011), which was about 7 days shorter than that of the common culture time (Fujita et al. 1990a).

2.2 Inoculation Concentration of Inoculum

The resistance or susceptibility of rice varieties to RFS could be effectively differentiated when rice plants were inoculated with 5×10^5 conidia/mL suspension of *U. virens* (Fujita et al., 1990a, 1990b). The concentration of 7.5×10^5 conidia/mL could also identify the resistance level of rice varieties to RFS, but it would result in that 30% of susceptible varieties without heading. The concentration of 5×10^4 conidia/mL could not differentiate the varieties' resistance to RFS (Ashizawa et al., 2011). Increasing the dosage of inocula (0.2, 0.5, 1, and 2 ml of a mixture of hyphae fragment and 2×10^6 conidia/ml suspension) caused more severe infections. There were small differences for different inoculation sites, for example, at the base, apex and mid of rice panicle. The optimum inoculation condition was 1-2 ml inoculum of hyphae fragment and 2×10^6 conidia/ml mixture suspension injected into the mid-point of rice panicle (Hu et al., 2014). If the concentrations of 1, 2 and 4×10^6 conidia/mL were used for inoculation, the average numbers of the diseased grain per panicle were 10.25±1.33, 20.92±1.69 and 24.38±2.05, and the highest numbers of the diseased grains per panicle were 19, 32 and 42 grains, respectively (Yang et al., 2011).

2.3 Inoculation Time and Method

2.3.1 Inoculation Period

Different rice varieties were artificially inoculated at different growth stages, the incidences of RFS were different. The experiments of nine times repetition in three years, it was proved that the rice plants during the young panicle formation period and middle booting stage was most vulnerable to be infected, and there was basically no infection after the heading stage (Chen et al., 1994). The comparative studies were conducted in greenhouse and field natural conditions. Rice plants were inoculated with chlamydospores and conidia, it was found that the RFS incidence of infected hills, panicles and grains during the booting stage inoculation were significantly higher than those of the begin heading and full heading stages, and also significantly higher than those of the control (Lu et al., 1996). Therefore, to identify the rice varieties' resistance to RFS, the most suitable inoculation time was booting stage of rice. If the chlamydospores of *U.virens* stored at -20 °C for a long time and was used for injection inoculation, it could not cause RFS (Zhang et al., 2003), whereas the inoculation of the thin-wall conidia obtained from the potato sucrose liquid medium could cause diseases. The higher the concentration of spores, the higher of the infected panicle rate is. The inoculation performed at 6-9 d before

begin heading stage, and the best inoculation time each day was 4-6 pm, the incidence of RFS was the highest. The addition of the potato juice into the suspension of inocula liquid could significantly increase the incidence of inoculation.

Identification of the rice growth stage by phyllula distance: The distance of phyllula here refers to the distance from the flag leaf phyllula to the phyllula of the next leaf (or the penult leaf, where the flag leaf is the last one). The susceptible variety "LYP9" was taken as an example, under room temperature conditions, the phyllula distance at the begin heading stage is 12-14 cm (the flag leaf phyllula is above 12-14 cm the penult leaf phyllula, it also called positive phyllula distance, the flag leaf phyllula is below he penult leaf phyllula, it was call negative phyllula distance) (Figure 1).

Inoculation with suspension of "conidia + mycelium fragments" was conducted at 0-13 cm of positive phyllula distance, or just begin heading stage of rice during booting period. When the inoculation periods were at 0-1, 2-3, 4-5, 6-7, 8-9, 10-11 and 12-13 cm (containing begin heading) of positive phyllula distance during the booting period, the average number of diseased grain per panicle were 5.42, 10.70, 16.8l, 26.07, 35.10, 28.82 and 23.06. Among these, inoculation at 8-9 cm positive phyllula distance of rice booting period was the best effect of RFS incidence, and the highest diseased grain number reached up to 87. In the injection inoculation, if the positive phyllula distance is short, for example, the early and middle booting stages, the number of the diseased grains is less, the lower glume necrosis is easily caused, and it even affects the heading. Therefore, it was believed that the optimal injection inoculation stage for LYP9 was 8-9 cm positive phyllula distance, and the positive phyllula distance of 6-11 cm was appropriate, which is the middle to late booting stage of rice (Yang et al., 2011).

