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Full Length Research Paper

Assessment of bacterial wilt (*Xanthomonas campestris* pv. *musacearum*) of enset in Southern Ethiopia

Mekuria Wolde[1]*, Amare Ayalew[2] and Alemayehu Chala[3]

[1]Madawalabu University, School of Agriculture, Plant Science Course Team, P. O. Box 247, Bale Robe, Ethiopia.
[2]The Partnership for Aflatoxin Control in Africa (PACA), Department of Rural Economy and Agriculture (REA), P. O. Box 3243, Roosevelt Street, Addis Ababa, Ethiopia.
3Hawasa Collage of Agriculture, School of Plant Science, P. O. Box 5, Hawasa, Ethiopia.

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Enset (*Ensete ventricosum*) production and productivity is threatened by many biotic and abiotic factors among which bacterial wilt of enset (BWE), caused by *Xanthomonas campestris* pv. musacearum is one of the major factors. There were no reports on the intensity and distribution of bacterial wilt of enset in South Nation Nationalities and Peoples' Regional State (SNNPRS). Hence, the objective of this study was to determine the distribution and incidence of bacterial wilt of enset in relation to age, altitude and clonal variation in major enset growing districts of South Nation Nationalities and Peoples' Regional State (SNNPRS). Three major enset growing Zones namely Gurage, Hadiya and Sidama were included in the survey. In each Zone, three Districts were selected based on enset production status and altitudinal variation. The disease was detected in all agro ecologies and districts, but in varying extent. Bacterial wilt prevalence and incidence was highest in Hadiya and estimated at 42.22 and 5.56%, respectively, while both disease prevalence and incidence were the lowest (26.67 and 2.86%, respectively) in Sidama. At District level wilt prevalence varied from 6.67% at Aletachiko District to 76.67% at Lemo District. Bacterial wilt incidence also ranged from 0.74% at Aletachiko District to 10.31% at Lemo District. Wilt prevalence and incidence were at the highest (50% and 5.81%, respectively) in the altitude range of 2000-2500 masl. The disease varied according to the crop growth stage, with severe (4.75%) in Cycle 4 (an age greater than 4) and less severe (0.2%) at Cycle 1 (age of less than one year). Highest disease incidence and prevalence (30 and 6.65%) were noted with a plant age of 4-5 years. The disease was highly associated with administrative Zone, District, altitude, number of clones and spacing in a logistic regression model.

Key words: Bacterial wilt of enset, wilt incidence, wilt prevalence, South Nation Nationalities and Peoples' Regional State (SNNPRS), enset clones

INTRODUCTION

Enset [*Ensete ventricosum* (Welw.) Cheesman] is a staple food for over 15 million people in Ethiopia (Brandt et al., 1997). The plant is a drought tolerant and multi-purpose crop of which all parts are utilized for different

*Corresponding author. E-mail: hendawysaber@yahoo.com.

purposes. Enset production is largely for human food, fiber, animal forage, construction materials, medicine and for cultural practices (Tsehaye and Kebebew, 2006). The major foods obtained from enset are *kocho*, *bulla* and *amicho*. The energy yield of enset is by far higher than those of several cereals and also reported to be higher than potato, sweet potato and banana (Pijls et al., 1995). More than 20% of Ethiopia's population depends upon enset for human food, fibre, animal forage, construction materials and medicines (Azerefegne et al., 2009).

Although the economic importance of enset is great, its production is affected by several factors, including biotic and abiotic agents, such as diseases, insect pests, weeds, wild animals and soil nutrient depletion, which contribute to low yield and low quality of enset production. Diseases are collectively the most severe biological problem for enset production. Enset diseases are caused by several bacteria, fungi, viruses and nematodes. Among these, bacterial wilt of enset (BWE), caused by *Xanthomonas campestris* pv. *musacearum* (Xcm) is the most important constraint to enset production (Brandt et al., 1997).

Enset bacterial wilt was first reported by Yirgou and Bradbury (1968) in Ethiopia in 1968 and is currently found in all the enset growing regions and on wild enset plants (Brandt et al., 1997). The disease also attacks banana and other *Musa* spp (Viljoen, 2010). BWE is currently restricted to Africa (Fenta and Karamura, 2012). Bacterial wilt attacks enset at any stage of growth, including full maturity (Brandt et al., 1997). Once established in an area, the disease spreads rapidly and results in total yield loss (Welde-michael, 2008a). The initial symptoms of the disease occur on the central leaf and spread to all parts. Bacterial ooze exudes, when non-dry part of the plant is removed. The disease mainly spreads through infected farm tools, infected planting materials (since the plant requires repeated transplanting that damage the corm and roots), animals that fed on infected plants and possibly insects feeding on the foliage (Welde-michael et al., 2008b). Survival of the pathogen is mainly through infected plant debris and infected soil (Mwebaze et al., 2006). Handoro (2014) also reported that Xcm can survive in *Kocho* for more than 14 weeks.

Bacterial diseases of plants, once established, are difficult to control owing to the lack of an effective chemical or other curative treatments (Biruma et al., 2007). Handoro et al. (2012) reported cultural practices and sanitation control measures are the most principal control measures for BWE. On the other hand, good sanitation (removal of infected plant and plant parts), curative mechanisms, use of disease free sucker for planting material, crop rotation, use of resistant clones can serve as viable management options for bacterial wilt of enset. The identification and early removal of infected plants a key part of the control system (Karamura et al, 2008).

Bacterial wilt is a destructive disease in all enset growing areas of Ethiopia. However, the current status and distribution of the disease in Ethiopia is not well assessed. Furthermore, farmers mention that bacterial wilt is more severe at high altitudes (Brandt et al., 1997), but this has not been scientifically tested. Even though the pathogen attacks all stages of the plant (Brandt et al., 1997), the comparative importance at different stages is not determined yet. Enset is propagated by suckers or shoots rather than by seeds. Enset suckers are ready for transplanting into the permanent field from three to five years after propagation, depending on agro-ecological condition and locations. Farmers commonly grow the plant in 3 to 4 cycles (growth stages) with 2-3 transplanting (for every year or two) in different field in a crop's life time by increasing spacing in each cycle or stage. Research to determine the incidence of the disease at these stages would generate information that would contribute towards targeted management of the disease. Hence, this study was proposed with objectives of, determining the distribution and incidence of bacterial wilt of enset in relation to age, altitude and clonal variation in major enset growing districts of South Nation Nationalities and Peoples' Regional State (SNNPRS).

MATERIALS AND METHODS

Sampling procedures

To determine the incidence and distribution of bacterial wilt of enset in major enset growing areas of SNNPRS, particularly to assess the situation after the recent upsurge of the disease, a reconnaissance survey was made from enset growing farmers' fields. Three administrative Zones, namely Gurage, Sidama and Hadiya were covered in the study (Figure 1). They were selected purposively by their potential enset production. Districts were stratified into three agro-ecological groups, based on altitudinal range and one District was selected in each agro-ecology. For the ease of this research work ago-ecologies were categorized into three altitudinal ranges (groups), namely less than 2000 masl, 2000-2500 masl and greater than 2500 masl. In each District three representative kebeles were selected and ten enset farms were assessed at a distance of fixed interval from one to two kilometers from each kebele based on enset availability. Thus, a total of 270 enset fields were assessed in the survey.

Disease assessment

The status of BWE at each field was assessed and recorded through direct field observations. In each enset field, the plants were classified into three to four stages based on the crop age (growth stages) and cropping systems used by the farmers. Based on these, stage one is less than one year old sucker developed from a single corm, stage two is two years old which are transplanted from stage one, stage three is three to four years old and stage four is four years to harvesting (maturity) stage in which all are grown in separate field and different spacing. Based on the stages, random sampling was made for row selection. Within the row, two consecutive plants were assessed at an interval of five successive plants. In each stage, the total number of plants and the number of plants showing typical bacterial wilt symptoms were recorded. Disease incidence for each stage was calculated as

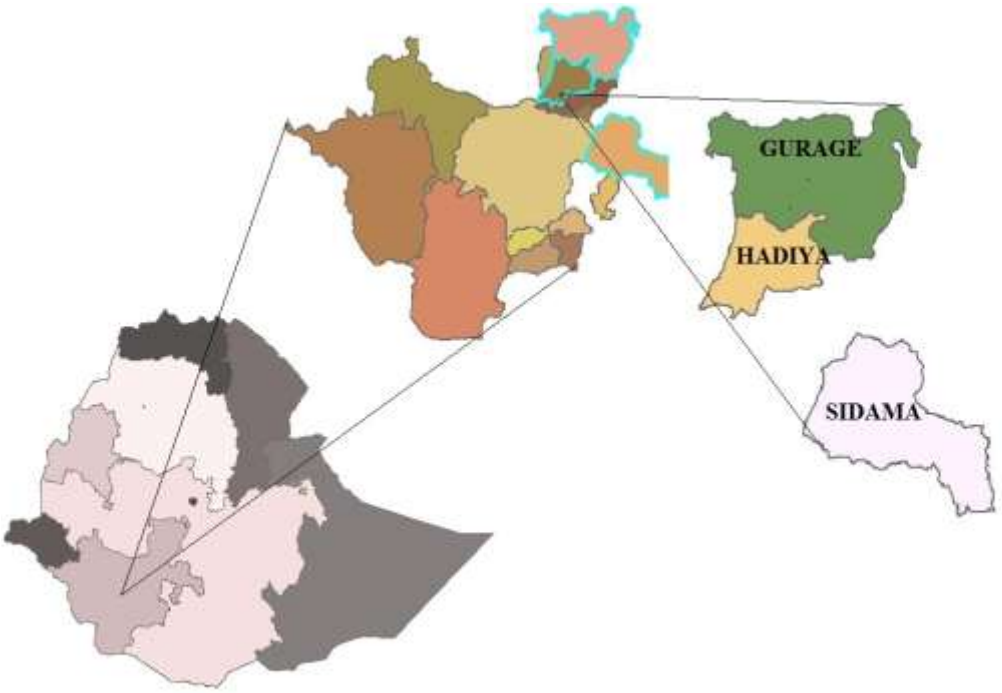

Figure 1. Map of Ethiopia showing locations of SNNPRS and Zones surveyed for BWE disease.

number of plants showing wilting symptom divided by total number of plants assessed multiplied by 100. Average wilt incidence for the field was obtained by summing up the percentage wilt incidence for each stage divided by three or four (based on the number of stages used). Prevalence of the disease was calculated using percentage of fields encountered with bacterial wilt disease.

Prevalence = (NWF/NTF) × 100

Where, NWF = the number of fields with bacterial wilt symptom and NTF = the total number of fields.

Wilt Incidence = (NWP/TNP) × 100

Where, NWF = the number of plants infected by bacterial wilt symptom and NTF = the total number of plants assessed.
In addition to bacterial wilt incidence, supplementary information was also recorded through direct field observation, interview with enset growers and global positioning system (GPS).

Data analysis

Simple descriptive statistics were used to summarize data obtained from field surveys after being entered in SPSS computer program version 20.0 for Windows. Summary of wilt incidence was presented for each independent variable and variable classes. The association of BWE incidence and incidence at Cycle 4 with independent variables were analyzed using logistic regression as described by Yuen et al. (1996) and Hosmer and Lemeshow (1989) with the SAS Procedure GENMOD (SAS for Windows, 2002-2003, SAS Institute). The wilt incidence and wilt incidence at Cycle 4 were classified into distinct groups of binomial qualitative data. Thus, ≤15 and >15% were chosen for wilt incidence yielding a binary dependant variable. Class boundaries of ≤20 and >20% were chosen for incidence at Cycle 4.

The logistic regression model allows evaluating the importance of multiple independent variables that affect the response variable (Yuen et al., 1996). The GENMOD (generalized linear models) Procedure gives parameter estimates and the standard error of the parameter estimates. Exponentiating the parameter estimate yields the odds ratio, which is interpreted here as the relative risk (Yuen et al., 1996). The importance of the independent variables was evaluated in two ways. First, the association of an independent variable alone with BWE incidence or wilt incidence was examined. In the other method, independent variables with high association to both parameters were added to reduce multiple variable models. The odds ratio shows the strength of association between a predictor and the response of interest.

RESULTS AND DISCUSION

Prevalence of BWE

From 270 enset fields assessed in the three Zones, on average 34.81% enset of the fields were affected by the disease. However, Africa RISING (2014) reported that 80% of enset farms during the 2013 growing season were infected with BWE. During the survey period, it was recognized that the disease was widely distributed and detected in all agro-ecologies and locations. This was in agreement with reports by Ashagari (1985); Anita et al. (1996) and Spring et al. (1996). The disease was most prevalent in Hadiya Zone with 42.22% BWE prevalence, while only 26.67% and 35.56% of enset fields were affected by the disease in Sidama and Gurage Zones, respectively. On the contrary, Brandt et al. (1997) reported that higher wilt prevalence was occurred in

Table 1. Mean incidence and prevalence of BWE for different production locations in SNNPRS.

Variables	Variable class	NIF	Prevalence (%)	Incidence (%)				
				Max.	Min.	Mean	SD.	SEM.
Total		94	34.81	28.57	0.00	3.89	6.15	0.37
	Gurage	32	35.56	16.67	0.00	3.21	4.93	0.52
Zones	Hadiya	38	42.22	28.57	0.00	5.56	7.44	0.78
	Sidama	24	26.67	22.22	0.00	2. 89	5.49	0.58
	<2000	15	16.67	17.24	0.00	1.91	4.37	0.46
Altitude (masl)	2000-2500	45	50	28.57	0.00	5.81	7.27	0.77
	≥2500	33	36.67	20.00	0.00	3.93	5.87	0.62
	Edja	14	46.67	16.67	0.00	4.10	5.57	1.02
	Cheha	6	20	10.34	0.00	1.52	3.17	0.58
	Gumer	12	40	15.79	0.00	4.00	5.39	0.98
District	Aletachiko	2	6.67	11.43	0.00	0.74	2.81	0.51
	Wonsho	9	30	22.22	0.00	3.05	5.67	1.03
	Lemo	23	76.67	28.57	0.00	10.31	8.21	1.49
	Hula	13	43.33	20.00	0.00	4.86	6.61	1.20
	Misha	7	23.33	16.3	0.00	2.93	5.56	1.02
	Gibe	8	26.67	17.24	0.00	3.46	6.05	1.10

SD, Standard Deviation; SEM, Error mean square; NIF, Number of infected fields; Max., maximum; Min, minimum.

Gurage Zone, followed by Hadiya, while similar report was for Sidama Zone. Similarly, Anita et al. (1996) also reported the disease was devastating in these areas during 2013 growing season.

There was variation in BWE prevalence across altitudes with the disease being most prevalent (50%) in at an altitude of 2000-2500 masl. This was followed by >2500 and <2000 masl, which had BWE prevalence averaging on 36.67% and 16.67%, respectively (Table 1). Brandt et al. (1997) found out that the disease was more severe in highlands than in lowlands. A study by Maina et al. (2006) also reported that the disease is severe at midland in banana plant. When comparisons were made across seasons, some farmers responded that the disease is more severe in summer than in winter. This indicates the pathogen may require high moisture and lower temperature. There was slight variation in BWE prevalence, when comparisons were made between cropping practices. About 30.58% of intercropped fields were affected by the disease and 36.93% of monocropped fields were infected with the disease, which was statistically insignificant.

At the District level, the highest BWE prevalence (76.7%) was registered in Lemo District (Hadiya Zone). It was followed by Hula, Edja, Gumer and Wonsho Districts with 43.33, 46.67, 40 and 30% disease prevalence, respectively. Aletachiko, Cheha, Misha and Gibe Districts were less affected by the disease, with 6.67, 20, 23.33 and 26.67% BWE prevalence were registered, respectively.

Even though bacterial wilt could infect enset at all cycles and growth stages, minimum disease prevalence occurred in Cycle 1 where only 1.11% of the surveyed fields were affected by the disease. Likewise, 20% of Cycle 2, 20.56% of Cycle 3 and 31.48% of Cycle 4 enset fields were affected by BWE disease. Disease data for Cycle 4 was categorized into two age groups, with an age of four to five years and age greater than or equal to six years for analysis. Hence, higher (30%) disease prevalence was recorded at age of four to five and less (14.07%) in an age greater than or equal to six (Table 2). The present finding is in agreement with the findings of Brandt et al. (1997) where the disease was severe at middle age. However, Welde-michael et al. (2008a) indicated in an experiment involving cutting of plants with contaminated knife that older plants were less vulnerable to infection than young plants. On the other hand, higher disease prevalence (36.89%) was recorded on fields with fewer than or equal to five clones diversity per enset fields. Similarly, 33.53% of enset fields containing more than five clones were affected by the disease. However, during the survey period some farmers, which have the disease in their farm, grew only few clones, in which they believed that these clones are resistant to the disease.

Nearly similar wilt prevalence was recorded for spacing greater than 1.5*1.5 m and less than or equal to 1.5*1.5 m, with 32.9 and 37.2% of enset fields respectively, being affected by the disease. Correspondingly, 30.3% and 33.1% wilt prevalence was registered in Cycle 4. On the other hand, spacing data for Cycle 3 were grouped in to

Table 2. The mean incidence and prevalence of BWE for different variables.

Variables	Variable class	NIF	Prevalence (%)	Incidence (%)				
				Max.	Min.	Mean	SD.	SEM.
Cropping Cycle	Cycle 1	3	1.11	20.0	0.00	0.20	1.87	0.11
	Cycle 2	54	20	40.00	0.00	2.48	6.95	0.42
	Cycle3	37	20.56	40.00	0.00	4.13	8.93	0.67
	Cycle 4	85	31.48	37.50	0.00	4.75	7.92	0.48
Age (year)	4-5	81	30	57.14	0.00	6.55	11.32	0.69
	≥6	38	14.07	33.33	0.00	2.37	6.25	0.38
Cropping Sys	Intercrop	29	30.85	21.62	0.00	3.61	6.01	0.62
	Mono crop	65	36.93	28.57	0.00	4.04	6.23	0.47
Spacing at C4 (m)[a]	>1.5*1.5	50 (43)	32.9 (30.3)	21.62 (23.81)	0.00 (0.0)	3.39 (4.30)	5.46 (7.17)	0.44 (0.58)
	≤1.5*1.5	44 (41)	37.2 (33.1)	28.57 (37.5)	0.00 (0.0)	4.53 (5.33)	6.9 (8.79)	0.63 (0.81)
Spacing at C3 (m)[c]	≥1*1	18	19.35	33.33	0.00	3.70	8.16	0.85
	<1*1	19	21.87	40	0.00	4.58	9.71	1.04
Enset FS (ha) Priority of Enset	>0.25	43	30.71	25.81	0.00	3.22	5.72	0.48
	≤0.25	51	39.23	28.57	0.00	4.60	6.52	0.57
	1st	80	32. 92	28.57	0.00	3.66	6.02	0.38
	2nd	12	54.54	22.86	0.00	6.45	7.36	1.57
	3rd	2	40	10.71	0.00	3.62	5.09	2.28
No. of clone	≤5	38	36.89	22.2	0.00	4.19	6.22	0.61
	>5	56	33.53	28.57	0.00	3.70	6.11	0.47

[a] data in parenthesis are for Cycle 4 only; [c] data for only Cycle 3; SD, Standard Deviation; SEM, Error mean square; NIF, Number of infected fields; Max., maximum; Min, minimum.

two, ≥1*1 m and less than 1*1m. Based on this, about 19.35% of enset fields at Cycle 3 cultivated in wide spacing (≥1*1 m) were infected with the disease, while 21.87% of enset fields at Cycle 3 with a spacing of less than 1*1 m were infected with BWE. This indicated that close spacing increase the disease prevalence at both cycles.

Incidence of BWE

The mean incidence of BWE varied for different variables and variable classes (Tables 1 and 2). The overall mean incidence of the disease during the survey time was 3.89%. Mean BWE incidence varied from 2.89% in Sidama Zone to 5.56% in Hadiya Zone. Mean incidence of the disease in Gurage Zone was 3.21%. The high BWE

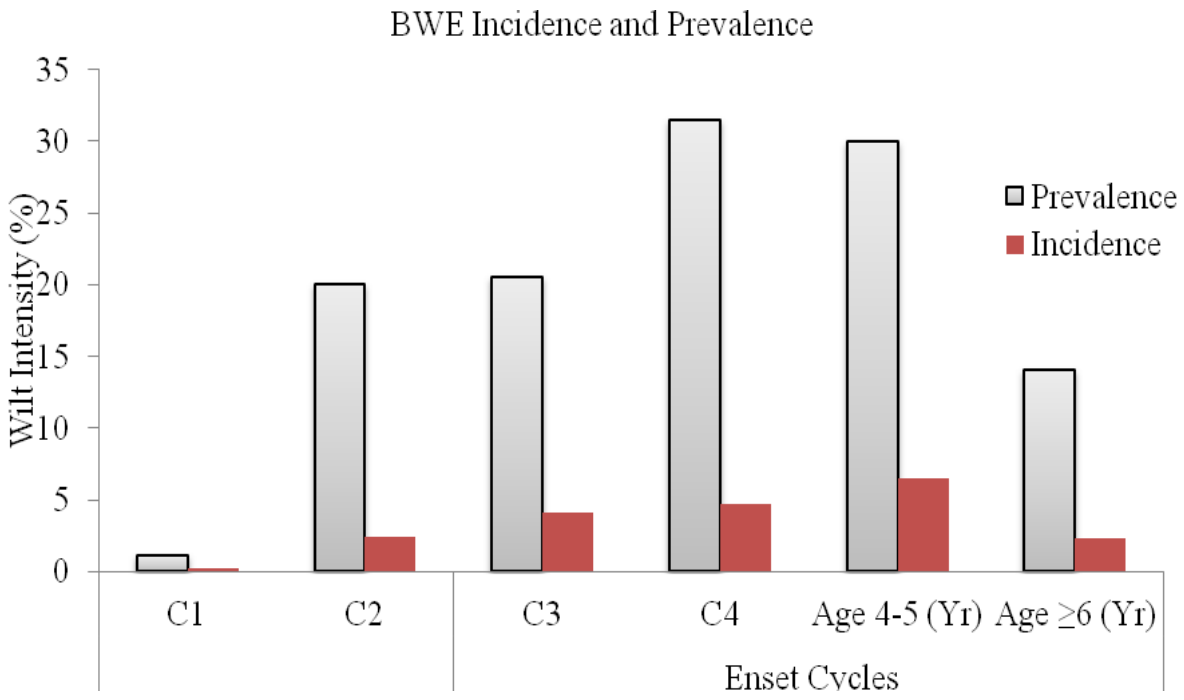

Figure 2. Mean BWE prevalence and incidence at different cycles and ages of enset in SNNPRS. C1 = Cycle 1; C2 = Cycle 2; C3 = Cycle 3 and C4 = Cycle 4.

incidence in Hadiya Zone might be attributed to management practices, environmental factors and awareness of the farmer for transmission and management. A maximum mean incidence of 5.81% was recorded in the altitude of 2000-2500 masl, while minimum mean incidence of 1.91% was recorded in an altitude of less than 2000 masl, and the BWE mean incidence in high altitude (>2500 masl) was intermediate and was estimated at 3.93%.

Among the enset fields surveyed in nine Districts, the least mean incidence (0.74%) was recorded in Aletachiko District, followed by Cheha and Misha District with mean incidence of 1.5 and 2.93%, respectively. Likewise, the highest mean incidence (10.31%) was recorded in Lemo District, followed by Hula and Edja Districts with mean incidence of 4.86 and 4.10%, respectively. During the survey, Lemo District was found as the most affected area. Africa RISING (2014) has also reported high infestation at Lemo District. The farmers in Lemo District responded that the disease was lower at winter (dry) time and they grew enset continuously without rotation, since the disease is soilborne and it was severely affected, rotation with other crop might be better for this area. The other reason for high incidence of the disease at Lemo may be due to the virulence of the pathogen. Haile et al. (2014) reported there is a huge variation for Xcm isolates in their pathogenicity.

Minimum mean BWE incidence (0.20%) was recorded in Cycle 1, followed by Cycle 2 with 2.48% mean incidence. Maximum mean incidence (4.75%) was registered in Cycle 4, while BWE mean incidence at Cycle 3 was 4.13% (Figure 2). This indicates that the disease was more destructive in Cycles 3 and 4 and less destructive in Cycles 1 and 2. But it does not indicate that at younger age of the plant it is immune to the disease. However, this might indicate that suckers had no or little significant role in the transmission of the disease, but they might cause latent infection. The highest wilt incidence at middle age might be due to long exposure time of the host to the pathogen and crop management practices. Moreover, higher cycles are more prone to frequent cut by infected farm tools for different purposes. In this connection, BWE incidence was higher at an age of 4 to 5 years with mean incidence of 6.55% and minimum (2.37%) at an age of greater than 5 years in Cycle 4 during the survey time (Figure 2). This indicates that wilt incidence was higher at mid stage than at juvenile or sucker stage. However, Hayward (2006) reported that suckers are an important means of spread for systemic bacterial diseases. On the other hand, farming instruments may play great role in disease transmission during transplanting and other management practices.