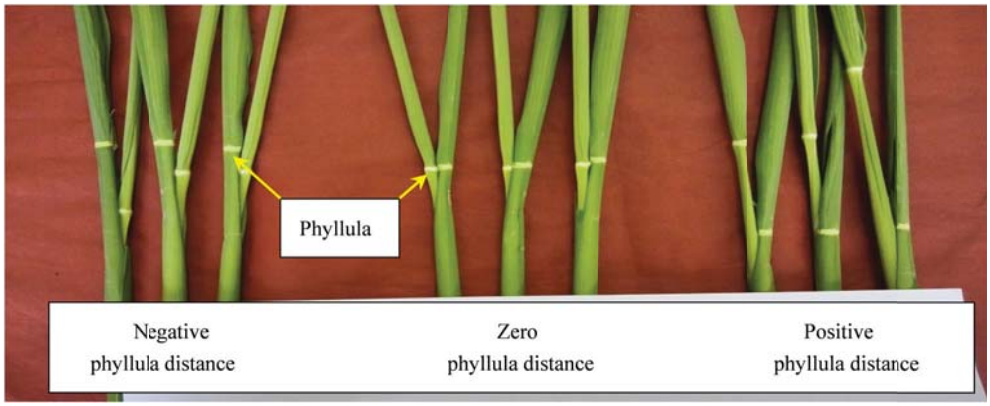

Figure 1. Sketch map of rice phyllula and phylulla distance

2.3.2 Inoculation Method

The artificial inoculation of RFS methods mainly include the injection method and the spraying method. Spraying inoculation with *U. virens* conidia suspension at 7-10 days before heading, it could identify the differences of varieties' resistance (Liao, 1993). Wang et al. (1996) believed that the best effect was injection inoculation with chlamydospore, followed by spray inoculation. Rice plants were inoculated with *U. virens* conidia suspension by using injection and spray method during booting stage and heading stage, both of which could induce RFS on rice. However, the injection inoculation easily caused rice panicle deformity or heading difficulties, which needs to be improved (Li, 1996). The research results of Lu et al. (1996) showed that the incidence of the conidia inoculation method at booting stage was the highest. It was higher than that of the chlamydospore inoculation no matter in the field or greenhouse, and the incidence of the injection inoculation was higher than that of spray inoculation for the same inocula. The incidence of RFS was higher when spray inoculation with conidia suspension at rice heading stages and preserve moisture. The conidia or chlamydospores of *U. virens* were used as inocula, the incidence of the same inocula by injection inoculation method was higher than that of the spraying inoculation both in the field and indoor. The incidence of RFS reached up to 100%, the highest disease index was 93.96, and the number of diseased grains per panicle reached up to 110. The inoculation technique could distinguish the resistances differences of rice varieties Dai et al. (2005).

3. Management of RFS

At present, the management of RFS basically adopt the strategy of "prevention first, integrated management". Agricultural management was the basis and chemical control used as assistance or the emergency measures. The rice varieties with resistance (tolerance) to RFS should be planted, and seeds sterilization and field management should be done very well. Rice production practice have proven that the RFS could only be prevention but could not be treated. It was very important to choose the optimum rice growth stage for chemical application and select special agents in order to obtain better protective effect. The treatment effect is very poor (limited tiny) even spraying multiple with high concentration fungicide once the appearance of smut ball of RFS. Biological control is becoming a hot topic gradually, and it will likely be the development trend in the future.

3.1 Agricultural Control

According to the description of "Conditions affecting incidence of RFS" in part III, the targeted farming operation should be carried out, which has a good auxiliary effect on the prevention and control of RFS.

3.1.1 Resistance of Varieties

Different rice varieties have significant differences in the resistance responses to RFS. Selecting and planting resistant (tolerant) rice varieties is the most economic and effective measures for prevention and control of RFS, and the susceptible varieties need to be eliminated in rice production practice.

3.1.2 Cultivation Techniques

The early maturing (or short growth duration), short tillering and booting stage of rice varieties should be selected and/or sowing and planting time need to be adjusted according to the rice planting areas, so that the sensitive or KGSR (the late booting to heading stage) could avoid local climate conditions which was favorable the occurrence of RFS, and could reduce the incidence of RFS (Yashoda et al., 2000; Dodan et al., 1995). Rational close planting, for example, the wide row and short distance of hills planting pattern should be used. Rice seeds from the infected areas should be avoided, and the infected rice plants should be removed early in order to eliminate the primary infection source to avoid the spread of RFS. Sclerotia of *U. virens* need to be pick out and deeply buried or burned, plough and bask the field should be carried out after harvesting in the RFS occurred areas, and timely sowing and transplanting.