BWE incidence was greater in monocropping than in intercropping with mean incidence of 4.04 and 3.61%, respectively. But it was not significantly different from each other. Bacterial wilt incidence at whole field was at maximum in narrower spacing (less than or equal to

Table 3. Independent variables used in logistic regression modeling of BWE incidence and incidence at Cycle 4 and likelihood ratio test for 6 variables.

Independent variable	DF	Wilt incidence LRT >15%		Incidence at Cycle 4 LRT >20%	
		Deviance	Pr > x^2	Deviance	Pr > x^2
Zone	2	2791.91	<0.0001	3625.00	<0.0001
District	8	2417.09	<0.0001	3133.77	<0.0001
Altitude	2	2687.62	<0.0001	3450.02	<0.0001
Cropping system	1	2405.32	0.1765	3091.89	0.50
Number of clone	1	2344.15	<0.0001	3056.68	<0.0001
Spacing at Cycle 4	1	2324.45	<0.0001	3046.37	0.0013

DF, degrees of freedom; Pr, Probability of a x^2-value exceeding the deviance; LRT, likelihood ratio test.

1.5*1.5 m) than in wider spacing (greater than 1.5*1.5 m) with an incidence of 4.53 and 3.39%, respectively. Likewise, the mean incidence in Cycle 4 with narrow (less than or equal to 1.5*1.5 m) spacing was at maximum with 5.33% wilt incidence, while only 4.30% of enset plants at Cycle 4 were infected in widely spaced (greater than 1.5*1.5 m) enset fields (Table 2). This might be attributed to higher disease transmission in narrow spacing, because of suffocation, humid microclimate and physical contact, which aggravate disease spread. That is why it had strong influence on wilt incidence and little influence on wilt prevalence. Similarly, spacing at Cycle 3 had influence on wilt incidence. Maximum wilt incidence (4.58%) was recorded in narrow spacing (less than 1*1 m), while 3.58% wilt incidence was recorded in enset farms with spacing greater than or equal to 1*1 m at Cycle 3.

Data on the field size were grouped into two ranges (≤0.25 and >0.25 ha). Incidence of 4.60% were recorded in enset field size of less than or equal to 0.25 ha and 3.22% incidence was noted in enset farm size with greater than 0.25 ha. It appeared that the field size and cropping system had an influence on BWE incidence. To this effect, maximum wilt incidence (5.73%) was recorded in enset farms where less than 30 ensets per year were harvested, followed by farms where 31-49 ensets were harvested per year with correspondingly 3.88% incidence, while minimum wilt incidence (1.28%) was recorded in enset farm fields where greater than 49 ensets were harvest per year. Higher wilt incidence (4.19%) was registered from enset fields which posses less than or equal to five clones per enset field, while lower (3.70%) incidence was from diversified enset fields.

Association of bacterial wilt of enset with independent variables

Enset bacterial wilt incidence and wilt incidence at Cycle 4 were significantly associated with most of the independent variables in the logistic regression (Table 3). Both disease parameters were significantly associated ($p<0.0001$) with five variables, namely, administrative Zone, District, altitude range, number of enset clones and plant spacing at Cycle 4. However, both BWE incidence and incidence at Cycle 4 have no significant association ($p<0.0001$) only with enset farming system. The likelihood ratio test showed that the associations of the administrative Zone, altitude and District with infection of BWE were the highest as evidenced by higher deviance reductions and *x*2 value.

The variables that showed significant associations in likelihood ratio test were tested in reduced multiple variable models with wilt incidence and incidence at Cycle 4 as a dependent variable. Low wilt incidence (≤15%) had a high probability of association to Aletachiko, Cheha and Gibe District, to lower altitude (<2000 masl) and wider planting space at Cycle 4 (>1.5*1.5 m) (Table 4). Similarly, Aletachiko, Cheha and Gibe District, to lower altitude (<2000 masl) and narrow spacing had a high probability of association to lower wilt incidence (≤20%) at Cycle 4.

On the other hand, high wilt incidence (>15%) had a high probability of association to Gurage Zone, Lemo, Edja and Hula Districts, mid altitude (2000-2500 masl) and diversified enset fields (>5 clones). Likewise, high incidence (>20%) in Cycle 4 had high probability of association to Hadiya Zone, Lemo District, altitude of 2000-2500 masl and less diversified fields (≤5 clones per enset farm). There were about four times greater probabilities that wilt incidence would exceed 15% in the Lemo District as compared to Wonsho District. Similarly, the probability of occurrence of high wilt incidence in an altitude of 2000-2500 masl and less number of clones was about four and two times greater than in altitude of <2000 masl and more diversified enset fields, respectively. On the other hand, the probability of occurrence of high wilt incidence (>20%) at Cycle 4 in Lemo District and at altitude range of 2000-2500 masl was about five and four times greater as compared to Wonsho District and at an altitude of <2000 msal, respectively.

There were about seven and two times less probabilities that wilt incidence would exceed 15% in

Table 4. Analysis of deviance, natural logarithms of odds ratio, parameter estimate and standard error of added variables in logistic regression model analyzing BWE incidence and incidence at Cycle 4.

			Wilt incidence			Incidence at Cycle 4		
Variable	**DF**	**Variable class**	**Parameter estimate**	**SE**	**Odds ratios**	**Parameter estimate**	**SE**	**Odds ratios**
Intercept			-1.94	0.15	0.14	-1.94	0.13	0.14
Administrative Zone	2	Gurage	0.26	0.17	1.3	0.008	0.15	1.08
		Hadiya	0.09	0.17	1.09	0.062	0.14	1.06
		Sidama	0.00[a]	0.00[a]	1	0.00[a]	0.00[a]	1
District	8	Aletachiko	-1.91	0.25	0.15	-1.64	0.20	0.19
		Cheha	-0.82	0.19	0.44	-0.95	0.17	0.39
		Edja	0.40	0.15	1.49	0.21	0.14	1.2
		Gibe	-0.24	0.16	0.79	-0.52	0.15	0.59
		Gumer	0.26	0.17	1.3	0.008	0.15	1.01
		Hula	0. 40	0.15	1.49	0.31	0.13	1.36
		Lemo	1.45	0.13	4.26	1.53	0.12	4.62
		Misha	0.09	0.16	1.09	0.06	0.14	1.06
		Wonsho	0.00[a]	0.00[a]	1	0.00[a]	0.00[a]	1
Altitude (masl)	2	<2000	-0.33	0.17	0.72	-0.58	0.16	0.56
		2000-2500	1.45	0.14	4.26	1.46	0.12	4.31
		≥2500	0.00[a]	0.00[a]	1	0.00[a]	0.00[a]	1
Number of clones	1	≤5	0.49	0.09	1.63	0.46	0.08	1.58
		>5	0.00[a]	0.00[a]	1	0.00[a]	0.00[a]	1
Spacing at Cycle 4 (m)	1	>1.5*1.5	-0.31	0.07	0.73	-0.20	0.06	0.82
		≤1.5*1.5	0.00[a]	0.00[a]	1	0.00[a]	0.00[a]	1

[a] Reference group; DF, degrees of freedom; Pr, Probability of a x^2-value exceeding the deviance; SE, standard error.

Aletachiko and Cheha Districts as compared to Wonsho District, respectively. However, there were five, three and two times lesser probabilities that wilt incidence at Cycle 4 would exceed 20% in Aletachiko, Cheha and Gibe Districts as compared to Wonsho, respectively. Similarly, the probability of occurrence of high wilt incidence at Cycle 4 in an altitude of <2000 masl was about two times lesser as compared to 2000-2500 masl area (Table 4).

Thus, the incidence of BWE and incidence at Cycle 4 appeared to be influenced by different independent variables. The association of wilt incidence to altitude could be attributed to the preferences of the pathogen for moisture and temperature requirement or might be for the difference in production system in different agro-ecologies. Ashagari (1985) and Maina et al. (2006)

also reported that the pathogen requires humid condition for survival. A similar report by Smith et al. (2008) indicated that altitude and environmental factor as the two major factors influencing the pathogen. Similarly, the association of BWE incidence with enset growing areas (administrative Zone and District) could be attributed to enset production and management system, type of clone grown and environmental effect. The association of wilt incidence and incidence at Cycle 4 to spacing might be related to pathogen transfer in contact with healthy plant in close or narrow spacing. On the other hand, the association of number of clones to both disease parameters could be, the farmers grow only some clones which tolerate the disease on their farm.

CONCLUSION AND RECOMMENDATIONS

Bacterial wilt of enset is one of the major biotic constraints of enset production in major enset producing parts of Ethiopia and it is widely distributed in all enset producing areas. It can result in up to 100% yield loss when causing complete wilting. The field survey in three major enset growing Zones, namely Gurage, Hadiya and Sidama of SNNPRS revealed the wide distribution of BWE although at varying intensity. It was noted during the survey that the disease has been reducing the yield by about 3.9% of enset in the survey area.

The average prevalence and incidence of the disease across the survey areas in the three Zones were 34.81 and 3.89%, respectively. The disease was more destructive in four Districts, namely Lemo (Hadiya), Edja (Gurage), Hula (Sidama) and Gumer (Gurage). Aletachiko and Cheha Districts were the least affected Districts by BWE. The distribution of the disease also varied greatly with altitude groups, with the mid- and high-altitudes having higher disease pressure than the low altitude. Variation in BWE was also observed due to growth stages. The disease was severe at Cycle 4 and at an age of 4 - 5 years and lowest at Cycle 1. However, the pathogen could attack the plant at any growth stage. In the survey areas, the farmers depended on enset for their food security. Besides, the disease has been threatening their economy and food security. The disease has also been risking the genetic diversity of the plant. The traditional cultivation practices, like cutting during propagation and agronomic practices of enset contributed to infection and spread of the disease.

Understanding disease epidemiology as affected by different variables is useful to design sustainable BWE management strategies. The present study identified that the disease was influenced by agro-ecology, plant population, growth stage and type and number of clones in enset fields. The result of this study confirmed that including these variables in developing management strategies for the disease is essential. The disease was most severe at Cycle 4, which was mostly prone to contamination by cutting with infected instruments. Therefore, reducing the enset cutting frequencies when the disease is suspected to prevail is important. Close spacing of enset had influence on increasing the disease spread, so wider spacing of greater than 1.5*1.5 m is recommended for reducing the disease spread.

Awareness creation among the farmers about the disease transmission, waste disposal methods and management options is essential. The present one season study is not complete in terms of sample size and area coverage. However, enset is important crop in other areas as a major food crop and the disease is devastating in such locations. Therefore, the status and distribution of the disease should be further determined. The effect of the disease at different growing seasons should also be assessed.

Conflict of Interests

The authors have not declared any conflict of interests.

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Morphocultural and molecular characterization of papaya tree *Colletotrichum* spp.

Juliana Stracieri[1]*, Fernanda Dias Pereira[2], Amanda Letícia da Silveira[2], Hélida Mara Magalhães[3] and Antonio de Goes[2]

[1]Universidade Estadual de Maringá – UEM Campus Regional de Umuarama PR, Brazil.
[2]Universidade Estadual Paulista "Júlio de Mesquita Filho" UNESP Jaboticabal SP, Brazil.
[3]Universidade Paranaense - UNIPAR Umuarama PR, Brazil.

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Papaya cultivation has great economic importance in tropical and subtropical countries, and Brazil is one of the largest producers of papaya (*Carica papaya* L.) in the world. However, productivity is hampered by plant health problems, particularly the pathogen *Colletotrichum* spp., which causes anthracnose and great damages on postharvest handling. This study characterized the morphocultural and genetic diversity of 21 isolates of papaya *Colletotrichum* spp. from different Brazilian states. The species were identified using the taxon-specific primers for *Colletotrichum gloeosporioides* and *Colletotrichum acutatum*. Eleven ISSR molecular markers were used to investigate the genetic diversity of the isolates. Moreover, the mycelial growth rate, pathogenicity, average diameter and colony color parameters were used in the morphocultural characterization. Of the 21 isolates, 19 were identified as *C. gloeosporioides* and two as *C. acutatum*. The ISSR markers showed great genetic diversity between the *C. gloeosporioides* and *C. acutatum* isolates, especially those from different locations. The morphocultural aspects displayed high polymorphism, and Linhares-1 and Linhares-2 isolated stood out for having unique characteristics when compared to other isolates. The pathogenicity test was positive for all isolates, but with different severity degree. *C. gloeosporioides* and *C. acutatum* displayed high genetic diversity among the isolates from different locations, and great morphocultural variability among isolates of papaya *Colletotrichum*.

Key words: Inter simple sequence repeat, *Carica papaya* L. *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*.

INTRODUCTION

Brazil is the third largest fruit producer in the world and ranks second in papaya production (*Carica papaya* L.), accounting for approximately 12.5% of world production. Over 1.5 million tons were produced in 2012 (FAO 2015). In recent years, however, the industry has been facing complex problems of a different nature, particularly

*Corresponding author. E-mail: juliana.uem@outlook.com.

regarding plant health. In this context, anthracnose, a disease caused by fungi of the *Colletotrichum* genus, stands out.

Anthracnose results in pre- and post-harvest losses. In general, pathogens associated with complex papaya anthracnose symptoms are classified as *Colletotrichum gloeosporioides*. However, recent studies have shown that this species is not always the only one involved in the disease (Prihastuti et al., 2009; Phoulivong et al., 2010). There are several species of the *Colletotrichum* genus that cause numerous diseases in various hosts, whose symptoms are not always visible until the fruit begins the maturation process (Prusky, 1996). The hosts include more than 470 genus of plants, where the fungi coexist as pathogens or endophytes (Cannon et al., 2008; Lu et al., 2004). Therefore, it is essential to identify the prevailing species since the epidemiology of fungal species and the control methods vary (Torres-Calzada et al., 2013). In addition, the precise determination of the *Colletotrichum* species is critical to the quarantine programs, for plant genetic improvement, and even to control these pathogens (Freeman et al., 1998; Cai et al., 2009).

The species most commonly associated with fruit anthracnose are *C. gloeosporioides* and *C. acutatum*, which are disseminated worldwide, infecting a broad range of subtropical and tropical crops such as papaya (*Carica papaya*), mangoes (*Mangifera indica*), avocado (*Persea americana*), passion fruit (*Passiflora edulis*) and banana (*Musa paradisiaca*) (Alahakoon and Brown, 1994; Abdul, 2001; Johnston et al., 2008; Hyde et al., 2009; Prihastuti et al., 2009). In recent years, however, there has been much disagreement over the definition of the taxa associated with anthracnose symptoms (Phoulivong et al., 2010).

There are many fungal species considered cryptic, forming a complex, including *C. gloeosporioides* and *C. Dematium,* among others (Shivas and Cai, 2012). According to Crous and Groenewald (2005), this complex can be extended to virtually every species of plant pathogenic fungi. The cryptic species are important due to the diversity of symptoms, host range, and geographic distribution.

Several works on the *Colletotrichum-Carica papaya* pathosystem have been published in recent years, including epidemiological aspects (Vásquez-López et al., 2012), genetic diversity, phylogeny, population structures (Andrade et al., 2007; Phoulivong et al., 2010; Rampersad, 2011), control (De la Cruz, et al. 2014; Barrera-Necha et al., 2008; Al-Eryani-Raqeeb et al., 2009). However, given the importance of the problem, and despite the great contribution of information created, there is a need for additional information regarding taxonomic analysis, to help define control strategies and other purposes.

Molecular methods based on DNA sequences are indispensable especially for cryptic species (Shivas and Cai, 2012). In general, studies of this nature are becoming popular for *Colletotrichum* spp. since the methods based on morphological and cultural tests have not always been satisfactory (Freeman et al., 1998). The alternative inter simple sequence repeat (ISSR) technique characterizes complex genomes, allowing the detection of polymorphisms in regions flanked by microsatellite DNA without isolating and sequencing specific DNA fragments (Cançado et al., 2012).

The ISSR marker is multilocus and, therefore, does not require prior knowledge of the DNA to be evaluated. It is a highly reproducible technique, low cost, and easy to use (Sserumaga et al., 2013). The ISSR markers are very effective in understanding the phylogenetic relationships of fungi in genetic diversity studies due to their high polymorphism (Schwarzenbach et al., 2007). When studying the several pathosystems, it is important to investigate the existence of genetic structures among the isolates of a particular pathogen from different producing regions, as well as those from specific niches, and their possible genetic divergence levels. The study on the species complex is important because it contributes to understanding its population dynamics. Genetic structure and gene flow influence the generation and distribution of new genotypes that affect the evolutionary and adaptive potential of the pathogen under changing selection pressure, and contribute to population viability and survival (Meng and Chen, 2001; Rampersad, 2013).

It is assumed that isolates from papaya that have never been treated with a fungicide may have specific characteristics compared to those from intensive production areas. Genetic data can contribute to quantifying genetic diversity and, also, lead to results that can contribute and guide the breeding programs. The molecular information complements the knowledge about the morphocultural aspects of the isolates, assesses, validates and facilitates comparisons to species that have already been described (Gaiero et al., 2011). To date, the literature on the genetic diversity of *Colletotrichum* from papayas in Brazil is somewhat generic and scarce. Studies in this field have been published by Peres et al. (2002) and Andrade (2007). This study characterized the morphocultural and genetic diversity of 21 Colletotrichum spp. isolates of papayas from different Brazilian states.

MATERIALS AND METHODS

Origin, cultivation and preservation of *Colletotrichum* spp.

A total of 21 isolates of *Colletotrichum* were collected from two types of papaya, 'Formosa' and 'Solo' (Sunrise Solo, Improved Sunrise Solo cv. 72/12 'or Tainung 1 or 2) in Itápolis/SP, Jaboticabal/SP, Maringá/PR, Umuarama/PR, Linhares/ES and Freitas Teixeira/BA, in 2014 (Table 1). The fruits were collected, stored in coolers and transported to the Phytopathology Laboratory of FCAV/UNESP, in Jaboticabal, where the pathogen was isolated. Most of the fruit was collected from producing areas, where all

Table 1. *Colletotrichum* spp. isolates, the origin of the papaya fruits, number of isolates and variety.

Origin	Number of isolates	Variety
Itápolis SP	8	Formosa
Jaboticabal SP	2	Solo
	2	Formosa
Linhares ES	1	Solo
	2	Formosa
Umuarama PR	2	Solo
Maringá PR	2	Solo
Teixeira de Freitas BA	1	Solo
	1	Formosa

Table 2. List of primers used, their respective sequences, amplification temperatures and number of fragments observed in *Colletotrichum* sp. isolates from papaya.

Primer	Sequences (5'– 3')	Amplification temperatures (ºC)	Number of fragments
CaInt2	GGGGAAGCCTCTCGCGG	64	1
CgInt	GGCCTCCCGCCTCCGGGCGG	54	1
P7	ACAACAACAACAACA	48	08
P8	AACAACAACAACAAC	48	09
P10	AAGAAGAAGAAGAAGAAG	48	07
P12	GACAGACAGACAGACA	48	10
P14	GACACGACACGACAC	53.8	14
P20	CTGAGAGAGAGAGAGAGA	48	12
P22	GAGCAACAACAACAACAA	53.8	09
AF80820	AGAGAGAGAGAGAGAGT	48	08
AF80821	AGAGAGAGAGAGAGAGC	48	10
AF80822	GAGAGAGAGAGAGAGAT	41.7	07
AF80824	GAGAGAGAGAGAGAGAYG	48	08

agricultural practices indicated for the culture are common, including the use of pesticides. However, the fruit collected in Jaboticabal originated from family farms where commercial and agronomic practices, including the use of pesticides, were not used.

The harvested fruits were maintained in the laboratory at room temperature under 12/12 h photoperiod, and at the onset of the symptoms, the pathogen was isolated. Symptomatic tissue fragments of approximately 5 mm^2 were removed, disinfected, rinsed in sterile water, dried on filter paper and placed in Petri dishes containing potato dextrose agar (PDA) medium. Subsequently, the petri dishes were incubated in BOD incubators at 25°C and 12 h photoperiod. After seven days, the typical *Colletotrichum* spp. colonies were selected, followed by subculturing, and subsequent identification based on the morphophysiological aspects of the culture (Sutton, 1992). From the typical and pure colonies of the pathogen, monosporic cultures were obtained and stored in mineral oil. The *Colletotrichum* spp. isolates were used in subsequent studies.

Molecular identification of *Colletotrichum* spp. isolates

For DNA extraction, the discs with the isolates' colonies were transferred to 110 ml glass bottles containing PD (potato-dextrose) liquid culture medium and kept for ten days. After this, the mycelium was sieved out from the culture medium, washed with distilled water, drained and dried on a Petri dish, and kept at room temperature for approximately 12 h. The dried mycelium was then macerated to form a dry powder in liquid nitrogen, which was transferred to 2.0 ml Eppendorf tubes.

DNA extractions were based on the Kuramae-Izioka protocol (1997). The quantity and quality of extracted DNA were assessed by measuring the absorbance of each sample using a NanoDrop 100 spectrophotometer (Uniscience).

The DNA samples extracted from *Colletotrichum* spp. were subjected to polymerase chain reaction (PCR) reaction with eleven ISSR primers (Table 2) and with two specific primers designed and developed by Mills et al. (1992), for amplifying the bands of about 500 bp for CaInt2 and 450 bp for CgInt, specific for *C. acutatum* and *C. gloeosporioides*, respectively, which were used in conjunction with the universal primer ITS4 to amplify the ITS region (White et al., 1990). Two isolates, one from *C. acutatum,* and one from *C. gloeosporioides* were used as amplification standards. They originated from tissues containing typical anthracnose symptoms, previously identified by sequencing the ITS2-ITS1-5.8S region, using the primer pair ITS1/ITS4 (White et al., 1990). The

Table 3. Grades attributed to edge and reverse coloring of colonies while determining the presence of sectors as much as possible.

Grades	C_C	C_E	C_R	S	P
1	Gray	White	White	+	Asymptomatic
2	White	Gray	Salmon /White	-	Small dark lesions
3	Salmon/White	Salmon	Salmon/ black		Lesions with waterlogging
4	Salmon	Salmon/ White	Salmon/ black/ White		Lesions with sporulation
5	Salmon/black	Salmon/ black	Salmon/ White /Green		-
6	Salmon/ black/ White	Salmon/ black/ White	Salmon/ black/Green/ White		-

C_C: Coloration of the colonies; C_E: edge coloring; C_R: Reverse coloring; B: Presence (+) or absence of sectors (-); Q: Pathogenicity.

sequencing of standard samples was carried out in the Laboratory of Biochemistry of Plants and Microorganisms, Department of Technology, FCAV/UNESP, Jaboticabal, SP.

PCR reactions were performed using 1X buffer (50 mM KCl, 200 mM Tris-HCl, pH 8.4); dNTP's (dATP, dTTP, dGTP, dCTP, 2.5 mM each) 0.2 mM, 2.0 U Taq polymerase, 2 mM $MgCl_2$, 5 pmol primer, 80 ng DNA and sterile pure water q.s.p. 20 µl. The amplification reactions with primers were performed in a Nexus thermal cycler (Eppendorf), using one cycle at 95°C for 3 min, 35 cycles of 94°C for 40 s, temperature-specific primer for amplification for 1 minute and 72°C for 1 min, and ending with 1 cycle at 72°C for 10 min. The PCR product was revealed by electrophoresis in TEB 1X buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) using 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) and visualized under the UV light of the Gel Doc XR (Bio-Rad) photographic equipment. The molecular size standard used was the 100 bp "DNA Ladder Plus" marker (Fermentas). The primers used were Invitrogen Life Technologies. The choice of primers was based on work carried out with species of the *Colletotrichum* genus or that presented high polymorphism (Gupta et al., 1994; Ratanacherdchai et al., 2010; Rampersad, 2013).

Morphocultural characterization of Colletotrichum sp.

All isolates used in molecular identification were previously grouped based on their origin. The appearance and color of the colonies were assessed. To this end, colonies' discs of 5 mm diameter were extracted from the margins of 7 day cultures, grown on PDA medium and transferred to new dishes containing the same medium. These cultures were then incubated at 25°C under a continuous fluorescent light. After ten days of incubation, period in which the colonies uniformly reached the edges of the plates while color stabilized, edge and reverse were evaluated, and the possible presence of sectors, as well. Three replications were used for each isolate. Each sample unit was represented by a Petri dish containing their isolates, the color and the presence of sectors were graded qualitatively (Table 3).