3.1.3 Fertilization and Water Management

Fertilization technology should be improved, N, P, K fertilizers should be applied reasonably. Most of the fertilizer should be applied in early growth stage of rice for promoting tillering, and less fertilizer should be used in the late growth stages of rice. Panicle fertilizer, especially N fertilizer, should be used modestly. The dosage of potassium fertilizer and organic fertilizer should be increased due to they could increase the resistance of rice varieties. Based on the "edge effect" of RFS, only a small amount of fertilization should be used at the edges of the field.

Water management should be conducted scientifically, and intermittent irrigation methods should be used to keep the paddy field dry and moisture alternative. The specific procedures were as follows: shallow water transplanting, deep water (3-5 cm) for protecting the seedlings, tiller seedlings with 2-3 cm deep of water. If the seedlings were enough and the field drying was needed. Keeping the paddy field dry and wet alternating in the late rice growing stage (Bhagat et al., 1999).

3.2 Chemical Control

RFS can be prevented and controlled by fungicides (Ahonsi et al., 2002; Tsuda et al., 2006), but it has been very difficult to forecast the epidemics of RFS due to no consistent correlation has been established between environmental factors, development of the *U. virens*, susceptibility of rice cultivars and RFS. Consequently, chemical control of RFS is ineffective because farmers usually cannot predict when they should spray fungicides before symptoms (smut ball) emerge, whereas it is too late to spray chemicals after symptoms have appeared. Therefore, identification of favorable alleles and improvement of rice resistance to RFS would be a cost-effective and practical strategy (Zhou et al., 2014). The application of chemicals is an effective and emergency measure for prevention and control of RFS, and the key factor for obtaining good control effect is "selection of specific fungicides, and determining the optimum time or KGSR to apply fungicides".

3.2.1 Seed Treatments

Before sowing, rice seeds without disease should be selected, and seed disinfection need to be conducted. It can reduce the quantity of the *U. virens* in seeds and reduce the occurrence of RFS. The diseased seeds were treated with seed disinfectant, and the preventive effect on RFS was 70.0-90.5%. Different reagents were used for

soaking seeds for 48 h, and the effect orders was as follows: bayleton > thiophanate methyl > pentachloronitrobenzene > carbendazim, but none of these could completely prevent the occurrence of RFS (Li, 1996).

3.2.2 Control Practice of RFS

Selection of fungicides: A large number of experiments on *U. virens* in indoor and RFS in paddy fields were carried out by Chinese and foreign scholars, and numerous of fungicides with good inhibition and control efficiency on *U. virens/*RFS have screened out (Hegde et al., 2000; Singh et al., 2002; Ahonsi et al., 2003; Sehly et al., 2004). The indoor tests demonstrated that prochloraz, difenoconazole, propiconazole and tebuconazole had good inhibitory effects on *U. virens*, and the average EC_{50} values were 0.32±0.08, 0.45±0.08, 0.19±0.03 and 0.21±0.06 μg mL^{-1}, respectively. The inhibitory effects of the fungicides trifloxystrobin, picoxystrobin, azoxystrobin on mycelium were strong, and the EC_{50} values were 0.0328, 0.0826 and 0.1001 μg mL^{-1}, respectively. The synergism effect of the difenoconazole and jinggangmycin A mixed at a ratio of 1:2 was very significant, the toxicity coefficient of mixture was 174. 8, and the EC_{50} value was 0.2263 μg mL^{-1}. The field control effects of 43% tebuconazole SC, 25% azoxystrobin SC, 25% prochloraz EC, 8% validamycin A + 4% difenoconazole WP and 25% propiconazole EC were 83.27%, 82.8%, 82.7%, 80.7% and 80.3%, respectively (Ruan et al., 2013).

50% propiconazole EC at 300 g a.i./ha was first applied at 10 days before begin heading of rice, then 10 d later a second application was carried out. The control efficiency on RFS of two years' were 71.5% to 74.3% (Chen et al., 2013). The results of the paddy experiments showed that the following chemicals had a good control efficiency on RFS: 30% difenoconazole propiconazole (Armure) EC and 12.5% diniconazole (Hu et al., 2010; Zhang et al., 2010); 12.5% epoxiconazole suspension agent (Wang, 2013; He et al., 2013); 27.12% basic copper sulfate suspension (Zhang et al., 2012; Zhou et al., 2013); 25% difenoconazole EC (Wei et al., 2009; He et al., 2013); 50% kresoxim-methyl suspension, and MJ2006 (prochloraz + validamycin compound) (Zhang et al., 2010).