For the pathogenicity test, healthy papaya cv. Formosa fruits were subjected to the disinfection method according to Sanchez (1990). The fruits were superficially wounded with a sterile needle, and a drop of a suspension containing 1-2 x 10^6 conidia.mL of each isolate (7-day cultures on PDA), was placed over the wound using a 20 µl pipette. The fruits were placed in plastic bowls with polystyrene and water film at the bottom, covered with plastic wrap and kept in a cold chamber at 25°C and a 12 h photoperiod. The isolates were evaluated at 2, 4 and 6 days after inoculation (Table 3) (Andrade et al., 2007 modified). The experiment was conducted in a completely randomized design with four replicates per isolate. Each repetition consisted of a fruit inoculated at one point. As a control, fruits were inoculated with sterile water.

Colony diameters were measured daily perpendicular to each other, up to when the first colony of an isolate reached the edge of the plate. From these data, the mycelial growth rate (MGR) was determined according to Nechet (1999), using the formula employed by Oliveira (1991).

Data analysis

The amplification products, visualized in the gel, produced by each primer were used for preparing a genetic similarity array: present (1) or absent (0). The binary matrices were used to obtain estimates of genetic similarities, using the XLSTAT software (Addinsoft®, 2014 version) and the Jaccard coefficient. The Unweighted Pair Group Method with Arithmetic Average (UPGMA) was used to group the genotypes.

The results of IVCM and diameter of the colonies after 7 days of culture were analyzed statistically first as individual characteristics. The statistical design was completely randomized with 21 isolates and three replicates per isolate. Each sample was represented by a petri dish, where the *Colletotrichum* isolates were cultivated. Means were compared using the Tukey test ($P \leq 0.05$). Multivariate exploratory analysis, cluster analysis by the hierarchical method and principal component analysis, which allowed group evaluation of all variables, were also performed as a complement.

The hierarchical clustering technique links the samples by their associations, producing a dendrogram where similar samples are grouped together according to the chosen variables (Moita Neto and Moita, 1998). The similarity between the centroids of each isolate was measured using the Euclidean distance (a measure of dissimilarity) for a set of seven variables while the method of Ward was adopted for the grouping strategy. The result of the analysis to help characterize the groups is presented as a dendrogram.

The principal component analysis allows summarizing the greatest amount of original information contained in p variables (edge and reverse coloring of the colonies, presence or absence of sectors, colony diameter, and mycelial growth rate, MIGS) in orthogonal latent variables called major components, which are linear combinations of the original variables created with the eigenvalues of the data covariance matrix (Hair, 2005). The Kaiser criterion is used to designate the main components. The eigenvalue preserves the relevant information when it is greater than one.

RESULTS AND DISCUSSION

Only two of the 21 isolates subjected to PCR with the CaInt2/ITS4 primer pair molecular identification (T from F BA1 and Linhares ES1) were amplified for the 450 bp specific band of *C. acutatum*. The remaining isolates

Table 4. List of *Colletotrichum* spp. isolates according to the origin of the papaya fruit, isolates reference, analyzed variety, and species.

Origin	References	Variety	Species
Itápolis SP	1	Formosa	*C. gloeosporioides*
	2	Formosa	*C. gloeosporioides*
	3	Formosa	*C. gloeosporioides*
	4	Formosa	*C. gloeosporioides*
	5	Formosa	*C. gloeosporioides*
	6	Formosa	*C. gloeosporioides*
	7	Formosa	*C. gloeosporioides*
	8	Formosa	*C. gloeosporioides*
Jaboticabal SP	1	Solo	*C. gloeosporioides*
	2	Formosa	*C. gloeosporioides*
	3	Formosa	*C. gloeosporioides*
	4	Solo	*C. gloeosporioides*
Linhares ES	1	Solo	*C. acutatum*
	2	Formosa	*C. gloeosporioides*
	3	Formosa	*C. gloeosporioides*
Umuarama PR	1	Solo	*C. gloeosporioides*
	2	Solo	*C. gloeosporioides*
Maringá PR	1	Solo	*C. gloeosporioides*
	2	Solo	*C. gloeosporioides*
Teixeira de Freitas BA	1	Solo	*C. acutatum*
	2	Formosa	*C. gloeosporioides*

were amplified for the 500 bp specific band, corresponding to *C. gloeosporioides* when subjected to PCR with CgInt/ITS4 specific primers (Table 4).

In recent years, ISSR primers have become an important tool in molecular research of the genetic diversity of *Colletotrichum* (McKay, 2009; Rampersad, 2013; Ratanacherdchai, 2010) and other fungal genera due to the high reproducibility compared to other markers based on non-specific PCR, such as RAPD.

Studies related to the taxonomy of fungi species of the *Colletotrichum* genus associated with papaya have been conducted in different parts of the world. According to the literature, eight species of *Colletotrichum* have already been reported. *C. gloeosporioides* is the prevalent species and distributed worldwide (Prihastuti et al., 2009). *C. acutatum* was first described in papaya by Simmonds (1965), in Australia, *C. truncatum* (syn. *C. capsici*) (Damm et al., 2010), was described in Mexico, United States, Japan, Thailand, and Trinidad and Tobago (Tapia-Tussell et al., 2008; Tarnowski and Ploetz, 2010; Yaguchi et al., 1995; Sepiah, 1994; Rampersad et al., 2011), *C. brevisporum* (Vieira et al., 2013), *C. karstii* (Sharma and Shenoy, 2013) and *C. magna*, in Brazil (Nascimento et al., 2010).

In this study, two species of *Colletotrichum* spp. associated with the symptoms of anthracnose in the analyzed fruits have been identified: *C. gloeosporioides* and *C. acutatum* similar to the results in the literature. These results demonstrate the feasibility of using ISSR molecular markers in the taxonomy of *Colletotrichum* spp. Species associated with papaya. However, it is acknowledged that follow-up studies with *Colletotrichum* isolates from more locations and in greater numbers may help to detect additional new species, which have not yet been found in studies conducted under Brazilian conditions.

Generally, it is accepted in various parts of the world that the symptoms of anthracnose in papaya result from *C. gloeosporioides* infections (*strictu sensu*). However, the results of recent studies related to the taxonomy of the causative agents associated with the anthracnose-type symptoms, in several species of plants, including tropical fruits, should bring deep modifications in this scenario. Among these studies, there are analyses related to the diversity of fungal species (Phoulivong et al., 2010; Torres-Calzada et al., 2013), which obviously

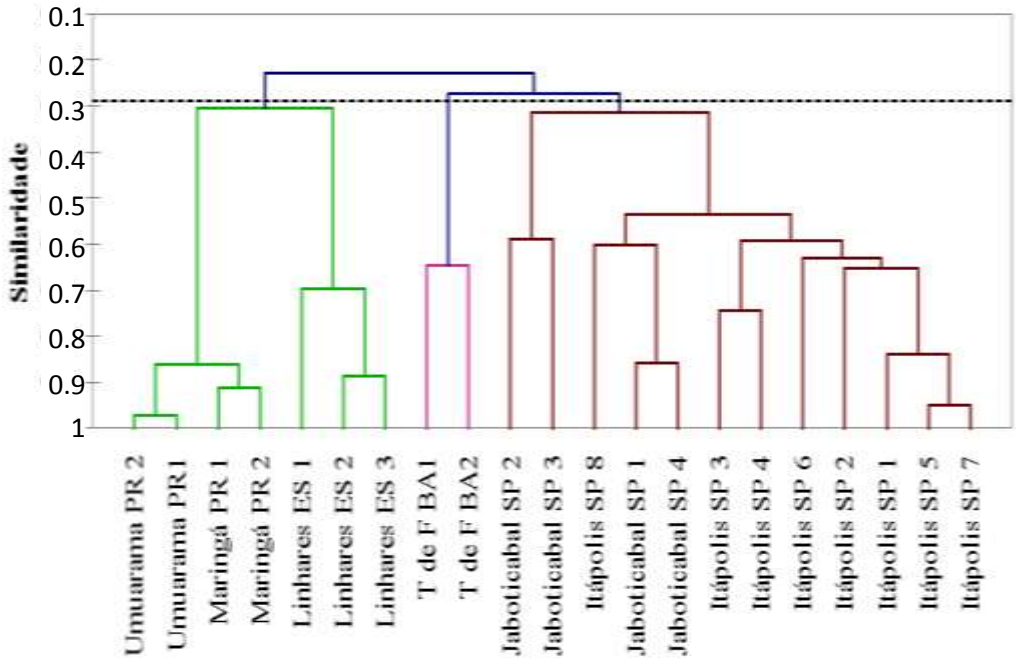

Figure 1. Different *Colletotrichum* spp. represented by UPGMAa and genetic similarity by Jaccard coefficient.

are going to contribute significantly to better understanding the pathogen-host relationship, including the development and validation of new and efficient control strategies of their causative agents. According to Andrade et al. (2007), to develop appropriate control strategies it is essential to thoroughly understand the various epidemiological relationships.

The 11 ISSR primers used allowed the coding of 103 loci, ranging from 7 (P10 and AF80822) to 14 (P14). The obtained dendrogram shows four distinct groups with common traits within the groups, the origin of the isolates. Group I consists of isolates from Paraná (Maringá PR1, Maringá PR2, Umuarama PR1 and Umuarama PR2); Group II, from Espírito Santo (Linhares ES1, Linhares ES2 and Linhares ES3), and Group III, from Bahia (Teixeira de Freitas BA1 and Teixeira de Freitas BA2). Last, Group IV consists of the isolates from papaya fruits collected in São Paulo (Figure 1). This study displayed a mean genetic similarity of 0.289, which shows a great genetic diversity among the isolates studied. Group I, the Umuarama PR1 and PR2, *Colletotrichum* spp. isolates from Paraná displayed the greatest genetic similarity of this study (0.971). The lowest genetic similarities (0.135 to 0.2) were found between the isolates from Linhares ES and Jaboticabal SP.

Agronomically, the culture managements between these two areas, Jaboticabal SP and Linhares ES, are completely different. In Linhares, Espirito Santo, papaya is cultured under intensive management, with various applications of pesticides to control insects, mites, and fungi. Unlike Jaboticabal, SP, where the plants were cultured in backyards as extractive exploration, without any technical and agro-economic concerns. Possibly, the ISSR markers were also efficient in detecting the biological nuances resulting from the effects associated with responses from these two ecosystems. The excessive use of systemic pesticides are factors that increase the strength of selection exerted on the populations of pathogens, favoring new virulence genes and polymorphism in the structure of these isolates (Araya, 2003).

The Linhares ES1 and TdeF BA1 access belong to the species *C. acutatum* and this particular trait/feature may have influenced the low similarity (less than 0.29), compared to the isolates from other locations. Rampersad (2013) used only 5 ISSR primers to evaluate *C. gloeosporioides* genetic structure from papaya fruit, and obtained two distinct groups, one from the South and the other from the North regions of Trinidad and Tobago. In this study, the *Colletotrichum* grouping showed an evident relationship with the geographical distribution. The genotype separation according to geographic distribution is not uncommon for ISSR, and has been done in Mexico (Torres-Calzada et al., 2013), Trinidad and Tobago (Rampersad, 2013) and Malaysia (Mahmodi et al., 2014).

Results of studies using either a limited number of isolates or from the same geographical site are usually inadequate to detect possible genetic diversity, which is better represented by a numerically and geographically better set of isolates. Generalized conclusions derived from a group of isolates of the same location, and without

Table 5. Mean mycelial growth rate (MGR), diameter of the colonies (DC) and notes corresponding to the color of the colonies (C_C), the edge (C_E) and reverse (C_R), and the absence or presence sectors (S) and the pathogenicity of *Colletotrichum* spp. isolates in BDA medium, at 25°C.

Isolates	MGR		DC		C_C	C_E	C_R	S	P
Itápolis SP 1	1,615	cde	8,021	de	2	1	3	-	3
Itápolis SP 2	1,596	cde	7,885	de	2	1	2	-	2
Itápolis SP 3	1,811	efg	8,447	de	2	1	1	-	4
Itápolis SP 4	1,504	cd	7,473	cde	1	2	3	-	4
Itápolis SP 5	1,613	cde	7,875	de	1	1	1	-	3
Itápolis SP 6	1,619	cde	8,062	de	2	1	2	+	4
Itápolis SP 7	1,895	fg	8,518	de	2	1	1	-	3
Itápolis SP 8	1,934	g	8,837	e	2	1	1	-	4
T de F BA1	1,650	def	5,248	a	2	1	2	-	4
T de F BA2	1,105	ab	5,835	abc	2	1	2	-	4
Umuarama PR1	1,363	bcd	7,011	Cd	2	1	4	-	4
Umuarama PR 2	1,095	a	6,872	bcd	2	1	1	+	4
Maringá PR 1	1,329	abc	5,965	abc	2	1	1	-	4
Maringá PR 2	1,194	ab	5,832	abc	2	1	1	-	4
Jaboticabal SP 1	1,713	defg	8,203	De	2	1	1	-	2
Jaboticabal SP 2	1,613	cde	7,904	De	2	1	1	-	3
Jaboticabal SP 4	1,813	efg	8,749	E	2	1	1	-	2
Jaboticabal SP 3	1,848	efg	8,326	De	2	1	1	-	3
Linhares ES 1	1,157	ab	5,309	Ab	3	2	5	-	4
Linhares ES 2	1,543	cd	8,064	De	1	1	6	+	4
Linhares ES 3	1,635	def	7,996	De	2	1	5	-	2

Means followed by the same letter do not differ. MGR: CV = 5.45 and DMS = 0.264 and DC: CV = 7.10 and = 1.66 DMS.

the backing of a molecular study, should be avoided. The colors of *Colletotrichum* colonies varied from gray to salmon/white/grayish (Table 5). The edge color ranged from white to gray while the reverse, from white to salmon/white. Sectors were observed in some isolates. Analysis of variance of both the IVCM data and diameter of the colonies after seven days of culture showed a significant difference in the behavior of the isolates (Tukey at 5%).

The isolates T de F BA2, Umuarama PR2, Maringa PR1, Maringa PR2 and Linhares ES1 displayed the lowest MGR and differed significantly from the isolates from Jaboticabal SP, Itápolis SP, Linhares ES2, Linhares ES3 and TdeF BA1 (Table 4). On the other hand, the isolates from Itápolis SP8, SP7 and SP3; Jaboticabal SP1, SP3, and SP4 displayed the highest values for this variable.

The isolates Maringa PR1 and PR2; TdeF BA2; and, Linhares ES1 displayed the lowest value for the diameter of the colony parameter while the largest was recorded for Jaboticabal SP4 (Table 4).

The hierarchical cluster analysis method applied to the variables, coloration of the colonies, board and reverse, presence or absence of sectors, mycelial growth rate and diameter of colonies after seven culture days showed a great variability among the isolates, that were clustered in three different groups: Group I (GI), group II (GII) and group III (GIII) (Figure 2).

GI group consisted of the isolates from Itápolis (SP1, SP2, SP3, SP7, and SP8) and Jaboticabal (SP1, SP2, SP3, and SP4). A high degree of similarity was observed among them, given the existence of common features, especially for the Jaboticabal SP3 and Itápolis SP3 isolates, whose Euclidean distances were close to zero. The isolates of this group displayed white colored colonies and edges, lack of sectors, and the largest MGR and diameter of the colony. In the molecular analysis, these genotypes also shared the same grouping.

GII consisted of the isolates Itápolis (SP4, SP5 and SP6), Umuarama PR2 and Linhares ES2, which were characterized by the presence of sectors and the isolates Itápolis SP4 and SP5, which displayed gray colonies. The similarity in this group was low, with high Euclidean distances, as shown in Figure 2.

The isolates of GIII displayed predominantly white colonies, white edge, and no sectors. The exception was the Linhares ES1 isolate, which presented salmon/white colony and gray edge. T de F BA1 and BA2 isolates had gray reverse colonies while Maringa PR1 and PR2 were white. Linhares ES1 and ES3 isolates had salmon/white/green reverse while Umuarama PR1 isolate, salmon/white. Only two of the 21 studied isolates were

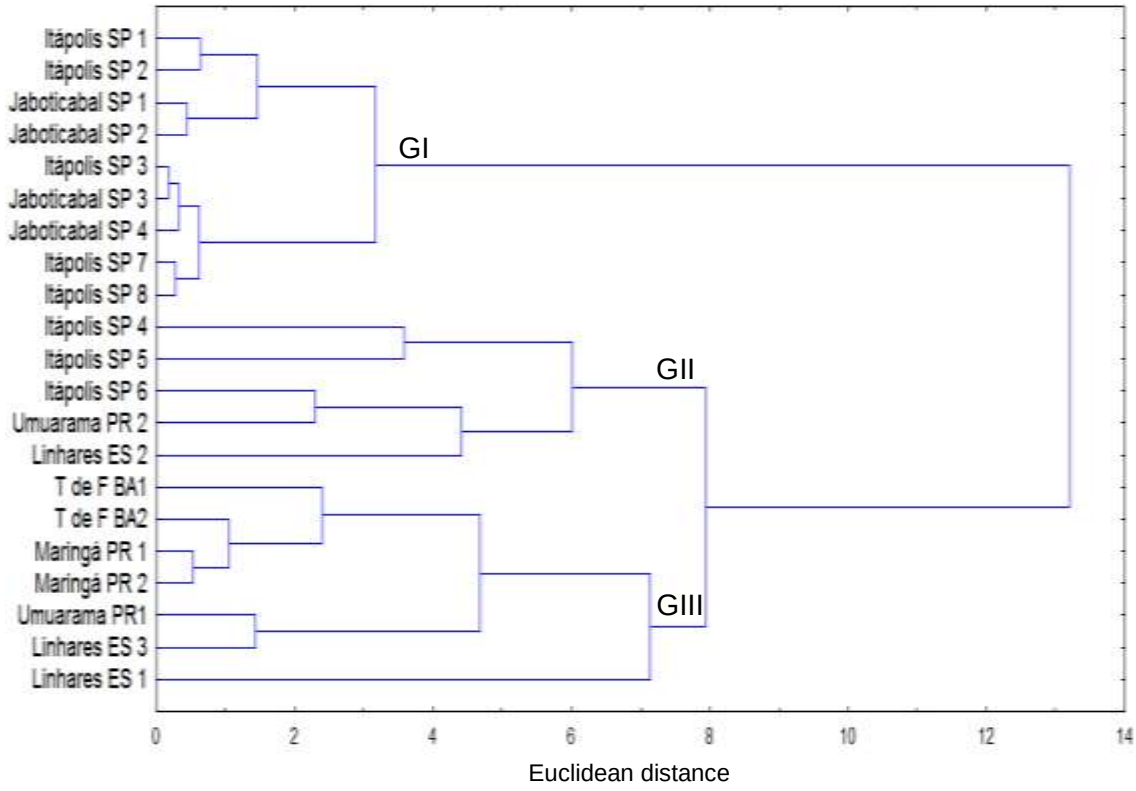

Figure 2. Dendrogram resulting from the hierarchical cluster analysis showing the groups formed according to the variables: color of colonies, edge and reverse, presence or absence of sectors, mycelial growth rate, diameter of colonies after seven days cultivation.

identified as *C. acutatum*, and both are in this group (Linhares ES1 and TdeF BA1).

Two eigenvalues were greater than unity, which generated two main components, preserving 62.20% of the original variability (38.86% in CP1 and 23.34% in CP2). Thus, the initial set of six dependent variables was characterized by two new independent latent variables, located in two-dimensional figures (Figure 3) (ordering of isolates by the main components).

The graphical representation of the main components allowed to characterize the variables that discriminated the most when forming the Groups I, II and III. The mycelial growth rate (MGR), diameter of colonies after seven days of culture (DC) weighed the most in Group I, displaying the highest values of MGR and DC while in Group III, MGR and DC displayed the lowest values. The presence of sectors separated the isolates of Group II from the others. All isolates that displayed sectors are in Group II, but this group was not only discriminated by this feature. Isolate Linhares ES2 showed salmon/black/ green/white reverse color (C_R); furthermore, this is the only group in which all isolates have pathogenicity 4. The characteristic colony (C_C) and edge (C_E) colors also helped to separate the isolates of Group III, which displayed predominantly gray colony and white edge, only Linhares ES1 had special characteristics, salmon/ white colony and gray edge, and thus is away from the center of mass. It is known that this isolate belongs to *C. acutatum* species, and could, therefore, be responsible for its peculiar characteristics compared to *C. gloeosporioides.* The pathogenicity classification did not correlate with any of the characteristics of the colonies. The most pathogenic isolates were found in various geographic regions, all isolates from Paraná and Bahia displayed pathogenicity 4, depressed lesions with sporulation, and the only region that did not have any isolate with pathogenicity 4 was Jaboticabal, in São Paulo.

In terms of morphocultural aspects, there is, in general, high polymorphism among the species of fungi, especially between those belonging to the *Colletotrichum* genus. This behavior is, usually, due to environmental influences, particularly the aspects related to the types of culture media, ambient temperature and luminosity (Maia et al., 2011; Nechet and Abreu, 2002). Some species of fungi display cultures characteristics very different from the original, even when using sub-colonies that originated from the same colony. On the other hand, in the case of *Colletotrichum*, the morphology is greatly similar, resulting in complex taxon revisions (Weir et al., 2012). The morphological characteristics of the isolates are not sufficient to classify them as either *C. gloeosporioides* or

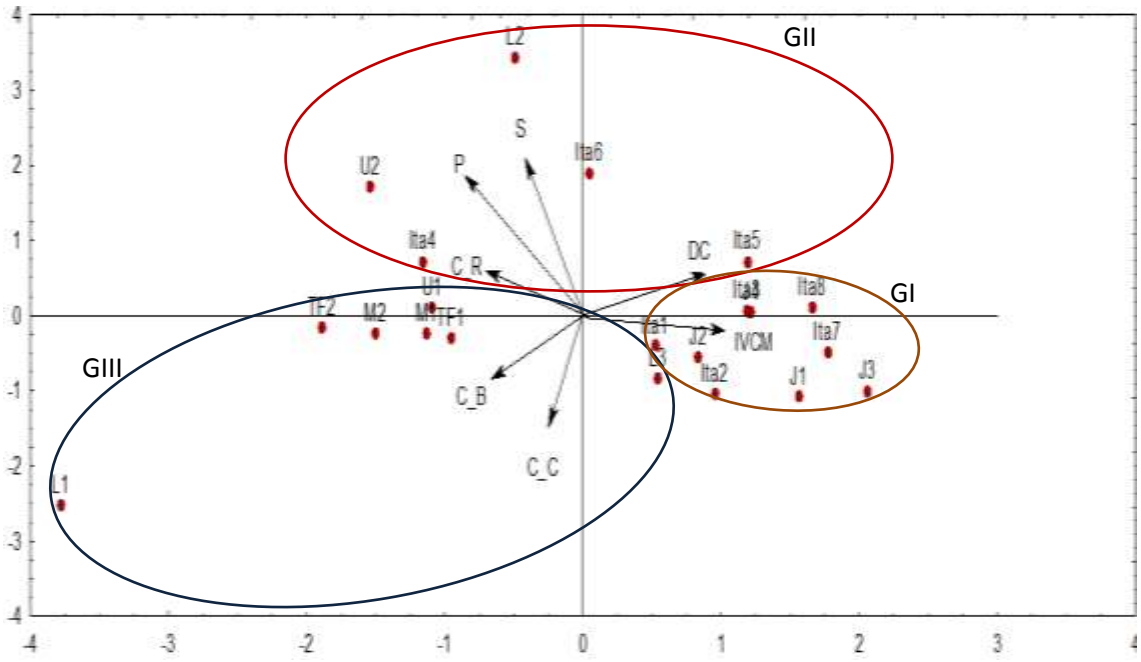

Figure 3. Distribution according to the results of the morphocultural analysis of isolates of *Colletotrichum* spp., in the principal components 1 and 2.

C. acutatum. Ferraz (1977), classified the isolates of the *Colletotrichum* genus into groups, according to their cultural characteristics, but in the present study there was a great molecular and morphocultural variability in individuals of the same species and, therefore, the classification in groups considering the cultural characteristics of the isolates was not possible.

CONCLUSION

(i) Isolates from 'Formosa' and `Solo' papaya from four Brazilian states were analyzed and classified as belonging to the *C. gloeosporioides* and *C. acutatum* species;
(ii) There was great genetic diversity among *C. gloeosporioides* and *C. acutatum* isolates from different locations, and in contrast, greater similarity among isolates from the same region;

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Levels of nitrate, pigments and thermographic analysis of lettuce under different temperatures of nutrient solution

Samuel Silva*, Ronaldo do Nascimento, Hallyson Oliveira, José Alberto Ferreira Cardoso, Diego Azevedo Xavier and Sonivagno de Sousa Silva

Federal University of Campina Grande, Academic Unit of Agricultural Engineering, Plant Physiology Laboratory, CEP 58429-140, Campina Grande, PB, Brazil.