Fungicides application frequency and time or rice growth period: Some scholars believed that three times application of fungicides could achieve the best control efficiency for RFS, which were applied in the late tillering stage, 3-7 d before begin heading stage, and the begin heading stage, respectively (Hu et al., 2010; Li et al., 2012), but some scholars believed that the control effect of two times applications of fungicides was also very good, namely 5-7 d before begin heading and full heading stage (Li et al., 2013a; Luo et al., 2013; Liu et al., 2013). For example, 250 g/L Azoxystrobin SC was sprayed at 7 d before begin heading and begin heading stage, the control effects for both the infected panicle rate and of disease index were 92.31%. Compared with the control efficiency of one time application of fungicide at 7 d before begin heading (80.8% and 86.5%, respectively), there was no significant difference. However, there were significant differences of control efficiency compared with that one time application of fungicide at heading period (42.31% and 55.77, respectively) (Zhou et al., 2013). For some rice varieties, the control effects were also very good for one time application of fungicides at 7-10 d before begin heading stage and during the heading period (Guo, 2013), or about 10 d before heading stage for the first time of chemicals application, and ten days later, a second application was carried out (Chen et al., 2013).

Japanese scholars used simeconazole granules (450-600 g a.i./ha) in a submerged application two to five weeks before heading was also highly effective against false smut, with treatment three weeks before heading being the most effective. The results also showed that the application of flusilazole at two to three weeks before heading could effectively control the panicle blight in the rice heading stage, RFS, rice kernel smut and sheath blight (*Rhizoctonia solani*) (Tsuda et al., 2006).

Precise timing of the optimum fungicide application time by "physical signs" of rice: It was difficulty for accurately determining the number of "days" before heading, especially for ordinary rice farmers, so it is very difficult to accurately grasp the optimum time for actual operations in the field. In addition, the growth process of rice was also associated with the rice varieties, climatic conditions and rice growth status. Rice physical signs here refer to phyllula, "the same level of phyllula, or "zero distance of phyllula, or distance of phyllula" of rice, Figure 1, *i.e.*, the phyllula of flag leaf and penult leaf hold the same level (the flag leaf is tailender leaf), which are equivalent to those of 5-12 d (there are differences among different varieties) before begin heading (Dong et al., 2004). If only one time application of fungicides, a better control efficiency for susceptible indica/japonica hybrid rice variety "Yongyou 12" can be acquired from the spraying at 10-12 d before begin heading (the physiological indicator of 10-12 d before begin heading of "Yongyou 12" was "Yezhenping", means zero distance of phyllula, the days of 10-12 determine by the climate conditions of this period of rice growing. The

days from zero distance of phyllula to begin heading there were differences among the different types of rice varieties, which were generally in the range of 5-12 d (Xu et al., 2005). After several years continuous experiments in the paddy field, a technology of "one soaking and two spraying, timing by phyllula distance" was developed. "One soaking" means rice seeds soaked with fungicides for sterillzation, "two spraying" means first spraying chemicals at 5-12 d (the days of different rice varieties are different) before begin heading and begin heading, or 1-3 cm negative phyllula distance, or 1/3-1/2 rice plants were at zero phyllula distance, the second spraying was at begin heading (5-10% rice plants heading) or 5-12 d after first spraying. The technology shows the characteristic of simple and convenient, easy to master, precision and high control efficiency (Rao et al., 2019).

3.3 Biological Control

Due to the limitations of chemical control, the biological control of RFS is the future direction of development. Antibiotics, antagonistic bacteria and plant source preparations have become research hot field in recent years (Liu et al., 2004). The prevention and control of RFS by means of antibiotics have been used for many years, and the most widely used agent is Jinggangmycin. The control effect of Jinggangmycin either alone or mixed with carbendazim was good (Li et al., 1990; Zhu et al., 1996). Wang et al. (2003) performed indoor and outdoor experiments on RFS with Ningnanmycin, and the indoor antibacterial effect was 67%, but the average spike control effect in the field was only 36.4%. The currently available biocontrol agents mixture, for example, Jinggangmycin and Bacillus mixture have been used in large production areas for the control of RFS, and good control effects have been achieved (Chen et al., 2003; Cai et al., 2011; Chen et al., 2011).