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The temperature of the nutrient solution in hydroponic crops of lettuce is a determinant of biomass yield and affects several physiological mechanisms of the plant. The aim of this study is to evaluate the influence of nutrient solution temperature on the levels of nitrate and pigments in leaves, as well as green mass yield and thermal flow in lettuce crop hydroponically. The experiment was conducted in a greenhouse at the Federal Technological University of Paraná. The nutrient solution was maintained at three temperatures (15 and 25°C and environmental temperature). The accumulated nitrate in leaf and photosynthetic pigment level were evaluated five times (28, 35, 42, 49 and 56 days after sowing). The total green mass yield and thermal flow behavior in the plants were assessed through thermographic analysis. The temperature of the solution influenced the levels of nitrate, chlorophyll and carotenoids in lettuce leaves, and green mass, which were larger at 25°C treatment. The root and stem had thermal equilibrium with the nutrient solution, while the temperature at the middle and upper part of the plant is similar to that of the environment.

Key words: *Lactuca sativa*, pigments, nitrate, heat conduction, green mass.

INTRODUCTION

Hydroponic is a production technique that allows the control of many variables. With the current available technologies, it is of great benefit to conduct research that enables the maximization of productivity in this cropping system.

Among the leafy vegetables that adapted more to this system, lettuce is mostly grown by farmers and consumed in Brazil because of its nutritional value, taste, and availability throughout the year (Geisenhoff et al., 2009). However, it is a very sensitive to adverse

*Corresponding author. E-mail: sam_capela@hotmail.com.

environmental conditions, as temperature of the nutrient solution is one of the factors that mostly influence the growth and development of plants.

Among the variables that can be affected by the temperature of the solution, there is the absorption of nutrients (Feltrim et al., 2009). Once the plant does not absorb excess water, the stomatal opening is not affected (Costa and Marenco, 2007). Thus, high transpiration flow carries more nutrients up to the leaves, mainly nitrogen (N) in nitrate form. With the satisfactory amounts of N accumulated in the leaves, the plant tends to produce more biomass and larger molecules formed by this element. Chlorophyll is one of the most present in the plant and most known. The amount of nitrate in the leaves must be accompanied often, because its residuality is an indicator of it being toxic to human health as a potential carcinogen (Luz et al., 2008).

Plants with high amount of heat in the leaves need transpiration to reduce their temperature (Taiz and Zeiger, 2009), and one of the tools that can be used in monitoring the changes associated with the pattern of leaf cooling model is thermal analysis, which is done remotely and instantly. Jones (1999b) suggests the potential use of thermographic images as estimation tools even for stomatal conductance. Thus, this work aims to evaluate the influence of the nutrient solution temperature on lettuce crop grown hydroponically.

MATERIALS AND METHODS

The experiment was conducted in a greenhouse at the Federal Technological University of Paraná - UTFPR (25 ° 17'58.06 " S and 54 ° 06'52.28 " O, 417 m altitude), in the City of Medianeira –PR. The climate is Cfa, humid with hot summers, based on Koppen classification. The average annual temperature is 21°C and rainfall is 1,880 mm per year.

The lettuce crops were placed in nutrient solution using NFT (Nutrient Film Technique) method. A wooden bench of 1 m and inclination of 3% were used. Two types of cultivation channels were used: Nursery channels with polyethylene of 6 m long and 40 mm in diameter and definitive cultivation channels with polyethylene of 6 m long and 75 mm in diameter. Definitive channels were placed 15 cm apart from each other.

Three boxes of polyethylene water with a capacity of 250 L were used as the nutrient solution reservoirs. They were installed below the level of the bench, and semi buried in the ground. This allows the return of the nutrient solution per gravity, forming a closed system. The nutrient solution pumping from the reservoirs to the cultivation channels was conducted using three electric pumps with 41 W powers. It was drowned and individually actuated by means of a timer. The flow at the channel inlet was 1.25 L min^{-1}, which was controlled per 1/4 valve and monitored twice a day (7h and 9h) by means of graduated cylinder and chronometer. The separation of the irrigation system was needed to ensure control of the nutrient solution temperature.

The control of the nutrient solution temperature was carried out by means of digital thermostat coupled to a sounder, which measured the temperature at the entrance of the discharge pump. This thermostat controls the cooling system as heat varies from 1°C more or less per minute.

The heating system is composed of a resistance heater encapsulated in glass. The cooling system was formed by a compressor (outer part) and an evaporator coil with copper (internal part). The copper coil was in contact with the nutrient solution and to avoid contamination of the solution with copper, it was coated with a polytetrafluoroethylene layer.

A completely randomized design with three treatments and 10 replications were used in this work. All the treatments used the following temperatures: 15, 25 and ambient temperature (21.8°C). Each plot consisted of a cultivation channel containing ten plants, wherein the first orifice channel was used to observe the flow, and the two as its surrounding. Also, a cultivation channel was used on each side of the borders.

Pelleted seeds of the cultivar Vera were used. The sowing was done in phenolic foam cells of 1.9 × 1.9 × 2.0 cm; they were washed and accommodated at the bottom of a corrugated pan, which was maintained in dark storage and moistened with water only for 48 h. After germination, the seedlings were exposed to light in the greenhouse and received diluted nutrient solution three times a day with electrical conductivity of 700 µS cm^{-1}. At 14 days after sowing (DAS), the seedlings were transferred to the nurseries channels; initially, they received nutrient solution of 700 µS cm^{-1} and then it increased from 100 µS cm^{-1} each day to 1,400 µS cm^{-1}. During this period, the plants received nutrient solution in ambient temperature with circulation time or resting time of 15 min from 7 am to 19 h. At night, the nutrient solution was supplied for 15 min with intervals of 105 min rest. At 21 DAS, the more uniform plants were transferred to the definitive cultivation channels, where the temperature of the nutrient solution was controlled and the circulation time was similar to that of the nurseries channels for 56 DAS.

The nutrient solution used in the experiment was recommended by Furlani et al. (1999). Its chemical composition of macro and micronutrients is presented in Table 1. The level of the reservoir, electrical conductivity and pH were monitored twice a day (7 and 19 h). The reservoir level was maintained above 50% of its capacity, the pH was maintained between 5 and 7 and the electrical conductivity between 1,000 and 1,400 µS cm^{-1}.

Nitrate concentration, chlorophyll content and carotenoid content were analyzed at 28, 35, 42, 49 and 56 days after sowing (DAS). For the determination of nitrate levels, we used the colorimetric method described by Cataldo et al. (1975), from the nitration of salicylic acid and read in spectrophotometer at 410 nm. For the extraction and quantification of chlorophyll and carotenoids, the method described by Lichtenthaler and Welburn (1983) was used. 10 leaf discs per treatment (one disk per plant) were used. They were collected around the middle of plant, avoiding the ribs. At the end of the cultivation (56 DAS), the total green mass with precision scale was analyzed. Analyses were done in Food and Water Analysis Laboratory, Campus Medianeira of the Federal Technological University of Paraná, accredited by the Secretary of State for Agriculture and Supply of Paraná.

The thermal images were obtained at 55 DAS by means of a brand imager of Testo® model 881, whose reading occurred from 19 to 20 h with the irrigation maintained in continuous flow. The reading started 10 min at the start of the irrigation. After removing the plants from the cultivation channel, they were accommodated in a support to reduce the thermal interference of the medium and the reading was realized by positioning the imager at a distance of 1 m from the plant in less than 30 s. When this time was extrapolated the plant was returned to the channel and a new measurement was realized. Four points in the plant were used for thermal comparison: Root temperature (Tr), stem temperature (Ts) temperature of the middle leaf (Tm) and temperature at the end of the leaf (Tex). The temperature and relative humidity during the measurement were 25.5°C and 84%, respectively. Image processing was carried out by Testo IRSoft® 3.2 software.

The averages of the variables: Nitrate concentration, chlorophyll content, carotenoid content and green mass yield were compared using confidence intervals overlap ($p<0.05$), while the temperature

Table 1. Chemical composition of the nutrient solution recommended for leafy vegetables for Furlani et al. (1999).

Macronutrients	**N-NO_3^-**	**N-NH_4^+**	**P**	**K^+**	**Ca^{2+}**	**Mg^{2+}**	**S**
(g 1,000 L^{-1})	174	24	32.7	193	183	39.4	52
Micronutrients	**B**	**Cu**	**Fe**	**Mn**	**Mo**	**Zn**	
(g 1,000 L^{-1})	0.3	0.004	3.6	0.3	0.08	0.11	

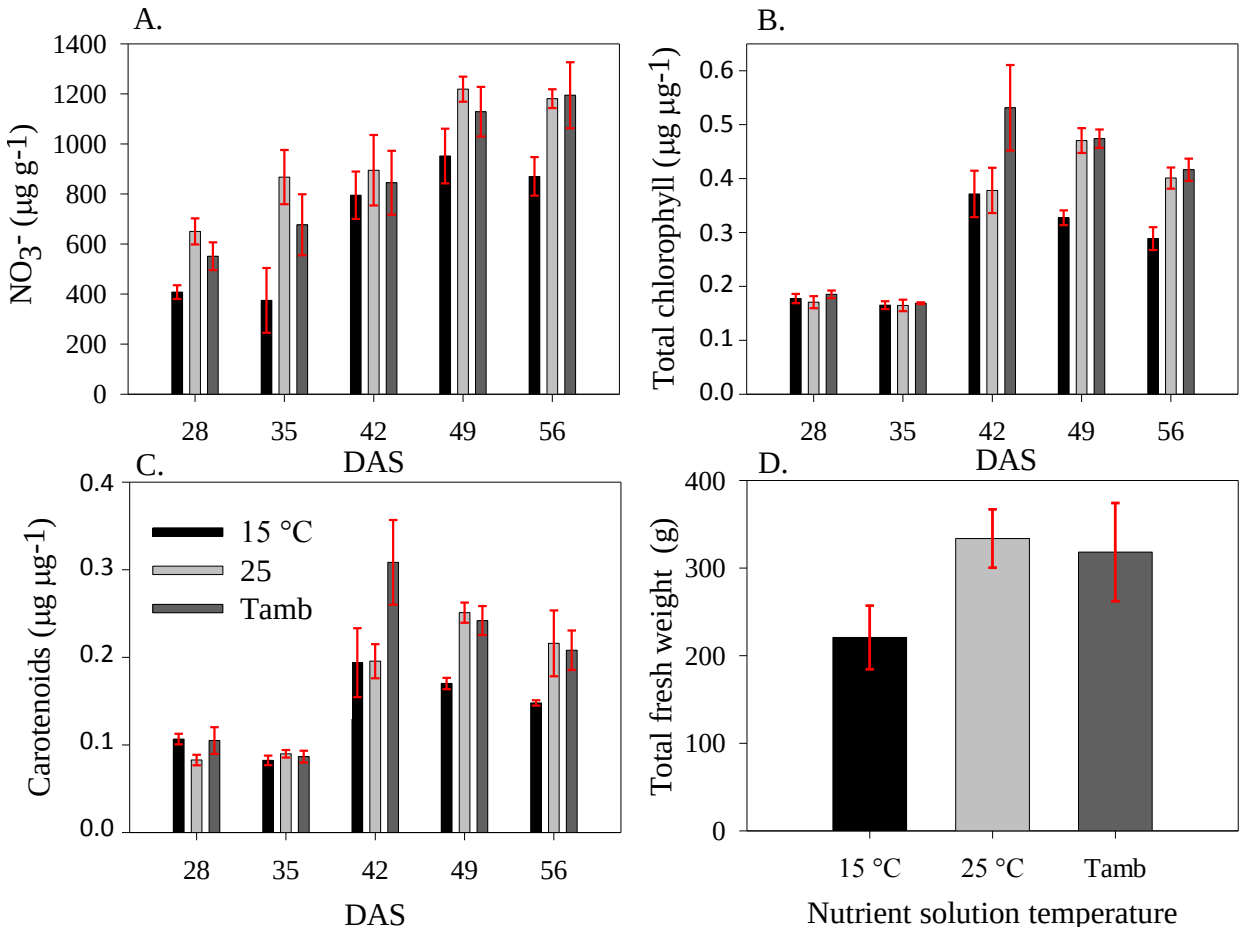

Figure 1. Nitrate levels (A), total chlorophyll (B), and carotenoids (C) leaves at 28, 35, 42, 49 and 56 DAS and total fresh weight (D) lettuce grown in nutrient solution temperature of 15 and 25°C and ambient temperature. The averages differ by no overlap of the bars of their respective confidence intervals ($p < 0.05$).

values measured in different parts of the plant in each treatment were submitted to Tukey test (5%).

RESULTS AND DISCUSSION

The nitrate content in lettuce leaves differed significantly between treatments at different temperatures of the nutrient solution during the cultivation. The maximum values ranged from 951.6 to 1,218.7 µg of NO_3^- per gram (g) of green mass leaf at 15 and 25°C, respectively (Figure 1A). Thus, plants irrigated with nutrient solution at 25°C accumulated, on average, 2 and 28% higher nitrate than plants with 15°C solution and ambient temperature (Tamb), respectively.

This behavior is due to the increase of nutrient solution temperature, which results in increased electrical conductivity thereof. A higher electrical conductivity favors increased nitrate content in lettuce leaves, because the conductivity of the nutritive solution does not only influence the absorption of water, but also the absorption of nutrients, both of which are closely linked (Steidle et al., 2005; Genuncio et al., 2012; Cometti et al., 2013).

Table 2. Test medium for the temperatures obtained in points of the plant and for the temperature of the nutrient solution [(1)].

Temperature of nutrient solution	Temperature at the measuring point in the plant (ºC)				
	Tr	Ts	Tm	Tex	CV (%)
15 °C	15.01[b]	14.85[b]	17.97[a]	17.96[a]	1.19
25 °C	24.96[a]	25.44[a]	23.26[b]	23.21[b]	3.89
T_{amb}	21.65[b]	22.09[b]	22.57[ab]	23.20[a]	6.12

Tr - Root temperature, Ts - Stem temperature, Tm - Temperature of the middle of the plant, Tex - Temperature of the extremity of the plant. [(1)] Means followed by different letters in the lines differ by Tukey test at 5% probability.

This association is due to the fact that as temperature increases in the leaf, the stomata of the lettuce tends to open more for the cooling process, transferring heat to the water and releasing it in vapor form, a process called transpiration. Thus, the higher the transpiration, the greater the cooling of the leaf; in other words, the higher the energy of the water in the leaf, the greater is the stomatal opening degree and time. As a result, the absorption and transport of nutrients to the shoot through transpirational flow occurs at higher rates, so that there is enhanced absorption in the root, transmission via the xylem and accumulation of nitrate in the leaves (Taiz and Zeiger, 2009).

The nitrate levels in leaf increase in the crop until it reaches its maximum in the last two collections. There is a tendency for these values to decrease when the plant is in its senescence period. Therefore, the values used for comparison with the limits tolerated for human consumption were the highest, which were observed at 49 DAS at 15 and 25°C and 56 DAS at Tamb. However, these values were lower than those recommended by the World Organization for Food and Agriculture (WHO, 2012) and the European Community legislation (CE, 2006), remaining within the standards allowed by standard-setting bodies and close to the values found in lettuce for several researchers (Escoín - Peña et al., 1998; Fernandes et al., 2002; Takahashi et al., 2007; Luz et al., 2008; Oshe et al., 2009; Aprigio et al., 2012).

Oshe et al. (2009) observed high nitrate content of 80.2 mg kg^{-1} in the shoot of the cultivar Vera, hydroponically without shading. However, Aprigio (2008) found 1,330 mg kg^{-1} and Takahashi et al. (2007) quantified 2,314 mg kg^{-1}.

Due to the variation in nitrate accumulation in the leaves at different temperatures of the nutrient solution, there were also significant changes in total chlorophyll content from the 49 DAS, when the plant was already well formed (Figure 1B). Considering the maximum levels of chlorophyll at 15 and 25°C treatments, there was 27% increase in the higher temperature treatment, a value which confirms the increase in nitrate concentration (28%).

As nitrogen is part of the constitution of chlorophyll molecules, there is direct relationship between the availability of N and the formation of chlorophyll in leaves (Taiz and Zeiger, 2009). Soratto et al. (2004) observed that the chlorophyll content is viable to indicate N deficiency in the plant. In some crops, the measurement of chlorophyll content through portable meters is a good alternative to indicate the amount of N to be applied (Argenta et al., 2011).

The content of carotenoids in the leaves, as well as chlorophyll, had significant change between treatments at 15 and 25°C from 49 DAS (Figure 1C). However, this difference is related to the need for heat loss by the plant, once carotenoids known as xanthophylls are responsible for heat dissipation. Since at 25°C treatment the water was already absorbed with a large amount of heat, it possibly might have reduced the cooling capacity for latent heat through transpiration. Thus, the plant could produce more carotenoids of this kind to convert the excess light energy absorbed into heat, preventing damage to the chloroplast photosynthetic apparatus, a process known as non-photochemical quenching (Taiz and Zeiger, 2009).

The green mass yield also increased significantly by increasing the temperature of the solution from 15 to 25°C, where the difference was equal to 51% in relation to treatment at 15°C. This could be due to the fact explained above, in which there was necessity for the plant stomata to have been opened longer for leaf cooling. Through the stomatal opening there is a control of CO_2 in the leaf, which directly affects photosynthesis (Liberato et al., 2006). With sufficient water and heat on the leaves, the stomata opening may have promoted greater absorption of CO_2, causing higher photosynthetic rates and consequently higher green mass yield.

Malorgio et al. (1990) observed an increase in the fresh weight of lettuce in NFT with temperature of 25°C in the area of the root system compared to lower temperatures.

The temperature readings realized in the several measuring points in the plant differed significantly according to Tukey test ($p<0.05$) for each treatment (Table 2). It is observed that in the treatment with 15°C the temperature tended to increase from the middle of the plant, while in the treatment with 25°C this effect occurred inversely.

In all treatments it was observed that the temperature of the root and stem is at equilibrium with the temperature of the nutrient solution. However, the temperature of the greenhouse and the plant tip tend to approach the

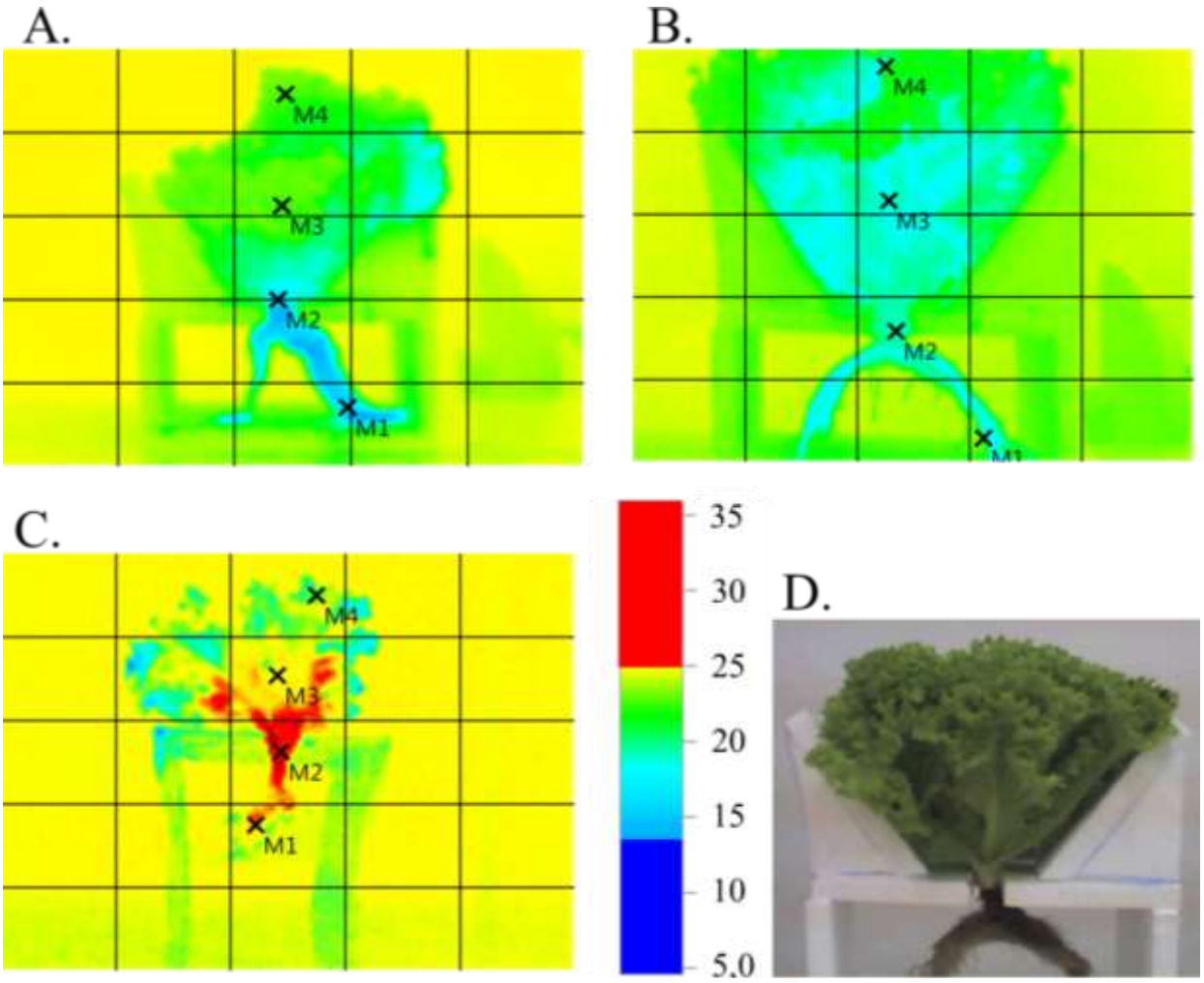

Figure 2. Thermographic images of lettuce plants submitted to nutrient solution at 15°C (A), ambient temperature (B) and 25°C (C), in detail for the accommodation of the plant for measuring the temperature in the several points (D); M1 - plant root , M2 - the plant stem, M3 - middle of the plant and M4 - extremity of the leaves

ambient temperature (Figure 2).

At 15°C treatment, the water of the solution was absorbed by the roots of low thermal level, but to be transported to the leaves, the temperature was increased. This is because it has received a great deal of latent heat derived from photosystems and cellular respiration (Figure 2A). Beyond that, as the leaf mass was higher than the root, the temperature at the upper part of the plant was increased by the need for thermal equilibrium solution-plant-atmosphere.

With the average thermal image of treatment with nutrient solution at ambient temperature, it is observed that the temperatures of the root, stem, middle of the plant and extremity of the leaves were maintained very close (Figure 2B). However, the plant temperature was below ambient temperature. This is due to the fact that for the plant to absorb water solution at ambient temperature, heat needs to be transferred to the water. It remained at equilibrium in its several parts. After losing latent and sensible heat to the environment, its temperature was lowered to absorb more water than this.

In thermography, average of the plant was irrigated with nutrient solution at 25°C. It is observed that temperatures of roots and stem were very close to the temperature of the nutritive solution (Figure 2C). The temperature of the middle of the plant, while achieving a smaller coverage area, was also close to the temperature of the nutrient solution, and the temperature at the end of the leaf had a tendency to become milder. In this case, the water was absorbed with a large amount of heat and reached the leaves. This heat may have been dissipated by the greater transpiration flow, making the leaf temperature lower than that of air. Furthermore, in this treatment there was a major yield of carotenoids, which may have contributed effectively to transfer the excess heat from the plant for the water and thereby reduce the

leaf temperature (Taiz and Zeiger, 2009).

Thermography is an effective technique for monitoring the heat content in plants. So it can be used as an important tool for the temperature of the nutrient solution to be adjusted carefully to avoid heat stress on the plant and accumulation of nitrate above the maximum level recommended for human health.

Conclusion

1. Increasing the nutrient solution temperature promotes greater accumulation of nitrate and higher levels of chlorophyll and carotenoids on lettuce leaves, as well as increased green mass yield.
2. Independently, the thermal condition, the temperature of the root and stem remain at equilibrium with the temperature of the nutrient solution, while the temperature of the middle and end of the plant tends to approach ambient temperature.

Conflict of Interests

The authors have not declared any conflict of interests.

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Foliar application of urea and bell pepper amino acids

Rodrigo Teles Mendes, Roberto Cardoso Resende, Marco Antonio Moreira Pereira, Rafael Umbelino Bento, Renan Cesar Dias da Silva, Sihélio Julio Silva Cruz* and Adilson Pelá

Universidade Estadual de Goiás, Câmpus Ipameri, Rodovia GO 330, km 241, s/n Anel Viário, Ipameri, Goiás, CEP: 75780-000 Brazil.

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The Bell peppers is a tropical culture and it is in the 10 most consumed vegetables of the world. The mineral nutrition is essential to the productivity and better quality of the gather fruits. In bell pepper cultivation, high portions of nitrogen are essential to a good performance of fruits. The foliar fertilizing is a complement to the ground fertilizing. The amino acids are a good source of nitrogen, once these are quickly incorporated to the plants metabolism. Therefore, this work aimed to estimate the sources and portions of N to the foliar application in Bell pepper cultivar. The Randomized blocks design was utilized in a factorial scheme 2 × 4, with four repetitions. The first factor corresponded in two sources of N: urea and amino acids. The second factor corresponded to the number of repetitions by foliar: 0, 1, 3, 5 and 7 applications during the culture cycle. The first foliar application occurred ten days after the transplant, and the subsequent in breaks of fifteen days after the first application. In the test there was not application. The baselines evaluated: Cross length of fruits, fruits diameters, number of total fruits and productivity in kg ha^{-1}. The results were submitted to variation and regression analysis. The application of amino acids by foliar in plants of Bell pepper, increased the diameter and the length of fruits. High sources of urea also provided morphological changes in fruits however lower amino acids utilization.

Key words: *Capsicum annuum* L., nitrogen fertilizing, fruit quality.

INTRODUCTION

The Bell pepper is from the family Solanaceae; it is a tropical culture. In an economic way, it is in the ten most important vegetables in the Brazilian market. It is a culture of fast return to investing, in a short period to the beginning of the production, which is why it is very explored by small and medium vegetables (Campos et al., 2008).

Into the factors of production of vegetables, the mineral nutrition is essential to raise the productivity and increase the quality of picked products (Marcussi et al., 2004). In bell pepper harvest, to a high quality and high performance of fruits, it is essential to provide high portions of nitrogen (N). In literature, there are recommendations raging in 221 to 400 kg of N ha^{-1}, with parcel applications during the sowing and then in cover, to reduce the lost by leaching and increase the efficiency of fertilizing usage (Stagnari et al., 2007; Campos et al., 2008; Araújo et al., 2009).

*Corresponding author. E-mail: sihelio@agronomo.eng.br.

Table 1. Average standards referring to the height of plants, length of fruits, diameter of fruits, number of fruits and productivity in function of the treatments with urea and amino acids.

Treatments	Length of fruits (cm)	Diameter of fruits (cm)	Total of fruits (units ha^{-1})	Productivity of fruits
Amino acids	12.5	68.8[a]	316.9[a]	48.5[a]
Urea	12.1[b]	67.2[b]	310.3[a]	47.5[a]
CV (%)	4.5	3.14	14.3	13.2

Means followed by the same letter in the column do not differ by Tukey test (P<0.05).

It is known that, in the field, the foliar fertilizing is a complement to the fertilizing done in the ground, related to the nitrogen provided to the cultures. This way, the incorporation of N by foliar fertilizing with amino acids can be an extra supply by ground in some growth stages. The usage of direct fertilizing in plants with free amino acids, decrease the chemistry transformation incorporated to the metabolism as it was synthesized by the plant adding to the process of growth and development (Lima et al., 2009; Gazola et al., 2014).

Besides that, there are other benefits of amino-acids application, quoted by Brandão (2007) and Kowalczyk and Zielony (2008). The better of photosynthesis, decreasing the phytotoxicity of some defensives, more tolerance to plagues and diseases, better absorbing and translocation of nutrients applied by foliar, making the root system more developed and with more strength, moderating the hormonal activities of the plants. Providing more tolerance to hydric and frost stress, more flowering of plants and increase the quality of picked products.

Although there are few works and researches published in Brazil about the effects of amino-acids pulverization on vegetables, this practice is too pervasive in horticultures, therefore, it is notorious the need in more information about the application of amino acids in agriculture, to obtain satisfactory improvement in the used culture. Therefore, the objective of this work is to evaluate the sources and portions of N to the foliar application in bell pepper culture (*Capsicum annuum* L.).

MATERIALS AND METHODS

The experiment was taken from August 2010 to April 2011, in the experimental area of Universidade Estadual de Goiás - UEG/UnU de Ipameri - GO (latitude 17º 43' 20" S and longitude 48º 09' 44" W) with a medium high level of 800 m. The weather of the region classified according to Koppen, is the Aw kind, with high temperatures, rain in the summer and dry winter.

The ground where the experiment was taken is the Latossolo Vermelho Amarelo Distrófico (Oxisol) (Embrapa, 2006). The chemistry analysis of the ground in layers from 0 to 0.2 m of depth. Showing the following characteristics: Organic Matter = 24 g dm^{-3}; pH = 6.4; CTC = 52 $mmol_c$ dm^{-3}; basis saturation = 14%; H+Al = 44 $mmol_c$ dm^{-3}; P = 0.9 mg dm^{-3}; K = 1.3 $mmol_c$ dm^{-3}; Ca = 50 $mmol_c$ dm^{-3}; Mg = 2 $mmol_c$ dm^{-3}.

To the elevation of basis evaluation of the ground to 80%, was applied 4600 kg ha^{-1} of limestone, parceled in two applications, on 80 and 60 days before the sowing, and a limestone with a portion of 810 kg ha^{-1}. The ground was prepared right after, with one plow and two light harrows.

The basis fertilizing was the same to all treatments, in furrow 15 days before the seedling transplantation, with 10.000 kg ha^{-1} of chicken manure; 340 kg ha^{-1} of P_2O_5; 45 kg ha^{-1} of N in 70% in basis and 30% in cover; and 95 kg ha^{-1} of K_2O in 70% in basis and 30% in cover. The fertilizing of cover was parcel in five applications in breaks of 15 days.

The experimental design was the randomized blocks design, in a factorial scheme 2 × 5, with four replications. The first factor corresponded to two sources of N: urea and amino acids. The second factor corresponded to the number of foliar applications: 0, 1, 3, 5 and 7 applications during the culture cycle, doses of a commercial product were use with concentration of 1% of N and 8% of an amino-acid complex. Each replication had dimensions of 3.60 × 2.00 m (7.20 m^2), where the seedling of the bell pepper hybrid Magali R in a tray, were transplanted in 30 days after the emergence in parallel lines, making the total of 20 plants per parcel, distant 0.40 m, and the space between the lines of 0.80 m. In which were consider useful to the plants of the centerlines, in six plants.

The first foliar application occurred ten days after the transplant, and the subsequent in breaks of fifteen days after the first application. In the test, there was not N application by foliar. To the foliar applications it was used a pressuring pulverization of CO_2 with pressure of two kgf and flow of 200 L ha^{-1}. The solutions to the foliar application were prepared with concentrations of N 2% in both treatments, mixture volume equivalent to 200 L ha^{-1}, corresponding to 0.5, 1.5, 2.5 and 3.5 g ha^{-1} of N. In the treatments with amino acids, a commercial product was use with concentration of 1% of N and 8% of an amino-acid complex.

The control of plagues and diseases was prophylactive and when showed incidence, to the control were utilized products based on thiophanate methyl 70%, mancozeb 80%, oxichloride of copper 84%, hydroxide of copper 54%, imidacloprid 70%, deltametrina 2%, metomil 21.5%, abamectina 1.8%. The control of weeds was manual. The irrigation was realized according to the need or in each three days, with a splitter of 8 mm.

The parameters evaluated were crossed length of fruits, diameter of fruits, total number of fruits and total productivity of fruits in kg ha^{-1}, being, and all the information collected, in plants of usage area of the parcel. The results were subordinated to a variance analysis, with medium comparison according to the Tukey (P<0.05) test and of regression, utilizing the software SISVAR.

RESULTS AND DISCUSSION

The treatments were different in the portions average, only for length and diameter of fruit, in the others the answers were not different (Table 1). In the treatments with amino acids, to each gram of N applicate, occurred an increase of 0.31 cm on the length of fruits, in the

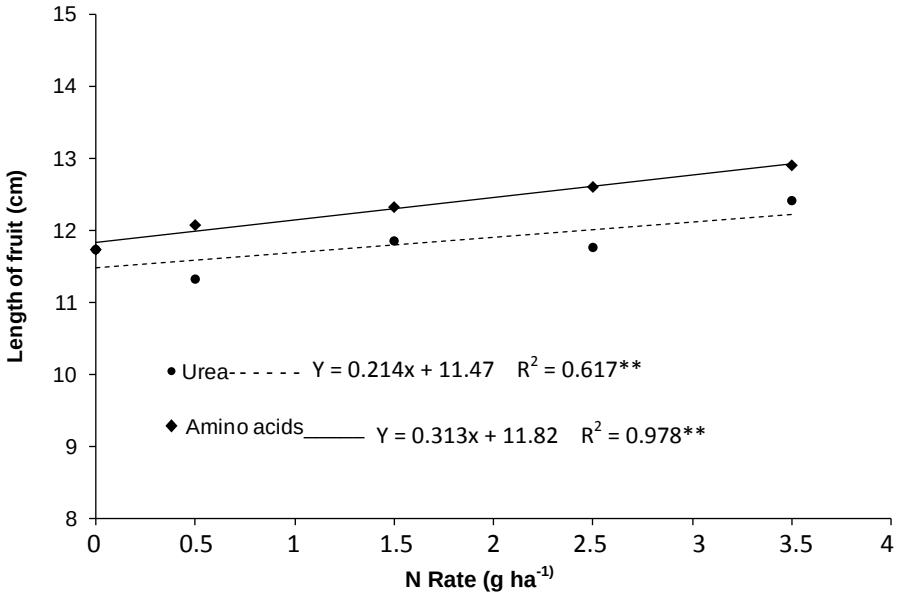

Figure 1. Length of fruits of bell pepper in function of the N portions, utilizing sources of urea and amino acids.

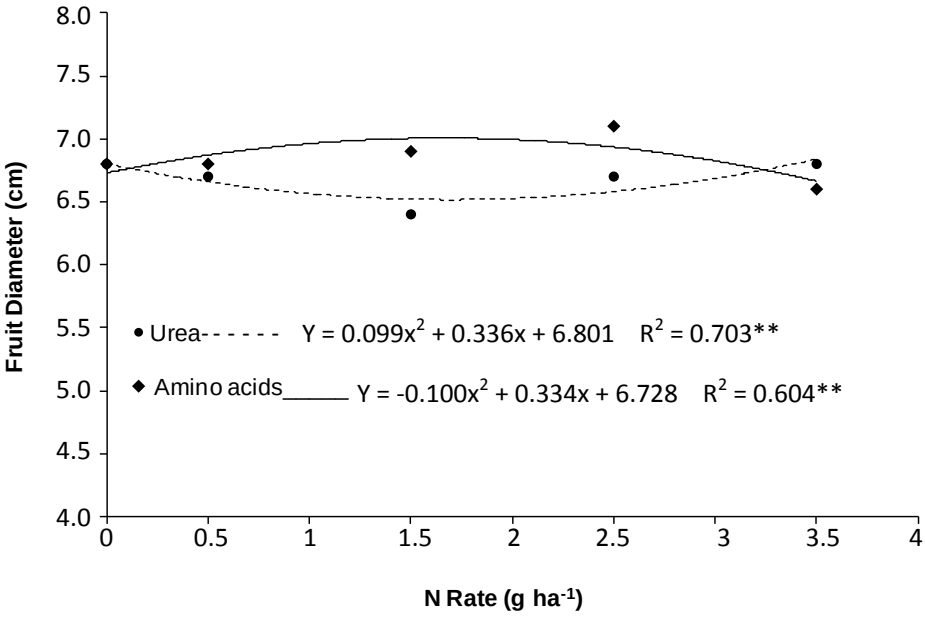

Figure 2. Diameter of bell pepper fruits in function of N, utilizing sources of urea and amino acids.

portion of 3.5 g ha^{-1} of N, providing an average of fruits with 12.91 cm, 8.4% superior when compared with the test without foliar N application (Figure 1). To the tests with urea, to each gram of N applied, there was an increase in 0.28 cm in the length of fruits, making possible the portion of 3.5 g ha^{-1} of N in an average of fruits with 12.41 cm, 7.8% when compared to the test without foliar N application (Figure 1).

Related to the diameter of fruits, in the treatments with amino acids, the maximum diameter of 7.6 cm was possible with 1.7 g ha^{-1} of N. In this portion, there was a shorter fruit of 6.6 cm, but, increasing the portion, the diameter increased, reaching 6.8 cm with 3.5 g ha^{-1} of N (Figure 2). However, the maximum diameter reached with

1.72 g of N ha^{-1} of amino-acids source was superior to 2.8% of the diameter reached with the source of 3.5 g ha^{-1} of N of urea source.

The amino acids as a source of N contributed to a better plant absorbing, which reflected in the difference of length and fruit diameter related to the urea as a source of N. Commercial amino acids as a source of N, proved more absorbing of vine leaves, where the plants showed air system growth and right N concentration (Albuquerque et al. (2008). According to Abd El-Aal et al. (2010), the basics components of live cells are the protein, and the main source of protein in vegetables tissues is the nitrogen or amino acids.

Several researches showed that amino acids could influence the physiologic activities during the plants growth, increasing the productivity. The foliar application of amino acids provided more growth and better quality of fruits in plants of potatoes, chili pepper, cucumber, garlic and pigeon pea (Kamar and Omar, 1987; Karuppaiah et al., 2000; El-Shabasi et al., 2005; Awad and Shall, 2007; El-Zohiri and Asfour, 2009; Moraditochaee et al., 2012). Others studies also showed that the foliar pulverization with urea during the phase of growing in many vegetables influence the better quality of fruits of cucumber, okra and pea (Xu-Fuli et al., 2004; Elizabeth et al., 2006; Shaheen et al., 2006, 2008).

In this work, the application of the treatments prorogued differences only to the length and diameter of fruits, not changing the number of fruits and the total productivity. Therefore the foliar applications of amino acids in bell pepper plants do not influence in quantity variations if plants but in quality variations of fruits, increasing the process of synthesis and translocation of sugars and empower the accumulation of the organ of supply, as the fruit.

Noticing the importance of biometrics variations to the benefice, and commercialization of vegetables deal in large warehouse of supply of agricultural products; the fruits produced in this study are classified as medium fruits - fruits of 12 to 15 cm of length and more than 6 cm of diameter, according to CEAGESP (2004). The size and shape are important, because the variation between the units of a product can affect the choice of the consumer.

In the same meaning, the results obtained by Luz et al. (2010), with applications of the foliar fertilizing Aminoagro Fruto® in plants of tomatoes, the treatments provided more productivity, and increased the production of better classified fruits in superior bunches, where, according to Fontes and Silva (2005), normally the production is shorter in the inferior bunches.

Conclusions

1. The foliar application of amino acids in plants of bell pepper increases the diameter and length of fruits.

2. High portions of urea also provide morphologic changes in fruits, however it is lower using amino acids.

Conflict of Interests

The authors have not declared any conflict of interests.

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Farmer participatory pest management evaluations and variety selection in diagnostic farmer field Fora in cowpea in Ghana

Mumuni Abudulai[1]*, Shaibu Seidu Seini[1], Mohammed Haruna[1], Adams Mashud Mohammed[2] and Stephen, K. Asante[1]

[1]CSIR-Savanna Agricultural Research Institute, P. O. Box TL 52, Tamale, Ghana.
[2]University for Development Studies, Nyankpala Campus, P. O. Box TL 1882, Tamale, Ghana.

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Participatory diagnostic farmer field fora (FFF) were conducted at two communities, Savelugu and Bukpomo, in northern Ghana to build the capacity of farmers on integrated pest management in cowpea production. The FFF involved a season-long comparative evaluation of farmers' practices (FP) and integrated pest management (IPM). Farmers' practices relied wholly on calendar insecticide sprays while IPM plots employed proven agronomic practices and treatment with neem (*Azadirachta indica* A. Juss) extract for insect pest control. Results showed that insect pest densities (Flower thrips, *Megalurothrips sjostedti* Trybom and pod-sucking bugs' for example, *Clavigralla tomentosicollis* Stal.) and percent damaged pods as well as grain yield were similar in FP and IPM plots at both Savelugu and Bukpomo. Partial budget analysis showed positive returns to investment in IPM and a near loss in FP. Post training ballot box test showed that 80% of the farmer participants across locations showed improved knowledge and skills in IPM after the training compared with about 30% before the training. An ancillary study to the FFF was conducted to expose the farmers to different cowpea genotypes in a participatory variety selection trial. Results showed that some of the genotypes selected by farmers as their most preferred genotypes at the vegetative stage were also selected at the podding stage but there were no significant correlations between these farmer preferences and yield. These findings are discussed in the context of sustainable cowpea production through farmer empowerment and involvement in technology generation and dissemination.

Key words: Farmer field fora**,** participatory variety selection, cowpea, *Azadirachta indica.*

INTRODUCTION

Cowpea, *Vigna unguiculata* (L.) Walpers, is an important food crop and a major source of protein for many families in Ghana and other countries in sub-Saharan West Africa. The dry grain with about 23 to 25% protein, supplies much of the protein needs of the rural poor who lack the needed capital to purchase animal protein.

*Corresponding author. E-mail: mabudulai@yahoo.com. Tel: +233-244-772209.

In addition to the food value of the grain, the foliage and stems are a good source of fodder for livestock, green manure and cover crop (Abudulai et al., 2006; Anonymous, 2012).

Despite its importance, cowpea production is faced with a lot of challenges, including the threat of insect pests that attack the crop throughout its growth, and low access of farmers to improved technologies (Jackai et al., 1992). Cowpea is very susceptible to many insect pests that need to be controlled to obtain economic yield. Insect pests, particularly aphids (*Aphis craccivora* Koch), thrips (*Megalurothrips sjostedti* Trybom) and a complex of pod-sucking bugs (PSBs) (for example, *Clavigralla tomentosicollis* Stal., *Aspavia armigera* F. and *Anoplocnemis curvipes* F.) inflict heavy damage to the crop and can cause complete crop failure in unprotected cowpea (Karungi et al., 2000; Abudulai et al., 2006).

Though a few cowpea varieties have shown slight to moderate levels of resistance to one or a few insect pests, there is no variety that has demonstrated resistance to the wide array of insect pests that attack the crop (Jackai and Dauost 1986). Consequently, most farmers use insecticides to control insect pests on their fields. However, because of lack of knowledge about the proper use of insecticides and their abuse, insect control is generally poor on farmers' fields. In addition, there are reported cases of insect resistance and detrimental effects of insecticides on the environment and human health (Jackai and Dauost, 1986; Karungi et al., 2000).

Scientists from both national and international research institutes have developed improved technologies including improved pest management options and high yielding cowpea varieties but only a few are adopted by farmers (Mulatu and Belete, 2001). The reasons for the low adoption rate include inadequate exposure to the new technologies or the technologies do not adequately satisfy farmers' needs (Richards, 1985; Ntega-Nanyeenya et al., 1997; Beshir, 2014).

Farmers operate in a heterogeneous environment that requires site-specific solutions to problems on their farms. Therefore, there is the need for a paradigm shift from merely transferring technologies to farmers to one that seeks to empower farmers with the requisite knowledge and skills to enable them make informed decisions about their farm operations. Scientists need to work with farmers and other stakeholders to diagnose and experiment for workable solutions suitable for the small-scale farmer (van Huis et al., 2007; Struik et al., 2014). Farmer empowerment through participatory technology development (PTD) recognizes the indigenous knowledge of farmers and puts them at the forefront of technology generation (van Huis and Meerrman, 1997).

Empowering farmers with the requisite knowledge about insect pests and control options such as those that are effective and environmentally friendly will improve their ability for effective insect control for improved yields. Also, participatory testing of crop cultivars with farmers helps in identifying cultivars preferred by farmers and accelerating their dissemination (Joshi and Witcombe, 1996).

The core objective of this study was to strengthen the capacities of small farmers on proper pest management practices for cowpea through participatory diagnostic farmer field fora (FFF). The FFF is one novel strategy that brings together scientists, extension officers and farmers in the technology development and dissemination process. The other objective aimed at improving farmers' access to quality seeds through participatory variety selection/testing.

MATERIALS AND METHODS

Farmer field fora (FFF)

This study involved a group training of farmers on integrated pest management (IPM) using farmer diagnostic exploratory approach in a farm setting. The study was mounted in two cowpea farming communities, Bukpomo and Savelugu, in the Guinea Savanna Zone of the Northern Region of Ghana from 2010 to 2011. Participating farmers were drawn from the two communities and also from nearby communities. The farmers in each community were put into five working groups (at least 8 farmers in a group) representing five replications to test the two treatments, viz Farmers' practice (FP) and IPM, of insect pest management in cowpea. Farmers said at the starting of the experiments that they use insecticides primarily the pyrethroid lambda-cyhalothrin to control insect pests, and this was documented and followed as their practice. For IPM, plots were treated with 10% (w/v) aqueous suspension of neem seed extract (NSE) with one round of lambda-cyhalothrin applied at 0.02 kg ai ha^{-1} at initiation of flowering. The cowpea cv Padi-Tuya was used. Plot sizes were 10 x 10 m. IPM and FP plots were laid side by side in each replicate. The plots were planted from 5 to 20 August in each year and farming community.

The training sessions were interactive and were held once a week. On each training day, farmers in each group or replicate met during Agro-ecosystem analysis (AESA) to diagnose problems on their plots, and also take records (e.g. pest/disease incidences) based on which they suggested possible solutions or interventions for their problems. Ten plants were randomly sampled in each plot during each AESA to record insect and disease incidences. The number of pods per plant was recorded at harvest. The total number of shriveled, unfilled pods and those with feeding scars were recorded as damaged by PSBs, and used to estimate percentage PSB damaged pods. The records from the groups were processed and presented at a plenary session, which were discussed by participants for consensus building on the appropriate interventions if any (for example, weed control, pest control, harvesting of plots) to apply to each field plot.

Researchers, technicians and staff of the Ministry of Food and Agriculture (MoFA) facilitated the training process. The farmers learnt crop growth habits, early preventive measures for insect pests and diseases, and acquired skills in the identification of insect pests and diseases and their management. Farmers' capacities also were strengthened through presentations of special topics on relevant areas of cowpea production by resource persons. Topics presented included:

1. Site selection, land preparation and soil management
2. Cowpea phenology and morphology.
3. Introduction to IPM/FFF

Table 1. Numbers and distribution by sex of farmers trained at farmer field fora at Bukpomo and Savelugu in 2010 and 2011.

Community	Year	Sex of facilitator		Total
		Male	Female	
Bukpomo	2010	65	25	90
Savelugu	2010	45	15	60
Bukpomo	2011	25	15	40
Savelugu	2011	35	25	60

4. Introduction to AESA.
5. Cowpea insect pests/diseases identification and management.
6. Safe and effective use of pesticides.
7. Preparation and application of botanicals
8. Natural enemies in cowpea production.

A pre- and post- training ballot box tests were conducted to evaluate the knowledge and skills of participants before and after the training, respectively. In these tests, farmers were asked certain key questions pertinent to the field ecology of cowpea such as

1. Identification of key insect pests of cowpea
2. Parts of cowpea plants attacked by these insect species
3. Type of damage they cause to plants
4. General methods of control if any
5. Timing of control practice
6. Natural enemies of insect pests and their importance.

Farmer participatory variety selection (PVS)

As part of the FFF, the participating farmer groups also evaluated 22 cowpea genotypes/varieties hereinafter referred to as genotypes in a participatory action research trial to expose them to the different genotypes of cowpea released or being evaluated for release by the Institute. Each genotype was planted in four rows, 5 m long by 2.4 m wide. There were 0.60 m spacing between rows and 0.20 m between plants in a row. The experimental design was a randomized complete block and treatments were replicated three times. Normal agronomic practices for cowpea production in northern Ghana were followed and the plots were protected against insect pests with Chlorpyrifos (as D-ban Super 48% EC) applied at 0.20 kg ai ha^{-1} at the vegetative stage and Lambda-cyhalothrin (as Lambda Super 2.5 EC) applied at 0.02 kg ai ha^{-1} during flowering and podding. The farmers' assessed the genotypes based on a preference score of 1 to 3, where 1 = poor or least preferred, 2 = average and 3 = good or most preferred. The assessments were done during the vegetative stage at 20 days after planting (DAP) and also during podding at 50 DAP. In all the assessments, farmers were asked to assign reasons for choosing or scoring a particular genotype higher over another.

Data analysis

The insect densities, damage and grain yield data for the FFF and the preference scores for the PVS study were analyzed using ANOVA for a randomized complete block design and when significant, treatment means were separated using Fisher's LSD test at $P < 0.05$ (SAS Institute, 1998). The pre- and post- ballot tests conducted at the FFF were analyzed using paired t-test to evaluate the knowledge and skills gained by the participants after the FFF training. Partial budgeting was used to estimate gross margin per hectare for IPM and FP. Gross margin for each treatment was estimated by deducting the total variable cost from the total revenue. The benefit-cost ratio was calculated by dividing the gross margin by the total cost of each treatment. The relationships between farmers' preference scores at the PVS and the agronomic performance of the genotypes were computed using simple correlation analysis.