Antimicrobial microorganisms have been isolated from RFS ball (Xu, 2002). A_1 and H-51 are two good strains among 114 bacteria isolated from paddy soil (Lan et al., 2004), the fermentation liquid of A_1 and H-51 had a good inhibition rates on mycelium growth, and conidia germination of *U. virens*. 1800 bacterial isolates were isolated from the soil, among which SF-62 and SF-3-38 of *Bacillus subtilis* grew quickly, and had a strong inhibition effect on the growth of *U. virens*, the inhibition rates reached 97.2% and 85.9%, respectively (Yin et al., 2011). The extract of some types of actinomycetes fermentation also could inhibit the growth of *U. virens* (Huang et al., 2000). All of the strains showed potential for the development of the biocontrol agents of RFS.

Some scholars have attempted to study and use plant extracts to control RFS. 10% mint extract had an inhibition rate of 100% on the germination of conidia, chlamydospores of *U. virens*, it could significantly inhibit the mycelial growth and cause variation of clony, which has a value for in-depth research and development (Jin et al., 2005).

Recently, it was found that some species of *Trichoderma* had a strong inhibitory effect on *U. virens*, which have potential to be developed into biocontrol agents (Ashish et al., 2014). Gliotoxin what secreted by *Trichoderma virens* strain TY009 could completely inhibit the conidia germination and formation of secondary spores of *U. virens* at a concentration of 1.0 μg mL^{-1} (Liu et al., 2010). Some viruses was isolated from chlamydospores (Zhang et al., 2013; Zhong et al., 2014; Jiang et al., 2014) and show some inhibition on *U. virens*, however, whether or not these viruses can be used as the virus agent need to be further studied (Yu et al., 2010).

4. Problems and Prospects

Rice false smut frequently and seriously occurred in rice production worldwide, and the damage both rice yield and grain quality caused by it is very serious. The relevant departments of the Chinese government and researchers pay high attention to this issue, and have also invested large amounts of manpower, financial resources and material resources in carrying out the basic research and development of prevention and control technologies. However, up to now, the life history of *U. virens*, its mechanism of infection, the interaction characteristic of the *U. virens*-host (rice) and other major basic theory problems have been not very clear or have no unified conclusion. There are key points need to be solved in the management technology of RFS, of which the infection mechanism of *U. virens*, including infection period, infection site, source of the pathogen, and its expansion pattern and other problems have been controversial (Gao et al., 2011).

The reports regarding the host range of *U. virens* are rare scientific results, and there is still lack of a systematic investigation. The pathogenicity variation of the pathogen and its molecular mechanism remain unclear, and the research regarding the mechanism of host and pathogen interaction, recognition and signal transduction in cells of rice, and rice defense response gene activation process are relatively weak (Zou et al., 2012).

Due to no uniform standards of the identification technology of rice resistance to RFS, the results of rice resistance to RFS obtained by different researchers are not consistent, not stable and the repeatability is poor, the results of different researchers unable to be compared. Recognized resistant materials of rice are not obtained in

screening, and the genetic breeding of rice with resistance to RFS has yet not to be carried out. Therefore, a technology system of artificial inoculation and identification of rice resistance to RFS should be established and improved as soon as possible. The standardized inoculation and identification can be carried out under controlled conditions. The resistant materials of rice could be screened out and provided guarantee for the breeding of rice resistance to RFS.

The use of ustiloxins produced by *U. virens* for simple and rapid screening of rice resistant materials is worthy of further research and development. Combination of molecular biology and genetic engineering to specifically confirm whether the rice resistance to RFS is the real genetic resistance or plant morphology or external environment influence are the pressing matters at present.

RFS primary infection sources, secondary infection source, infection period and location, infection pathway, pathogenic mechanism, cycle of infection and pathogen-host interaction require further clarification. The research and cloning of important genes related to *U. virens* pathogenicity by infection and virulence variation, along with a clear understanding of the genome and establishment of a genetic transformation system of mature pathogen. RNA interference, gene knockout method and other methods and techniques of reverse genetics should be used to establish some rice false smut pathogens (*U. virens*) mutants. The functional genomics and proteomics research methods should be employed in order to isolate and clone rice resistance genes to elucidate the mechanism of pathogenesis of *U. virens* from the molecular level. We studied on the interaction between RFS and rice, the regulatory mechanism of signal transduction of pathogenic elicitor and activation process of defense response genes, and establishing rice germplasm resources with resistances to RFS through the transgenic technology and breeding new varieties with lasting resistance to RFS.