RESULTS AND DISCUSSIONS

Farmer field fora (FFF)

A total of 130 cowpea farmers comprising 90 males and 40 females participated in the training at Bukpomo while a total of 120 comprising 80 males and 40 females participated at Savelugu during the two years of the FFF (Table 1). The influence of the two pest control technologies tested on insect pest densities, damage and grain yield is presented in Table 2. There were no significant differences between IPM and FP in the densities of thrips (*M. sjostedti*) and PSBs (*C. tomentosicollis, A. curvipes and A. armigera*) at the two locations tested. Also, percentage damaged pods due to the PSB complex was not different between the treatments. However, pod load or mean number of pods per plant was significantly higher in IPM than in FP at Bukpomo but there were no such differences observed between the treatments at Savelugu. There were no significant grain yield differences measured between the two treatments at both locations. Yields, however, were generally lower at Savelugu compared with those at Bukpomo. Planting of the trial at Savelugu coincided with the period of heavy rains which probably affected plant development and yield (Wright and Nageswara Rao, 1994).

These results demonstrated the insecticidal efficacy of neem in the IPM (Schmutterer 1990) comparable to the synthetic insecticide in FP. Neem also acts as an antifeedant to insects and probably prevented feeding in the neem-treated IPM plots (Mordue and Blackwell, 1993, Isman, 2006), which resulted in comparable damage to the synthetic insecticide control in the FP. The significantly higher number of pods per plant measured in IPM plots compared with FP at Bukpomo was also probably due to the antifeedant effect of neem which resulted in higher pod retention. PSB feeding in cowpea causes pod abscission (Jackai et al., 1992; Karungi et al., 2000).

Results of the ballot box tests showed that farmers gained more knowledge and skills about the cowpea ecology and management after the training than before the training (Table 3). About 80% of the participating farmers demonstrated improved knowledge and skills in IPM after the training compared with about 30% before the training. They exhibited adequate knowledge about the major insect pests of cowpea, their damage and how

Table 2. Influence of pest control technologies on insect densities, damage and cowpea yield at Bukpomo and Savelugu farming communities.

Parameter	Bukpomo			Savelugu		P-value
	IPM	FP	P-value	IPM	FP	
Thrips/20 flowers	37.6±9.3 a	27.6±6.7 a	0.4063	34.2±4.9 a	37.5±5.9 a	0.5731
PSB/m	2.1±0.5 a	1.4±0.2 a	0.1715	2.4±0.4 a	2.1±0.4 a	0.4853
% damaged pods	21.6±2.3 a	21.9±3.2 a	0.9387	15.2±1.6 a	18.3±2.4 a	0.3924
# of pods/ Plant	15.7±0.9 a	10.7±0.6 b	0.0039	7.8±0.8 a	6.4±0.6 a	0.1462
Yield	867.3±39.5a	934±69.6a	0.1664	595.2±45.9 a	651.9±52.7 a	0.3930

Values are pooled means of 2010 and 2011 seasons. Means in a row at a farming community with the same letters are not significantly different according to Fisher's LSD test at P < 0.05.

Table 3. Mean % of farmers' demonstrating knowledge and skills to do IPM assessed at pre- and post- ballot tests at Bukpomo and Saveleugu.

Location[1]	% (±SE) farmers showing knowledge and skills in IPM		t-value	P > t
	Pre-test	Post-test		
Bukpomo	29.6±4.6	80.0±4.5	7.90	<0.0001
Savelugu	34±3.9	81.0±4.8	7.41	<0.0001

[1]Savelugu, n =120; Bukomo, n = 130.

to manage them in their fields. Godtland et al. (1994) and Asiabaka (2002) reported that Farmer Field School training improves farmers' knowledge and skills for sustainable agricultural production. Farmers' participation in experimental groups increases their self-confidence and capacities through interacting with their peers and researchers to solve problems better on their fields (Sterk et al., 2013).

The cost of the IPM technology was relatively lower than the FP technology (Table 4). This resulted in positive returns to investment in the IPM and a near loss in the FP. For example, at Bukpomo farmers gained about 180% returns to their investment in IPM but made a marginal gain of about 5% with their own practice. Opare-Atakora et al. (2014) reported that the adoption of IPM technologies in yam production led to increased returns to farmers. The results further showed that farmers gain more when they reallocate their resources from their own practice to IPM. As opposed to calendar insecticide sprays in FP, some level of pest attack is tolerated in IPM which results in reduced use of pesticide and lower cost of production in IPM compared with FP (Mariyono 2007).

Only one round of chemical insecticide was applied in IPM as against six rounds of sprays in the FP. Further, IPM plots were protected against insect pests using mainly neem extract which is cheap because of abundance of neem trees in the wild in the trial area. There is therefore a high incentive for farmers to want to adopt the IPM technology because of its comparable yields with their own practice and lower cost of production (Timu et al., 2014). Struik et al. (2014) observed that smallholder farmers often capture limited benefits from appropriate and desirable technologies because of limited capital resources to invest in new technologies. Besides the economic benefits, adoption of the IPM technology that relies on the use of neem will lead to improved health of the farm family and the environment as opposed to the use of the more toxic synthetic pesticides. Neem has been reported to be relatively safe to humans and the environment (Schmutterer, 1990; Mordue and Blackwell, 1993).

Farmer participatory variety selection (PVS)

By consensus, plant vigor and/or weed competitive ability epitomized by the level of branching of a genotype were the key criteria used by famers for selection of a genotype at the vegetative stage. Genotypes with high levels of these traits were much preferred and scored higher than those with low levels of the traits. The criteria advanced for the podding stage assessment were earliness and pod load, with pod load being the overarching consideration for the selection of a genotype. Genotypes with heavy pod load were scored higher than those with low pod load. All the genotypes evaluated were white seed coated, which is the preferred choice of farmers in the study area (Etwire et al., 2013).

The preference scores given by farmers and grain yield for the 22 cowpea genotypes evaluated at Savelugu and Bukpomo are presented in Table 5. The scores at both the vegetative and podding stages were significantly

Table 4. Performance indicators of Farmers Practice (FP) and Integrated Pest Management (IPM) strategies in cowpea at Savelugu and Bukpomo.

Partial budget analysis	Savelugu		Bukpomo	
	FP	IPM	FP	IPM
Total variable cost (gh ¢ per ha)	541.32	377.38	740.77	256.65
Yield (kg/ha)	651.9	595.2	934	867.3
Price of grain (GHc/kg)	0.83	0.83	0.83	0.83
Revenue (GHc/ha)	541.07	494.02	775.22	719.86
Gross margin (gh ¢ per ha)	-0.25	116.64	34.45	463.21
Benefit-cost ratio/ total factor productivity(per ha)	-0.0005	0.30	0.05	1.80

FP; Farmers practices relied wholly on synthetic insecticide sprays to control insects; IPM: Combined monitoring of insects before control with neem seed extract (10% w/v) with one round of insecticide at flowering.

Table 5. Mean scores[2] of farmers' preferences[1] for cowpea genotypes at the vegetative and podding stages, and yields in a participatory varietal selection conducted at Bukpomo and Savelugu.

Genotype	Scores at vegetative stage		Mean	Scores at podding Stage		Mean	Yield kg/ha		Mean
	Savelugu	Bukpomo		Savelugu	Bukpomo		Savelugu	Bukpomo	
IT 95k-193-2	2.3 d-g	2.7 a	2.5	2.4 b-e	2.7 ab	2.6	847.2 a-d	759.7 a-e	803.5
SARC 3-129-2	2.4 c-f	2.2 a-e	2.3	2.1 e	2.1 c-f	2.1	605.6 e-g	654.2 c-g	629.9
IT 98K-506-1	1.7 i-j	2.6 ab	2.2	2.6 a-d	2.3 b-f	2.5	708.3 c-f	644.4 c-g	676.4
IT 97K-499-35	2.5 b-e	2.2 a-e	2.4	2.8 ab	2.7 ab	2.8	957.4 a	890.3 a	923.9
SARC 4-75	2.7 a-d	2.5 a-c	2.6	2.6 a-d	3.0 a	2.8	737.5 b-e	838.9 a-c	788.2
SARC 1-71-2	2.8 a-c	2.3 a-d	2.6	2.3 c-e	2.1 c-f	2.2	665.3 d-f	700.0 a-f	682.7
SARC 2-51-1	2.2 e-h	2.0 c-e	2.1	2.2 de	1.5 g	1.9	923.6 ab	825.0 a-d	878.8
SARC 3-154-1	1.9 g-j	2.0 c-e	2.0	2.9 a	2.3 b-f	2.6	875.0 a-c	827.8 a-d	851.4
SARC 3-90-2	1.9 g-j	1.8 de	1.9	2.7 a-c	1.9 fg	2.3	619.4 e-g	619.0 e-g	619.2
PADI-TUYA	3.0 a	2.0 c-e	2.5	2.7 a-e	2.7 ab	2.7	670.8 d-f	719.4 a-e	695.1
SARC 1-136-1	2.5 b-e	2.1 b-e	2.3	2.1 e	2.7 ab	2.4	600.0 e-g	500.0 fg	550.0
SARC 3-103-1	2.6 a-e	2.3 a-d	2.5	2.1 e	2.1 c-f	2.1	669.4 d-f	684.7 b-f	677.1
SARC 2-115-1	1.7 ij	2.1 b-e	1.9	2.8 ab	2.0 e-g	2.4	787.5 a-e	763.9 a-e	775.7
SARC 1-18-2	1.8 h-j	2.2 a-e	2.0	2.6 a-d	2.4 b-e	2.5	377.8 h	477.8 g	427.8
SARC 4-51	1.5 j	1.8 de	1.7	1.7 fg	2.1 c-f	1.9	727.8 c-e	691.6 a-f	709.7
SARC 3-74A-2	2.7 a-d	2.2 a-e	2.5	2.9 a	2.3 b-f	2.6	429.2 gh	497.2 fg	463.2
SARC 1-82-1	2.1 f-i	2.5 a-c	2.3	2.8 ab	2.5 a-d	2.7	666.7 d-f	627.4 d-g	647.1
SARC 1-13-1	2.9 ab	2.1 b-e	2.5	2.4 b-d	2.5 a-d	2.5	693.1 c-f	706.9 a-e	700.0
SARC 1-71-1	1.9 g-j	2.1 b-e	2.0	2.1 e	2.4 b-e	2.3	759.7 b-e	711.2 a-e	735.5
SARC 4-40	1.9 g-j	2.1 b-e	2.0	1.5 g	2.6 a-c	2.1	525.6 f-h	601.4 e-g	563.5
MARFO-TUYA	2.1 f-i	1.7 e	1.9	2.4 b-c	2.3 b-f	2.4	694.4 c-f	689.4 a-f	691.9
APAGBAALA	2.5 b-e	2.3 a-d	2.4	2.5 b-e	3.0 a	2.8	729.2 c-e	887.5 ab	808.4
P > F	< 0.0001	0.0425	-	< 0.0001	< 0.0001	-	<0.0001	0.0035	-
CV (%)	28.2	36.6	-	25.7	30.1	-	16.9	17.9	-

[1]Pooled data for two years (2010 and 2011); there were 3 replications in each year; [2]A score of 1 = least preferred and 3 = most preferred. Means in a column followed by different letters are significantly different according to Fisher's LSD mean separation test at P < 0.05.

different for the genotypes. At the vegetative stage, six genotypes including Padi-Tuya, SARC 1-13-1, SARC 1-71-2, SARC 4-75, SARC 3-74A-2 and, SARC 3-103-1 had the highest scores compared to most of the genotypes and thus were the most preferred by farmers at Savelugu. Among others, the genotypes SARC 4-75, SARC 3-74A-2, SARC 1-71-2 and SARC 3-103-1 ranked highest at Savelugu at the vegetative stage also were the most preferred at Bukpomo. At the podding stage, four genotypes including SARC 4-75, IT 97K-499-35, Padi

Tuya and SARC 1-18-2 were the most preferred at both locations. Grain yields differed significantly among the genotypes at both Savelugu and Bukpomo.

At both locations, significantly higher yields were recorded for the genotypes IT 95K-193-2, IT 97K-499-35, SARC 2-51-1, SARC 3-154-1 and SARC 2-115-1. Correlation analyses did not reveal any significant relationships between yields and farmers preference scores at the vegetative and podding stages for both Savelugu ($r = 0.00472$, $P = 0.9700$ and $r = 0.08476$, $P = 0.4986$) and Bukpomo ($r = -0.01011$, $P = 0.9358$ and $r = 0.01107$, $P = 0.9297$), respectively. This showed that the genotypes selected by farmers as their most preferred were not necessarily the highest yielding.

In addition to grain yield, farmers in the study area also make substantial use of cowpea leaves as vegetables in soup and stew, as snack and as fodder for livestock. These competing uses could have influenced farmer preferences for the genotypes resulting in the lack of significant correlations between the farmer preferences and yield. Moreover, different preferences between the sexes for germplasm have been reported (Defoer et al., 1997; Mulatu and Zelleke, 2002). In this study, the preference scores for males and females were assessed together, which divergent preferences could have resulted in the nonsignificant correlations observed between yield and farmers preferences. However, contributions of female and male farmers in a participatory selection program are necessary for addressing the overall needs of the household (vom Brocke et al., 2010). Farmers' preferences are important when developing a variety as their needs for attributes of a variety must be met to enhance its adoption (Richards, 1985; Mulatu and Belete, 2001). Among the genotypes evaluated, IT 95K-193-2 was released to farmers as Bawutawuta by the Institute for its relative resistance to the witch weed *Striga gesneroides*, IT 97K-499-35 as Songotra for aphid resistance and SARC 4-75 as Zaayura for its high preference by farmers.

Conclusion

The results of the FFF training showed that the participating farmers gained a lot of knowledge working to discover solutions they could apply to solve their site-specific problems on their farms. A post training ballot box test conducted at the end of the training showed that 80% of the participants across locations had improved knowledge in the ecology of the crop and skills in general pest management. The results of the participatory variety selection trial showed that some of the genotypes selected by farmers as their most preferred genotypes at the vegetative stage were also selected at the podding stage but there were no significant correlations detected between these farmer preferences and yield. Among the genotypes evaluated, IT 95K-193-2, IT 97K-499-35 and SARC 4-75 have been released to farmers for cultivation as Bawutawuta, Songotra and Zaayura, respectively.

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Conflict of interests

The authors have not declared any conflict of interests.

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Soil fertility status under smallholder farmers' fields in malawi

Joyce Prisca Njoloma[1]*, Weldesemayat Gudeta Sileshi[1,3], Bruce Geoffrey Sosola[1], Patson Cleopus Nalivata[2] and Betserai Isaac Nyoka[1]

[1]World Agroforestry Centre, International Centre for Research in Agroforestry (ICRAF), Chitedze Agricultural Research Station, P. O. Box 30798, Lilongwe 3, Malawi.
[2]Lilongwe University of Agriculture and Natural Resources (Bunda College),Crop and Soils Department, P. O. Box 219, Lilongwe, Malawi.
[3]1244 Ibex Hill, Lusaka, Zambia.

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Land degradation continues to contribute to the declining soil fertility especially in the smallholder farms. Thus, soil fertility depletion in the smallholder farms will continue to be the biophysical root cause for reduced food production if farmers do not implement best agricultural practices. It is expected that when farmers understand the current soil fertility status on their farms, they would make informed decisions considering appropriate soil fertility restoration and other conservation technologies. Soil fertility status of selected sites was determined in Northern, Central and Southern regions of Malawi. The overall objective of the study was to document current soil fertility status in smallholder farmers' fields. And that specifically, this study was meant to provide a basis for the promotion of soil fertility restoration interventions in Malawi. A total of 33 participating farmers' fields were selected for soil sampling and from each site soil samples were collected at two depths, 0 to 20 cm and 20 to 50 cm, using an auger. Soil chemical and physical analysis was carried out on all the sampled soils. Statistical analysis on the data was done using Genstat 14.1. The statistical analysis revealed that soils in all the selected sites are slightly acidic with most of the sites having pH <5.5. Another important finding is the low %OM (<2%) in most of the sites especially in Dedza and Mzimba. Soil organic matter (SOM) is important for healthy plant growing as it maintains favourable conditions supporting soil moisture retention, temperature, nutrient, pH, and aeration. The low %OM contributes to the low and moderate levels of N (<0.2%) in most of the sites. Sustainable soil management practices are therefore required to rebuild the soil fertility resource base.

Key words: Soil fertility, soil organic matter, acidic soils, nitrogen-fixing trees.

INTRODUCTION

The sustainable use of soil resource remains a critical determinant of agricultural productivity in Malawi for most farmers who have traditionally prioritised maize, the staple food over other food and cash crops. However,

*Corresponding author. E-mail: J.Njoloma@cgiar.org. Tel: +265 (0) 1 707 319.

despite the availability of improved varieties, a number of studies conducted in Malawi and other countries in the sub-Saharan Africa region reported declining levels of crop productivity that pose serious food security concerns for the region (Smale and Jayne 2003; FAO, 2008). Among other factors such as the climate variability, agricultural productivity is being threatened by land degradation resulting in declining soil fertility especially in the smallholder farms. Land degradation is not only negatively impacting on the future of smallholder agriculture in Malawi but also its economic growth prospects for a country whose economy is based on agriculture. It has been estimated that Malawi loses in excess of 30 kg of N and 20 kg of P per hectare per year through erosion on arable land (Henao and Baanante, 1999). Thus, soil fertility depletion in the smallholder farms will continue to be the biophysical root cause of reduced food production if farmers do not implement best agricultural practices (Vlek et al., 2008).

Soil fertility replenishment is one of the corrective measures that should be considered as an investment in natural resource capital (Onyango, 1997). It is therefore important to understand what is meant by soil fertility decline for a farmer to start planning for soil fertility restoration. However, defining soil fertility decline is relatively difficult because most soil chemical properties either change very slowly or have large seasonal fluctuations; in both cases, it requires long-term research commitment to understand the confounding factors that make assessment of soil fertility decline complicated. This calls for some considerable evidence of the overlaps between farmers' and researchers' perceptions of soil fertility decline (Murage et al., 2000). Over the years, researchers have strived to provide a breadth of the understanding of soil physical processes, research developments on soil fertility restoration and other conservation technologies. On the other hand, farmers are expected to have a context specific knowledge required to adapt the developed technologies to local biophysical and socio-economic conditions. Nonetheless soil fertility decline remains a major concern in most parts of the world especially in the developing countries (Acharya et al., 2000). A study was therefore conducted to determine the current soil fertility status levels in some selected maize based growing areas in Malawi to provide an understanding on the soil health status. The specific objective was to establish a scientifically based soil fertility status validation that will influence appropriate decisions on appropriate soil fertility enhancing interventions in the maize based farming systems.

MATERIALS AND METHODS

Soil sample collection sites

A total of 33 sites were selected from three districts in Malawi and these included Mzimba, Dedza and Thyolo. Twelve sites in three Extension Planning Areas (EPAs) were sampled in both Mzimba (Zombwe, Mpherembe and Emsizini EPAs) and Dedza (Mtakataka, Golomoti and Chafumbwa EPAs) whereas in Thyolo (Matapwata, Dwale and Thyolo Central EPAs) only 9 sites were assessed (Table 1).

Soil sampling and analyses (pp3 -6 re-worked as suggested by reviewers)

Soil sampling was conducted soon after the rainy season in the months between March and April, 2013. The sampling time was planned so in order to take care of sudden pulse-like events of rapidly increasing CO_2-efflux that do occur in soils under seasonally dry climates in response to re-wetting after dry periods (Jarvis et al., 2007; Griffiths and Birch, 1961). A well-mixed composite sample of 500g from three positions (the middle and two other random points) within a 6 m by 6 m plot was obtained. Top soil samples were taken at a depth of 0 to 20 cm and the sub soil samples were collected at a depth of 20 to 50 cm with an auger.

Processed air dried soil samples were analyzed at the Crop and Soils Laboratory, Bunda College Campus of the Lilongwe University of Agriculture and Natural Resources (LUANAR). Sub-soil samples determined through quartering process were analysed for pH, soil organic carbon (SOC), soil organic matter (SOM), total nitrogen (N), extractable phosphorus (P), exchangeable potassium (K) and soil texture. NPK were prioritized on the basis that they are considered as most limiting in the maize production systems. Determination of both the chemical and physical soil properties was carried out following the standard procedures (Mehlich, 1984; Anderson and Ingram, 1993; Wendt, 1996) as described in the subsequent paragraphs:

Soil pH was determined in water (1:2.5 H2O) (Wendt, 1996). Sieved soil samples each weighing 10g was placed into 50ml centrifuge tubes then 25 ml at room temperature distilled water was added to the tubes and the mixture was then placed in centrifuges, shaken for 5 minutes and pH was determined using a calibrated pH meter.

Total organic carbon was analysed using the Walkley and Black method as described by (Anderson and Ingrams, 1993). The amount of carbon was determined from a standard curve and percent organic carbon (OC) was calculated using the following formula:

$$\% \text{ Organic carbon (OC)} = \frac{M \times 0.39 \times mcf \times (v1 \times v2)}{S} \quad (1)$$

Where: M = molarity of ferrous sulphate solution, V1 = ml of ferrous sulphate solution, V2 = ml of ferrous sulphate solution required for blank, S = weight of air dry sample in grams, Mcf = moisture correcting factor (100 + %moisture)/100, 0.39 = 3 x 0.001 x 100% x 1.3 (3 = equivalent weight of carbon) 1.3 = a compensation factor for the incomplete combustion of the organic carbon.

$$\% \text{ Soil organic matter (SOM)} = \% \text{ OC} \times 1.72^* \quad (2)$$

*1.72 is the conversion factor commonly used for converting values of organic carbon to soil organic matter values (Anderson and Ingrams, 1993). Mineralisible Nitrogen was obtained through the following formula:

$$\% \text{ Mineralisable Nitrogen (N)} = \% \text{ OM} \times 0.05 \quad (3)$$

Available phosphorus (µg P/g soil) was analysed using Mehlick-3 extractant.

Exchangeable cations were determined after extraction with Mehlich-3 solution and analysis using an atomic absorption

Table 1. Study sites and locations of sampled fields.

District	EPA	Section	Village/Site	South	East
Mzimba (in the Northern region)	Zombwe	Kaluholo	Kenani Shonga	11°18'17.3''	033°54'22.9''
		Ekwendeni	Zinolema Khonje	11°20'59.5''	033°52'06.6''
		Zombwe 2	Bandawe Tembo	11°20'25.2''	033°50'38.5''
	Mpherembe	Doroba	Jailosi Mhlanga	11°21'58.1''	033°58'34.7''
		Kazuni	Tengasalu	11°09'11.3''	033°40'04.1''
		Elunyeni	Kazalawe	11°13'17.5''	033°41'22.7''
		Mpherembe	Chigagu jere	11°17'39.2''	033°36'49.2''
		Ezweleni	Ndindi	11°16'43.6''	033°39'30.5''
	Emsizini	Emtiyani	Chilenga Zamangwe	11°33'19.8''	033°44'25.4''
		Enyezini	Kaigwazanga Mphande	11°27'57.9''	033°51'20.7''
		Emsizini	Saulosi Nkosi	11°25'57.0''	033°50'05.0''
		Emsizini	Mkakangoma	11°27'39.2''	033°48'47.3''
Dedza (in the Central region)	Mtakataka	Nankokwe	Njolo	14°17'43.5''	034°31'30.7''
		Msekeni	Mphonde	14°15'12.5''	034°30'92.2''
		Mua	Kankhande	14°17'09.8''	034°31'15.7''
	Golomoti	Boolera	Asani	14°20'75.9''	034°33'63.5''
		Golomoti Centre	Msamala	14°26'04.3''	034°35'94.5''
		Chikololere	Somanje	14°25'06.3''	034°36'32.0''
		Kabulika	Liwengwa	14°22'67.4''	034°34'73.2''
		Mganja	James	14°24'03.8''	034°49'27.3''
	Chafumbwa	Ching'ombe	Chisani	14°21'25.0''	033°51'13.1''
		Magomero	Mnolo	14°22'76.7''	033°54'62.6''
		Abona	White	14°26'48.7''	033°54'09.7''
		Kadala	Kum'buka	14°25'44.7''	033°54'95.4''
Thyolo (in the Southern region)	Matapwata	Nachipere S.W	Chibwana	16°05'25.3''	035°16'72.6''
		Chingazi	Katundu	15°93'38.6''	035°18'87.7''
		Sharpe	Mtulo	16°01'55.3''	035°19'01.4''
		Makande South	Savala	15°99'06.0''	035°24'88.9''
	Dwale	Muonekera B	Supero	15°95'03.3''	035°09'12.8''
		Chandamale	Mpezo	16°08'03.6''	035°12'01.5''
		Dzimbiri	Mwanaphwa	16°07'83.7''	035°12'18.3''
	Thyolo centre	Nachipere centre site 1	Pemba	16°01'55.3''	035°19'01.4''
		Nachipere centre site 2	Pemba	16°01'55.5''	035°19'01.7''

spectrometer (AAS).

Soil samples each weighing 2.5 g were placed in separate 50 ml centrifuge tubes and then 25 ml of Mehlich 3 extracting solution was added. The tubes were capped and shaken for 5 min and let to stand for 10 min and then centrifuged for 5 min. The samples were then filtered through pure cotton that was previously rinsed with Mehlich 3 solution. Intermediate stock solution standards for K were used to obtain sample filtrates after dilution with lanthanum solution which was then passed through a flame photometer for potassium determination (Anderson and Ingram, 1993). Soil texture was determined using the hydrometer method. In this method the soil particles were dispersed in a 3% sodium hexametaphosphate (calgon) and then agitated. After dispersion, the amounts of each particle group (sand, silt, clay) were determined after the tubes were uncapped and left on racks undisturbed for 30 s. Then solutions from these tubes were poured into corresponding sets of tubes, leaving the sand that had settled. The second sets of tubes were left to stand for another 30 min to let the silt settle and so on. Volumes of the settled particles then were recorded. This is based on the principle of Stokes law, which states that particles will fall out of suspension at different rates over time, based on particle size, and is used to determine the amount of each particle size present in a soil.

All data collected was statistically analysed using GenStat 14.1 edition. The Analysis of variance procedure (ANOVA) was used to determine treatment effects and their significance levels. For the district comparative analysis a 3*3*9*12 factorial layout (the 3 districts, 3EPAs from the districts, 9 sites from each EPA and 12 sample plots per site) was used. Differences between and within treatments were separated using Least Significant Differences (LSD) tests at $P< 0.05$. Soil texture was analysed with the aid ArcView GIS 3.3 to determine the surface soil texture classification.

Table 2. Comparative soil analysis in the three districts (Dedza, Mzimba and Thyolo) in Malawi.

Soil parameter		Mean per district			LSD (5%)	SE	CV%
		Dedza	Mzimba	Thyolo			
pH	Top	5.14[a]	5.07[a]	5.50[b]	0.12	0.45	8.60
	Sub	5.10[b]	4.94[a]	5.44[c]	0.13	0.47	9.20
% Sand	Top	38.49[a]	43.99[b]	40.49[a]	2.40	9.00	22.10
	Sub	38.38[a]	42.56[b]	41.21[b]	2.50	9.20	22.60
% Silt	Top	18.45[b]	14.70[a]	27.95[c]	1.50	5.40	26.70
	Sub	18.70[b]	13.40[a]	28.07[c]	1.60	5.80	28.90
% Clay	Top	43.06[b]	41.31[b]	31.52[a]	2.90	10.80	27.80
	Sub	42.92[b]	44.04[b]	30.67[a]	3.00	11.20	28.50
% OC	Top	0.67[b]	0.44[a]	1.17[c]	0.15	0.55	72.60
	Sub	0.62[b]	0.48[a]	1.25[c]	0.14	0.52	66.60
% OM	Top	1.38[b]	0.76[a]	2.04[c]	0.25	0.92	66.00
	Sub	1.24[b]	0.82[a]	2.19[c]	0.23	0.85	60.10
% N	Top	0.04[b]	0.02[a]	0.06[c]	0.01	0.03	69.90
	Sub	0.04[b]	0.02[a]	0.06[c]	0.01	0.03	61.30
P(mg/kg)	Top	45.97[a]	140.65[b]	40.17[a]	12.70	47.40	62.70
	Sub	28.55[a]	129.44[b]	29.53[a]	8.90	33.20	53.00
K (mg/kg)	Top	255.50[c]	24.40[a]	155.60[b]	21.70	81.10	55.90
	Sub	197.30[c]	17.10[a]	124.10[b]	17.40	64.80	57.50
BD		1.42[a]	1.57[c]	1.50[b]	0.04	0.14	9.40

Means with different superscripts within a row are significantly different at P<0.001.

RESULTS

A comparative soil assessment of the sites in the 3 districts indicates that the soils in Mzimba are falling into the acidic levels more than the Thyolo sites. Generic soil texture classification shows that Dedza and Mzimba sites are more dominated by clay soils while sites in Thyolo are classified as being clay loam. This follows that the Thyolo soils have significantly higher %OM in relative terms though not reaching optimal levels (Table 2). The site specific soil assessment revealed that there is a lot of variability within and among the sites in the districts as described in the subsequent paragraphs.

Soils from Mzimba district sites (Table 3) show acidic reactions in both the top soil (pH 4.5 to 5.6) and sub soil (pH 4.3 to 5.4). The soil organic carbon varied from 0.2 to 1.5% with relatively higher values in the top soil. The %N for the top soils ranged from 0.007 to 0.042% for the top soil which was relatively lower than in the sub soils. Higher P (81.7 to 200.4 mg kg^{-1}) levels were obtained from the top soil. There were high variations on the exchangeable K across the sites ranging from 8 mg/kg to 65 mg/kg in the top soil and 4.3 to 56.9 in the sub soils. Soil organic matter (%OM) was also highly variable across the sites ranging from 0.50 to 1.44% in the top soil and relatively higher (0.7 to 2.10%) in the sub soils. Soil clay variations in the two soil depths were also determined.

In Zombwe EPA of Mzimba district, soils have a high proportion of clay ranging from 44.2 to 53.3%, followed by sand (35.3 to 45.1%) and some low proportions of Silt (loam) ranging from 9.4 to 15.0%. These soils have therefore been categorized as Sandy clay to clay. Soils in Mpherembe EPA sites have been categorized as Sandy clay loam with only Ndindi village having clayish soils. In Emsizini EPA the soils are mostly clayish.

Soil for the Dedza district sites (Table 4), shows less variation on the pH levels ranging from 4.6 to 5.5 and 4.6 to 5.8 in the top and sub soils respectively. Soil organic carbon is relatively high (up to 1.4%) in some sites while

Table 3. Soil chemical and physical properties for Mzimba district sites.

Site	pH		% OC		%OM		% N		P(mg/kg)		K (mg/kg)		% Sand		% Clay		%Silt		BD
	Top	Sub	Top	Sub	Top	Sub	Top	Sub	Top	Sub	Top	Sub	Top	Sub	Top	Sub	Top	Sub	
Kenani Shonga	5.4	5.4	0.9	0.7	0.50	0.70	0.03	0.04	151.5	149.1	30.5	20.2	40.8	40.3	44.2	47.8	15.0	11.9	1.52
Zinolema Khonje	5.2	5.1	0.8	0.7	1.40	1.07	0.02	0.03	175.4	159.4	24.9	11.7	36.2	35.3	51.0	52.5	12.8	12.2	1.55
Bandawe Tembo	4.6	4.5	1.4	0.9	0.95	1.22	0.04	0.05	102.0	93.5	13.1	7.8	37.5	35.3	49.6	53.3	12.9	11.4	1.67
Jailosi Mhlanga	4.7	4.5	0.5	0.2	0.79	1.15	0.02	0.01	82.4	74.8	8.8	4.3	45.1	42.8	44.4	47.8	10.6	9.4	1.65
Tengasalu	4.6	4.3	0.4	0.2	1.44	1.60	0.01	0.01	99.0	101.8	48.0	21.9	55.6	53.9	31.1	32.2	13.3	13.9	1.59
Kazalawe	5.4	5.3	0.4	0.3	0.50	0.37	0.01	0.02	161.8	156.7	65.0	56.9	52.5	48.1	32.5	37.5	15.0	14.4	1.46
Chigagu jere	4.6	4.5	0.3	0.2	0.43	0.30	0.01	0.01	81.7	79.1	14.2	8.2	45.8	47.2	35.3	42.5	18.9	10.3	1.56
Ndindi	5.0	5.0	1.0	0.7	0.36	0.52	0.03	0.03	143.8	137.1	8.0	4.6	39.2	38.9	44.2	47.8	16.7	13.3	1.56
Chilenga zamangwe	5.3	5.3	0.5	0.4	0.34	0.38	0.02	0.02	130.6	130.6	24.6	13.0	50.0	51.9	33.6	31.4	16.4	16.7	1.60
Kaigwazanga Mphande	5.6	5.3	1.5	0.6	0.13	1.13	0.04	0.03	211.7	167.2	30.2	17.2	41.4	39.7	41.7	43.6	16.9	16.7	1.51
saulosi Nkosi	4.5	4.3	1.4	1.2	1.44	2.10	0.04	0.06	159.2	151.9	11.7	5.5	24.7	19.2	65.3	72.2	10.0	8.6	1.51
Mkakangoma	5.0	4.7	0.2	0.2	0.23	0.43	0.01	0.01	200.4	176.9	24.7	22.0	43.6	38.9	46.4	44.7	10.0	16.4	1.52
Mean	5.0	4.9	0.8	0.5	0.7	0.9	0.02	0.03	141.6	131.5	25.3	16.1	42.7	41.0	43.3	46.1	14.0	12.9	1.6
CV (%)	7.7	8.8	59.8	62.5	68.5	61.6	52.8	62.6	30.8	26.9	67.1	89.7	19.2	22.4	22.3	23.5	20.8	21.6	3.9

traces were recorded in 33% of the sites. Traces of %OM and %N were also determined in the same areas. The P values across the sites were highly variable ranging from 6.8 to 137 mg kg^{-1} in the top soil and 3.7 to 61.7 mg kg^{-1} in the sub soil. Overall, almost all the sites have high K values with the lowest mean being 93.7 mg kg^{-1}. These soils in Dedza sites are predominantly clay especially in Chafumbwa and Golomoti ranging from 35.3 to 66.9% in the top soil and 38.1 to 63.9% in the sub soils. In Mtakataka, the clay content ranges from 19.2 to 44.8% in the top soils and almost similar values have been determined in the corresponding sub soils. Soil in Mphonde and Asani villages have high proportions (>55%) of sand in both the top and sub soils.

Sites in Thyolo districts (Table 5) show soil pH levels ranging from 5.3 to 5.8 in the top soil and 5.2 to 5.9 in the sub soil. In both soil depths, soil organic carbon ranges from 0.8 to 1.7%. Soil organic matter varies from 1.44% to 3.00% in the top soil and the sub soil showed almost same range (1.42 to 3.05%). Nitrogen level in both the top and sub soils is between 0.04 and 0.08%. Lowest levels of P (6.50 and 8.17 mg/kg) were obtained in the sub soil samples from Mwanaphwa and Katundu villages.

Low K values were recorded in Pemba (63.67 mg/kg) and Mtulo (75.92 mg/kg) villages while Supelo village recorded relatively higher K values, in the top (236.92 mg/kg) and 192.92 mg/kg (sub soils). Soil particle distribution in the assessed sites shows relatively high proportions of sand ranging from 36.42 to 44.42% in the top soil and 37.50 to 46.58% across the sites, clay and silt values range between 20 and 40% in both the top and sub soil depths. Bulk density determination showed evidence that the soils are not compacted with Mzimba soils averaging 1.57 g/cm^3, Dedza and Thyolo recorded 1.58 and 1.46 g/cm^3 respectively.

DISCUSSION

Current status of soil fertility in the study sites in general

This study shows that the current condition of most soils in the districts is of very low soil fertility status. The pH in the three districts varied considerably among sampled sites; values for Thyolo sites are higher than those of Dedza and Mzimba sites. Soil pH falling in the ranges below 5 are regarded as acidic (Brady and Weil, 1996) and not very good for production of most crops including maize. Many crops grow well in soils with pH close to neutral (pH 5.8 to 7.5) though some few crops would do better in a wide range of pH 5.8 to 9.0. In this study, only about 10% of the

Table 4. Soil chemical and physical properties for Dedza district sites.

Site	pH		% OC		%OM		% N		P(mg/kg)		K (mg/kg)		% Sand		% Clay		%Silt		BD
	Top	Sub	Top	Sub	Top	Sub	Top	Sub	Top	Sub	Top	Sub	Top	Sub	Top	Sub	Top	Sub	
Njolo	5.3	5.3	1.1	1.4	1.85	2.40	0.054	0.070	108.9	61.7	330.6	269.4	28.0	28.7	44.8	43.1	27.2	28.2	1.24
Mphonde	5.4	5.4	1.8	1.1	3.10	1.91	0.091	0.055	22.9	19.4	358.5	335.1	55.9	57.5	22.3	19.7	21.8	22.8	1.53
Kankhande	5.5	5.8	1.2	1.0	2.08	2.00	0.061	0.050	23.5	20.8	266.4	224.0	23.8	24.9	43.5	41.9	32.7	33.2	1.41
Asani	5.5	5.6	1.1	1.4	1.94	2.00	0.056	0.069	20.6	28.5	324.8	264.2	63.6	62.2	19.2	20.0	17.2	17.8	1.44
Msamala	5.2	5.2	1.2	0.6	2.01	1.00	0.058	0.029	58.6	39.9	270.1	212.1	19.2	21.4	66.9	63.9	13.9	14.7	1.37
Somanje	5.2	5.2	0.7	1.1	1.22	2.00	0.035	0.053	137.3	67.0	327.8	184.9	45.8	44.5	35.3	38.1	18.8	17.4	1.45
Liwengwa	5.1	5.2	Trace	0.5	1.09	1.00	Trace	0.025	8.2	1.7	174.9	107.0	35.3	35.9	45.8	48.2	18.9	15.8	1.50
James	5.2	5.2	0.7	1.3	1.12	2.00	0.033	0.066	80.1	53.6	239.2	216.2	35.3	34.7	43.9	43.8	20.8	21.5	1.38
Chisani	4.8	4.7	Trace	Trace	1.12	1.60	Trace	Trace	7.6	10.6	258.9	212.0	35.3	35.9	47.9	44.9	16.8	19.1	1.44
Mnolo	4.8	4.7	Trace	Trace	Trace	Trace	Trace	Trace	5.9	5.4	200.3	232.0	32.5	33.1	50.7	49.3	16.8	17.7	1.52
White	5.2	4.9	Trace	Trace	Trace	Trace	Trace	Trace	42.6	24.4	128.1	97.2	34.2	30.8	51.1	55.3	14.7	13.9	1.42
Kum'buka	4.6	4.6	Trace	Trace	Trace	Trace	Trace	Trace	6.8	3.7	125.8	93.7	29.2	28.3	54.2	53.1	16.7	18.6	1.38
Mean	5.2	5.2	1.1	1.1	1.7	1.8	0.1	0.1	43.6	28.1	250.5	204.0	36.5	36.5	43.8	43.4	19.7	20.1	1.4
CV (%)	5.5	7.1	33.3	32.6	38.6	27.1	34.7	33.1	100.9	81.0	31.7	36.2	35.1	34.1	30.0	29.9	27.5	28.3	5.5

Table 5. Soil chemical and physical properties for Thyolo district sites.

Site	pH		% OC		%OM		% N		P(mg/kg)		K (mg/kg)		% Sand		% Clay		%Silt		BD
	Top	Sub	Top	Sub	Top	Sub	Top	Sub	Top	Sub	Top	Sub	Top	Sub	Top	Sub	Top	Sub	
Chibwana	5.4	5.3	1.1	1.1	1.89	1.81	0.06	0.05	66.2	53.8	123.8	98.2	44.4	44.6	33.8	34.6	21.7	20.7	1.44
Katundu	5.6	5.5	0.9	1.2	1.60	2.00	0.05	0.06	15. 7	8.2	147. 7	127.7	40.3	40.8	24.4	23.3	35.3	36.0	1.55
Mtulo	5.3	5.3	0.9	0.8	1.58	1.42	0.05	0.04	55.5	39. 3	96.8	75.9	36.4	37.5	41.8	39.3	21.8	23.3	1.54
Savala	5.8	5.9	1.2	1.5	2.10	2.54	0.06	0.07	18.8	14. 7	164.0	137.4	40.3	40.8	24.4	23.3	35.3	36.0	1.38
Supelo	5.4	5.3	1.2	1.4	3.00	3.05	0.06	0.07	60.2	39. 3	236.9	192.9	44.4	44.6	33.8	34.6	21.7	20.7	1.61
Mpezo	5.4	5.2	0.8	1.0	2.65	2.61	0.04	0.05	28.8	17. 7	128.0	82.0	39.5	39.5	38.8	39.3	21.8	21.3	1.45
Mwanaphwa	5.8	5.8	1.7	1.7	2.07	2.32	0.08	0.08	10.5	6. 7	225.6	183.4	42.4	46.6	21.8	17.4	35.8	36.0	1.50
Pemba	5.3	5.3	1.2	1.4	1.97	2.28	0.06	0.07	92.4	79. 0	74.8	63.7	38.2	37.8	38.8	39.8	23.0	22.4	1.40
Mean	5.5	5.5	1.1	1.3	2.1	2.3	0.1	0.1	47.5	31.0	150.0	120.2	40.7	41.5	32.2	31.5	27.1	27.1	1.5

sampled sites would barely qualify to have adequate pH levels (pH> 5.8) for crop production and the rest fall in the acidic soil range. In very acidic soils (pH<5.0), some of the macro and micro nutrient elements including calcium and magnesium, nitrate-nitrogen, phosphorus, boron, and molybdenum are deficient; whereas aluminum, iron and manganese are abundant, sometimes at levels toxic to some plants (Belachew and Abera,

2010). Thus, soil pH influences the mobility of trace elements in the soil and it is a primary factor for the uptake of most nutrients by plants. Soil pH affects the soil's physical, chemical, and biological properties and processes, as well as plant growth. The nutrition, growth and yields of most crops decrease where pH is low and increase as pH rises to an optimum level above 5.8 (Karlen at al., 2003).

On the other hand soil bulk density also does play a major role in the nutrient accessibility by the plant roots. Critical value of bulk density for restricting root growth varies with soil type but in general bulk densities greater than 1.6 g/cm^3 tend to restrict root growth (McKenzie et al., 2004). Sandy soils usually have higher bulk densities (1.3 to 1.7 g/cm^3) than fine silts and clays (1.1 to 1.6 g/cm^3) because they have larger, but fewer, pore spaces. In clay soils with good soil structure, there is a greater amount of pore space, and many small pore spaces fit between them. Soils rich in organic matter can have densities of less than 0.5 g/cm^3. Bulk density increases with compaction at depth and very compact sub soils or strongly indurated horizons may exceed 2.0 g/cm^3 (Cresswell and Hamilton, 2002).

In order to understand the spread of the current soil status in the country, it is clear that while the soils of Malawi have been grouped into 28 classes, they are predominated by three major soil types: (1) The Leptosols, which occur in most hilly areas of the country; (2) The Luvisols, which are the red-yellow soils of the Lilongwe plain and some parts of southern region; (3) The Lixisols, which are the alluvial soils of lacustrine and river-line plains, the Vertisols of the lower shire valley and Phalombe plain and the Mopanosols in the Liwonde and Balaka areas (Dewitte et al., 2013). Other relatively dominant soils include Acrisols, Cambisols and Ferralsols (Sileshi et al., 2010). Studies have shown that most of these soil types are not as productive under poor agricultural management even when inorganic fertilizers are put into use. The high risks associated with inorganic fertilizer on Acrisols and Lixisols could probably be attributed to their inherent infertility (Bationo et al., 2006), low resilience and high sensitivity (Stocking, 2006). Lixisols become depleted quickly under agricultural use, though their physical characteristics are generally better than those of Acrisols (Bationo et al., 2006; Stocking, 2006). Acrisols are acidic, strongly leached, have low base status (Bationo et al., 2006) and they become degraded very quickly when utilized (Stocking, 2006). Even when soil cover is good, Acrisols do not continue to produce reasonable maize yields for more than 4 years (Stocking, 2006). Sileshi et al. (2010) also showed that yield and yield gaps for most of the crops in these soils did not significantly differ. On Cambisols yield risks were generally lower confirming the fact that these soils have high resilience to degradation, and low sensitivity to yield decline (Stocking and Tengberg, 1999). They suffer degradation under persistent mismanagement, while biological conservation methods can adequately maintain production (Stocking, 2006). This entails that all soil types can be as productive if they receive appropriate management and likewise they are vulnerable to degradation if not properly managed.

What are the implications for soil health and crop productivity based on the current soil status?

Soils with coarse textures may acidify easily compared to clay soils, because they have low organic matter content, a low buffering capacity, a low cation-exchange capacity (poor cation retention), and high rates of water percolation and infiltration (Buresh et al., 1997). However, in this study despite having most sampled soils with relatively high clay proportions, the soil analysis also revealed that %OM is low (<2%) in most of the sites such that the cation exchange buffer effect is limited. Soil organic matter (SOM) is important for healthy plant growing as it maintains favourable conditions supporting soil moisture retention, temperature, nutrient, pH, and aeration. SOM through the organic carbon also provides a steady food source for the decomposing soil biota. Unless the decomposition and loss of organic matter are halted in conventional tillage, the soil fertility will continue to decline and the system is not sustainable (Wolf and Snyder, 2003). From these results, the current soil fertility status is so low such that farming practices that ensure accumulation of organic matter needs to be encouraged in order to ensure improved soil productivity. Organic inputs have an important advantage over inorganic fertilizers with regard to fertility replenishment because they provide a source of C for microbial use. According to Palm (1995), recovery of N by the crop from the leaves of leguminous plants incorporated into the soil is generally lower (10 to 30%) than the recovery from N fertilizers (20 to 50%). Much of the remaining 70 to 90% of the applied organic N are not used by crops or leached and is incorporated into labile pools of soil organic N and C which support microbial growth. Soil microorganisms require C substrate for growth and subsequent release of the N to form soil N capital. Part of the bound N in recalcitrant fractions in the organic materials does also increase soil organic N (Giller et al., 1997; Buresh et al., 1997). Inorganic fertilizers do not contain C sources, and therefore much of the fertilizer N not used by crops is subject to leaching and denitrification losses in the absence of crop residue returns. Pieri (1987) reported that additions of N fertilizer alone did not increase soil C or N stocks in sandy soils but when complemented with organic inputs (crop-residue returns, manures, and composts) they increased soil N and C stocks, except in extremely sandy soils where there are very few clay particles to protect newly formed SOM from decomposition.

With respect to crop productivity, there is no doubt that

inorganic fertilizer gives higher yields than the organic inputs. Sileshi et al. (2010) emphasized that organic inputs from the legumes provide additional ecosystem services that cannot be provided by inorganic fertilizer. In addition, organic inputs from the legumes may have large impact on more sensitive and less resilient soils. Therefore, legume species should be targeted to niches where they can ensure soil cover, improve soil organic matter and maintain productivity. This ensures reduced yield risks, mitigated land degradation and enhances crop yields.

Is the current soil fertility status in Malawi reversible? Can agroforestry trees play a role?

When it comes to consistency on the use of inorganic fertilizer, it has been observed that many farmers switch back and forth between using and not using fertilizer from season to season (Duflo et al., 2008). It has also been reported that farmers are often risk-averse (Simtowe, 2006) as they find it difficult to recover the costs of fertilizer from their produce (Denning et al., 2009). Thus, the availability and use of inorganic fertilizers have also been low amidst growing land-use intensification and expansion of crop cultivation onto marginal soils. As a result, soil fertility has declined and it is widespread, particularly in sub-Saharan Africa (Henao and Baanante, 1999). It has been shown that most smallholder farming households in the sub-Saharan Africa are only able to afford fertilizer application of up to 8 kg/ha which is the lowest application rate to achieve increased yields (Morris et al., 2007) in the already nutrient deprived soils. On the other hand, continued and excessive use of N fertilizers cause problems of acidification and, the over-use of N and P fertilizers cause water pollution in the form of eutrophication among other negative effects on the environment (Brady and Weil, 2008; Olson, 1987). Studies have shown that the presence of nitrogen-fixing trees in a tree-crop farming systems (also commonly known as agroforestry systems) do improve nutrient use efficiency by providing a safety net to recover nutrients leached from the topsoil during intense rainfall and return them to the surface horizons on which crop roots primarily depend, in a manner analogous to the hydraulic lift of water (Sileshi et al., 2014). Furthermore, these tree-based systems enhance soil organic matter through production of good quality leaf biomass that decomposes easily releasing the most limiting nutrient which in nitrogen for subsequent support to crop growth. *Faidherbia albida, Gliricidia sepium* and *Tephrosia candida* are among the available tree species that shown significant increases in crop productivity and ensure food security (when integrated into maize cropping systems in rotational fallows or intercrops. Under good field management these nitrogen-fixing trees can be used singly or as a complement to limited inorganic fertilizer input (Akinnifesi et al., 2012).