The selection of the proper fungicides and precise timing to determine the optimal time of chemicals application is the key to increase the efficiency of the chemical control on RFS. Presently, the control effect for twice application of chemicals was the best: first application of fungicides was at 5-7 d before begin heading, and second time application was at heading stage, or second time application of the fungicide at 7-10 d after the first time application. In practical operation, it is difficult to accurately define the first time application of 5-7 d before begin heading or 7-10 d before the heading. Furthermore, different rice varieties with different weather conditions in the growth period (late booting to heading stage), different fertilization and water management, and different rice growth status and so on, all could affect the duration of the “late booting period and heading period”.

The “physical signs” method is used for precise timing of fungicide application to control RFS, namely the first time of chemical spraying should be carried out at phyllula in the same level (or zero distance between pulvinus) (*i.e.*, 5-7 d or 7-10 d before begin heading, and different rice varieties are different) of flag leaf and the penult leaf of most rice plants. The second time application of chemicals should be at begin heading stage of the most plants. The method of timing and fungicide application could achieve a better control effect on RFS.

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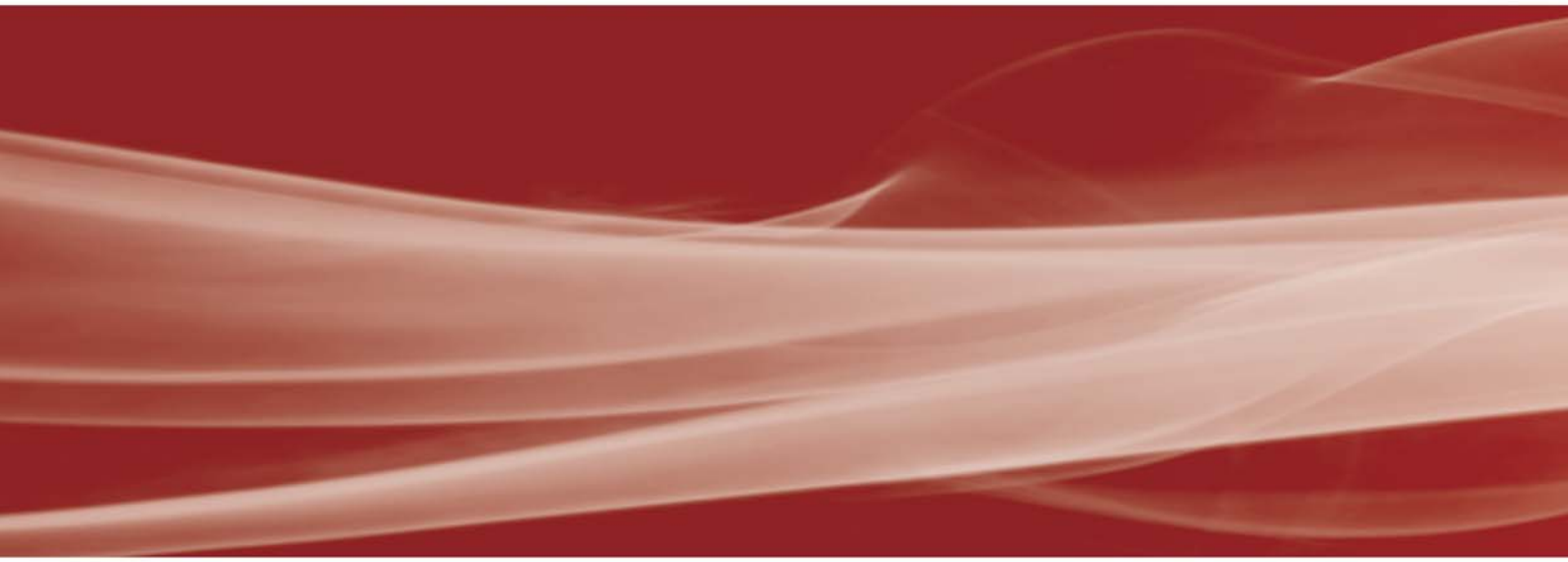

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