In conclusion, this study clearly shows that the soil fertility indicators include low to medium total nitrogen, available P and exchangeable K across the sites. The nutrient status is mostly affected by the low levels of %OM, associated %OC and pH. The fertility differences across the sites are due to the inherent soil texture properties. These results provide some basic understanding that soil health is varied in the different agro-ecologies such that for improved crop productivity is it paramount to consider site specific soil fertility management. The farmers in these areas must be encouraged to adopt the use of nitrogen-fixing tree-crop intercropping systems among other grain legume intercrops, as one way towards improving the soil organic matter for improved soil and crop productivity; consequently farmers' livelihood and resilience to weather variability is expected to improve over time.

Conflict of Interests

The authors have not declared any conflict of interests.

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Azospirillum brasilense promotes increment in corn production

José Roberto Portugal[1]*, Orivaldo Arf[1], Amanda Ribeiro Peres[2], Douglas de Castilho Gitti[3], Ricardo Antônio Ferreira Rodrigues[2], Nayara Fernanda Siviero Garcia[1] and Lucas Martins Garé[1]

[1]Departament of Plant Science, Food Technology and Socioeconomy, São Paulo State University "Júlio de Mesquita Filho", Ilha Solteira, São Paulo, Brazil.
[2]Departament of Plant Protection, Rural Engineering and Soils. São Paulo State University "Júlio de Mesquita Filho", Ilha Solteira, São Paulo, Brazil.
[3]MS Foundation, Maracaju, Brazil.

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The corn crop is one of the most cultivated in the world and one of the most need studies that seek alternatives on the use of *Azospirillum brasilense*. This bacterium produces growth hormones that may benefit the corn crop. The work objective was to verify the agronomical performance of corn crop, in function of foliar *Azospirillum brasilense* inoculation, associated with nitrogen doses. The research was performed in Selvíria, Mato Grosso do Sul State, Brazil, which is in the Brazilian Cerrado. The corn crop was cultivated during season (Spring/Summer) and late season (Summer/Autumn), under conventional tillage system. Experimental design of randomized blocks was used, with factorial scheme 4 × 2. The treatments were made in four nitrogen doses (0, 30, 60 and 90 Kg ha^{-1}) with and without the foliar applying (in stage V6) of the inoculant containing *A. brasilense*. The inoculant used had the strains AbV_5 and AbV_6 of *A. brasilense* ($2×10^8$ viable cells mL^{-1}) and, the dose 200 mL ha^{-1} was used. The nitrogen fertilization was made with ammonium sulfate, also on phenological stage V6. The following evaluations were made: Final plant population, foliar nitrogen content, foliar chlorophyll index, ear insertion height, plants height, stalk diameter, ears length, ears diameter, thousand grain weight and grain productivity. The foliar inoculation with the *A. brasilense* bacterium proved to be advantageous for the corn crop, and therefore, an option for the farmer. Because of the large volume of products used in seed (fungicide, insecticide, plant growth regulators) and the lack of information on the degree of interference of these products on the bacteria, it becomes pertinent the study of other inoculation forms on crops. Other studies that verify the influence of *A. brasilense* bacteria in foliar application on the physiology of the shoot and root of crops should be encouraged.

Key words: *Zea mays* L., inoculation, nitrogen, diazotrophic bacteria.

INTRODUCTION

The corn crop (*Zea mays* L.) has indisputable role in the world and Brazilian economy, due to its outstanding position among the agricultural species explored (Môro and Fritsche, 2015). Besides human feeding, corn is the main component in animal food, highlighting birds, cattle and pigs. Therefore, much research should be conducted in order to increase productivity (Purwanto et al., 2015).

Plant growth-promoting bacteria, called associative, as they do not make symbiosis with the host plant and belong to the genus *Azospirillum*, can help through

Table 1. Soil analysis result on depth of 0.0 - 0.2 m, before the experiment in the area, Selvíria, MS, Brazil, 2011/2012 and 2012/2013.

P (resin)	O.M.	pH	K	Ca	Mg	H+Al	CEC	BS
mg dm^{-3}	g dm^{-3}	$CaCl_2$	.....$mmol_c$ dm^{-3}.....					%
11	21	5.2	3.0	15.0	10.0	19.0	47.0	59

several mechanisms in the crops nitrogen nutrition (Bashan and Bashan, 2005). Among the mechanisms, the process of nitrogen biological fixation and the plant growth promotion stand out for the production of several vegetable hormones, which result in higher growth, water and nutrients absorption (Moreira et al., 2010) which may increase the productive potential of crops. According Bashan et al. (2004), the most common explanation for the effect of the bacteria in plants is the production of hormones that alter the metabolism and the morphology of the plant, improving the nutrient and water uptake.

Costa et al. (2015) studied inoculation with *Azospirillum brasilense* in seed and nitrogen doses in corn crop in the Cerrado region and reported that the use of bacteria in corn promoted greater plant height, stalk diameter, chlorophyll index, dry mass of stalk and root, ear length, thousand grain weight and yield. Similarly, Souza (2014) also in Cerrado region, noted that the use of bacteria in seed promoted greater plant height and ear insertion height, hundred grain weight and yield. Morais et al. (2016) researched several doses of inoculant containing *A. brasilense* applied in sowing furrow of corn and verified that the dose of 200 mL ha^{-1} promoted increased in grain yield. One of the most pronounced effects of inoculation with Azospirillum on root morphology is represented by the proliferation of root hairs (Saikia et al., 2012) and, according Fulchieri et al. (1993) the promotion of root growth can lead to better exploration of the soil and enhance the growth and development of plants by the greater water and nutrients uptake.

Studies that promote knowledge about bacteria of genera Azospirillum must be stimulated (Moreira et al., 2010; Lana et al., 2012) aiming not only low cost and low environmental impact agriculture, but also the biotechnological potential that these bacteria present (Moreira et al., 2010). Identifying the managing conditions which may contribute to the maximization of processes made by them is a challenge for the present research (Moreira et al., 2010), as an example, the study about foliar applying of the inoculant containing the bacterium *A. brasilense* can be mentioned. According to Fukami et al. (2016) little is known about the effects of pesticides used to treat seeds on *Azospirillum*. The authors report that alternative methods of inoculation in crops are needed and highlight the foliar application in the vegetative stage.

This way, the objective of the work was to evaluate the corn crop performance (season and late season) when submitted to foliar inoculation of *A. brasilense* and nitrogen doses applied in topdressing, in low altitude Cerrado region.

MATERIALS AND METHODS

The experiment was developed during the season in 2011/12 (Spring/Summer) and the late season in 2012/13 (Summer/Autumn) at the Teaching, Research and Extension Farm of Engineering College of Ilha Solteira - UNESP, located in Selvíria city, Mato Grosso do Sul State, Brazil, within the geographical coordinates of 22° 23' south latitude and 51° 27' west longitude and 335 m of altitude. Soil is classified according to Santos et al. (2013), in RED OXISOL typical dystrophic clayed. Before of experiment installation was collected soil in the layer from 0.0 to 0.2 m for the chemical characterization of all experimental area, with the same values for all experimental plots (Table 1).

The soil preparation was made with scarifier and leveling harrow. The conventional seeding system was used. The corn seeding on season period was made under soybean residues and on late season period under corn residues. The experimental area was cropped with soybeans on summer of 2010/2011 and remained fallow until the sowing of corn on season period (2011/12). After the cropped of corn on season period (2011/2012), the area remained fallow until the sowing of corn on late season period (2013). It was opted to sow the corn in the beginning of period that characterizes the late season (January) to match the period of much of the crop development with a part of the rainy period, providing better water condition. Agricultural activity in the Cerrado is concentrated in the rainy period, usually between the months of October to March, when it occurs between 80 and 90% of the annual precipitation (Assad et al., 1993), so there is a preference for sow crops in the period that provides better conditions for development.

The annual average rainfall in the region is 1,313 mm, maximum annual temperature is 31°C, minimum annual temperature is 19°C and average annual temperature is 25°C (Portugal et al., 2015) with climate type A_w, according to Köppen's classification. The daily rainfall values, maximum temperature and minimum temperature on the experiment leading period are on Figure 1.

*Corresponding Author. E-mail: jrp_agro@yahoo.com.

Abbreviation: CEC, Cation Exchange Capacity; **BS**, Base Saturation

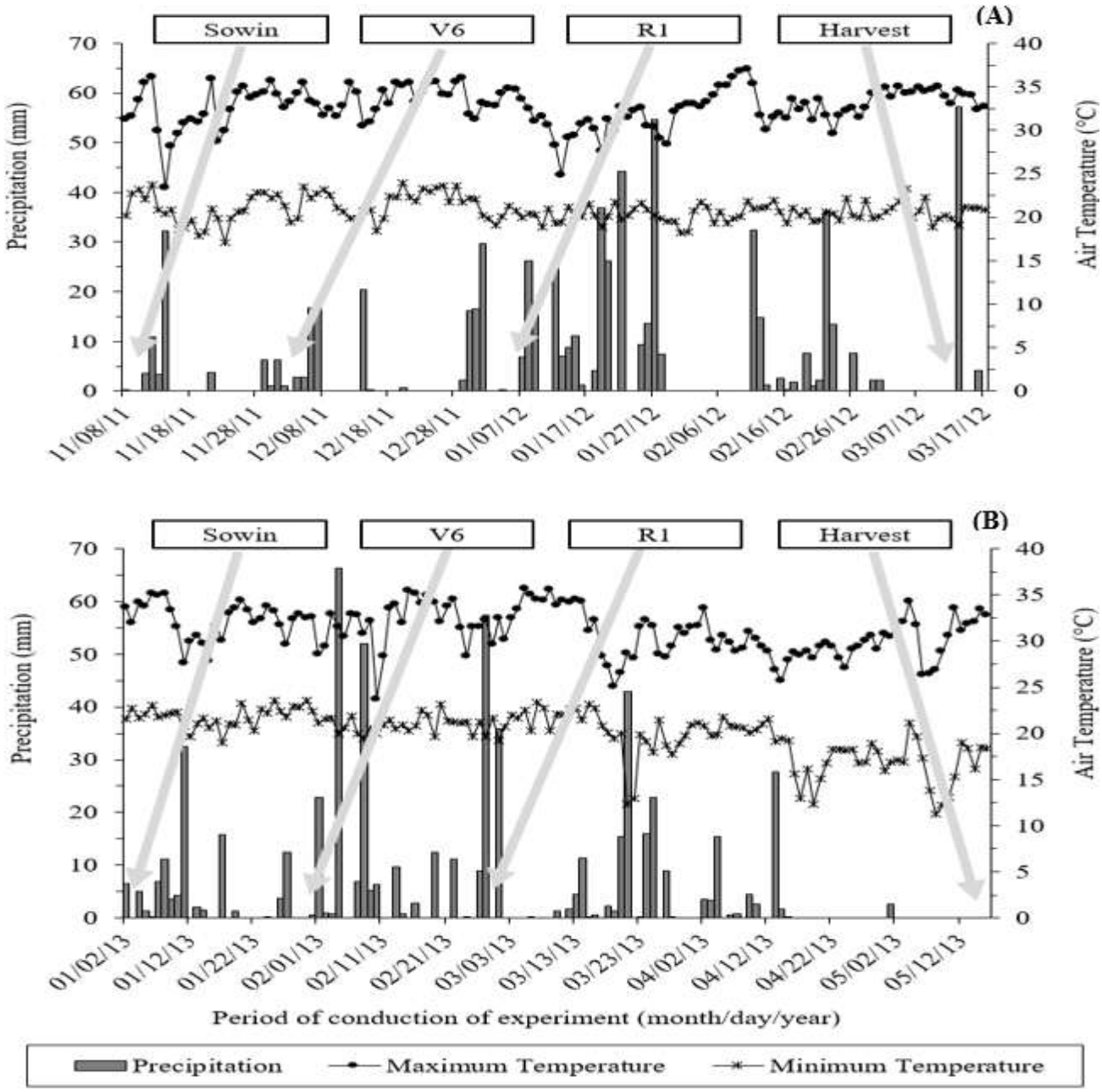

Figure 1. Daily values of rainfall, minimum and maximum temperature during the experiment leading period with season corn - 2011/12 (A) and late season corn - 2012/13 (B), in *Selvíria* - MS, Brazil.

The experimental design was of randomized blocks with 8 treatments, disposed in factorial scheme 4x2 with four replications. The treatments were composed by 4 nitrogen doses in ammonium sulfate form (0, 30, 60 and 90 Kg ha^{-1}) with and without the foliar application of the inoculant containing *A. brasilense*. The plots were composed by 5 lines with 5 m long, being considered as useful areas, the 2 central ones.

For the season corn the simple hybrid AG 8088 YG was used, of early cycle and hard orange seed; while for the late season corn, the simple hybrid DKB 390 VT PRO was used, of early cycle and semi hard yellow-orange seed (Cruz et al., 2013). The foliar inoculation was performed with Masterfix Gramineas® inoculant, with the strains AbV_5 and AbV_6 of *A. brasilense* (2×10^8 viable cells mL^{-1}) with dose of 200 mL ha^{-1} diluted with water only. The sowing was made on 11/9th/2011 (season corn) and 01/2nd/2013 (late season corn) with row spacing of 0.90 m and the initial population was established in 50,000 plants ha^{-1} for the corn in both cultivation seasons. According to Fornasieri-Filho (2007) the ideal population for the corn crop varies from 30,000 to 90,000 plants ha^{-1}. The mineral fertilization in the sowing furrow was defined based on the chemical soil analysis and expected productivity of 4,000 to 6,000 kg ha^{-1}, according to the recommendations of Raij and Cantarella (1997) using 250 Kg ha^{-1} of 08-28-16 (season) and 400 kg ha^{-1} of formulated 04-30-10 (late season).

The foliar inoculation with the bacterium, as well as the topdressing fertilization, were made when the plants were on developing stage V6 (six expanded leaves), according to the phenological scale proposed by Ritchie et al. (2003), at that phase the crop was with 17 DAE (days after the emergency) and 22 DAE on season and late season, respectively. The topdressing fertilization and the inoculant applying were made in the late afternoon, aiming mild temperature conditions, especially to favor the inoculation with the bacteria. For the inoculant applying, costal spray with cone jet tip and 180L ha^{-1} output was used.

Aiming to keep the crop free from competition with weeds, atrazine and tembotrione were used in doses of 1,000 and 100 g ha^{-1} of a.i., respectively, in tank mixture form. The adjuvant soy methylated ester was added to the application mixture (500 g ha^{-1} of a.i.). The application was made through the use of tractor powered spray with flat jet tips and adjusted to apply 200 L ha^{-1} of the mixture.

During the research, the following evaluations were performed: (a) final plant population, made through counting the plants in the useful area at the harvest moment and later extrapolated for hectare. This evaluation has an important role on the yield of corn crop, since small variations have great influence on the final yield (Cruz et al., 2015); (b) foliar nitrogen content, gotten through collect of the mid third of five opposite leaves and under the main corn ear on stage R1(flowering – at 52 DAE and 48 DAE of season and late season corn, respectively), according to the phenological scale proposed by Ritchie et al. (2003), after that the drying of leaves was made and the nitrogen content was determined in laboratory. The analysis of plant tissue as a dignostic criterion is based on the premise there is a relationship between growth and crops production and the nutrients content in their tissues (Coelho, 2008) among them, the nitrogen; (c) foliar chlorophyll index, made at the same moment and with the same leaf collect to obtain the foliar nitrogen content, using portable chlorophyll meter (model CFL 1030), which through sensors, analyzes three light frequency lines and through absorption relation of different frequencies, provides measurement of chlorophyll a, b, and total (a+b) levels, expressed in dimensioned units called FCI (Foliar Chlorophyll Index). The foliar chlorophyll index is a good parameter to indicate the nitrogen level in cereals (Argenta et al., 2001), because 50 to 70% of total nitrogen of the leaves is integral of enzymes that are associated to the chloroplasts (Lima et al., 2009); (d) plants height, gotten by measurement of five random plants per plot, using a graduated ruler, from the ground to the flag leaf, on R6 stage (physiological maturity), according to the phenological scale proposed by Ritchie et al. (2003); (e) ear insertion height, measured in the plants and at the same moment of plant height, however, the measurement was made from the ground to the ear insertion on the stalk. Higher plant height and ear insertion height on the stalk can contribute to the increase of crop lodging (Brachtvogel et al., 2012); (f) stalk diameter, measured at the second internode from the plants base, with digital caliper rule CD-6 CSX-B (Mitutoyo Sul Americana®), five random plants were considered. The stalk diameter is one of the characteristics that has been more related to the percentage of lodging and breakage of plants in corn crop (Kappes et al., 2013); (g) ear length, made after harvest, considering ten random ears without straw in each plot, which were measured from the base to the apex with graduated ruler; (h) ear diameter, was obtained by measuring the central third of the same ears used to measure the length, with the digital caliper ruler. The length and diameter of corn ears directly influence the yield of crop grain (Kappes et al., 2009); (i) thousand grain weight, obtained by counting a subsample of 250 grains per plot, which was submitted to weighting and humidity determination, making it possible to estimate the grains weight corrected to 13% of humidity (wet basis - w.b.). The grain mass depends entirely of the factors that control the supply of assimilates for grain filling (Fageria, 1989) and correlates positively with crop yield (Duarte et al., 2007); (j) grain productivity, it was obtained from the threshing and weighting of grains from the ears collected from the two central lines in each plot, the values were extrapolated to kg ha^{-1} and corrected to 13% humidity (w.b.). This is the most important variable to check if the crop was responsive to the different treatments in which it was subjected.

The corn ears harvest on the area was performed on 03/15/2012, at 120 DAE of corn on season and on 16/05/2013, at 127 DAE on late season.

The results were submitted to the F test of analysis of variance, comparing the treatments average with *A. brasilense* by the Tukey test at 5% de probability. The N doses average in topdressing were submitted to the regression analysis, adjusting meaningful equation models through the F test.

RESULTS AND DISCUSSION

During the corn crop cycle cropped in the season period (Figure 1A), the accumulated rainfall registered was 682 mm and on corn cycle cropped in late season (Figure 1B), the accumulated rainfall was 603 mm. In both cultivation seasons the precipitation was adequate to the corn crop because, according to Fancelli (2015) this crop requires 400 to 600 mm of rainfall during the cycle. It is noticed that in the period of late season occurred less rainfall and irregular distribution at the end of the reproductive phase.

The F test was significant at 5% to the inoculation in the final population variable only in the season period (Table 2). The foliar inoculation with *A. brasilense* provided higher final plant population. After 11 days of the inoculant applying, there was a 14-day dry period, what may have not favored the non-inoculated plants (Figure 1). At the initial development phase, the corn plants have fast absorption of nutrients and water; therefore, long periods without water may impair the development. It can be explained by the hormones production by the bacteria, which promoted the growth of the root system and, consequently the soil volume to be explored in search of water and/or nutrients (Okon and Labandera-Gonzalez, 1994), providing higher survival rate, while the non-inoculated plants did not have this competitive advantage. According Fornasieri-Filho (2007) in corn crops with excess of plants regarding the water supply capacity occur death of plants. Therefore, the dry periods are the main cause of mortality of plants in Cerrado region. What did not occur with the late season corn, because there was proper rainfall distribution in the vegetative and reproductive phases. For both corn crop periods, the F test showed that there was no influence of nitrogen doses (Table 2).

As to the foliar nitrogen content, it is noticed that on season corn, the F test was significant at 1% for inoculation and nitrogen doses, and the late season corn was significant at 5% only for nitrogen doses (Table 2). The inoculation had positive influence, resulting in higher foliar N content. However, both treatments obtained values within the suitable range, which, according to Cantarella and Furlani (1997) is between 27 and 35 g Kg^{-1}. In relation to the N doses, it's observed that in both crops,

Table 2. Average values of final plant population, nitrogen content and foliar chlorophyll index of season and late season corn according to the *A. brasilense* foliar inoculation and nitrogen applying in topdressing (Selvíria, MS, Brazil, 2011/12 and 2012/13).

Treatments		Final Population		Foliar Nitrogen Content		Foliar Chlorophyll Index	
		nº plants ha^{-1}		g kg^{-1}			
		Season	Late season	Season	Late season	Season	Late season
Inoculation							
Presence		43,595[a]	48,750	34.35[a]	28.06	70.93	55.94
Absence		40,972[b]	46,736	33.46[b]	27.49	71.75	58.43
M.S.D		2,450	2,370	0.67	1.09	3.29	2.54
N Dose (kg ha^{-1})							
0		43,518	49,027	32.20[(1)]	26.45[(2)]	67.59[(3)]	54.09[(4)]
30		41,666	47,083	34.65	27.64	71.90	56.86
60		41,512	47,777	33.65	28.30	74.20	58.61
90		42,438	47,083	35.13	28.70	71.66	59.19
F Test	I	4.90[*]	3.11	7.50[**]	1.21	0.26	4,15
	D	0.60	0.64	15.95[**]	3.54*	2.91[*]	3,49[*]
	I x D	2.60	0.56	1.55	2.89	0.86	0,29
General average		42,283	47,743	33.91	27.77	71.34	57.19
V.C. (%)		7.93	6.76	2.70	5.34	6.39	6.05

** and * - meaningful to 1 and 5% of probability, respectively; averages followed by distinct letter in the columns, differ by Tukey test at 5% of probability; V.C. - variation coefficient; M.S.D. - Minimum significant difference. [(1)]$y = 32.74+0.026x$ ($R^2= 0.60$**); [(2)] $y = 26.66+0.025x$ ($R^2= 0.94$**); [(3)]$y = 67.44+0.22x-0.0019x^2$ ($R^2= 0.98$*); [(4)]$y = 54.63+0.0567x$ ($R^2= 0.92$**).

the foliar N contents had a linear positive response, the same was reported by Farinelli and Lemos (2012), Mota et al. (2015), Lange et al. (2006), Moda et al. (2014) and Valderrama et al. (2014).

For the foliar chlorophyll index the F test was significant at 5% for nitrogen doses in both crops (Table 2). On season, it is noticed that the foliar chlorophyll index adjusted to the quadratic equation, which had higher values with the approximate dose 58 kg ha^{-1} of N. Maestro et al. (2014), working with N doses in corn at the same region of the present work, it was observed a higher foliar chlorophyll index with doses of 85 and 96 kg ha^{-1}, in the years 2009 and 2010, respectively. Torres et al. (2015) observed higher values for the chlorophyll index with the dose of 142 kg ha^{-1}, the authors report that above these doses, there was chlorophyll content reduction due to the fact that they stopped responding to the increase of N offer.

The foliar chlorophyll index is used to predict the nitrogen content in the leaves, however, making comparison it's observed that the chlorophyll index did not reflect on the foliar N content on season. According to Blackmer and Schepers (1995) the luxury consumption of nitrogen by the plant, in nitrate form is not detected by the chlorophyll meter. It happens because the N does not associate to the chlorophyll molecule (Sangoi et al., 2015). On late season, the foliar chlorophyll index adjusted to the positive linear equation with the nitrogen dose increase, reliably reflecting the foliar nitrogen level, the same was reported by Costa et al. (2012) and Mota et al. (2015).

In relation to plant height, the F test was significant at 1% for inoculation only on late season corn (Table 3). The plants height was benefited by the foliar inoculation with inoculant containing *A. brasilense*, representing increase a little above of 7 cm under inoculation treatment. The growth hormones production characteristic, given by the bacteria, may be the reason for such superiority in plants height. According to Bashan and Bashan (2010) and Vacheron et al. (2013) these hormones change the plants metabolism and morphology, leading to better mineral and water absorption, consequently higher plants. Kappes et al. (2013), researching inoculation, applying nitrogen foliar and in topdressing in Cerrado region, obtained increase of 12.6 cm in corn plant height. Puente et al. (2009), working with seeds inoculation with *A. brasilense* (without inoculation, 12 mL kg^{-1} of seeds and 0.41 mL kg^{-1} of seeds in three study locations) in Argentina, observed that at the second location, the plant height with treatment 0.41 mL kg^{-1} of seeds was superior to the ones without inoculation.

It is observed that for the ear insertion height the F test was significant at 5% for the interaction between inoculation and N doses on season corn (Table 3). According to the Table 4 values, it is noticed that when the nitrogen in topdressing was not applied (0 kg ha^{-1}), the presence of inoculation with *A. brasilense* provided superior ear insertion height. The same thing did not happen when N in topdressing was made. This way, it is possible to infer that the inoculation with the bacteria *A.*

Table 3. Average values of plants height, ear insertion height and stalk diameter of season and late season corn according to the *A. brasilense* foliar inoculation and nitrogen applying in topdressing (Selvíria, MS, Brazil, 2011/2012 and 2012/2013).

Treatments		Plants height		Ear Insertion height		Stalk diameter	
		cm		cm		mm	
		Season	Late season	Season	Late season	Season	Late season
Inoculation							
Presence		187.26	199.18[a]	104.34	115.40[a]	25.25	19.52
Absence		189.41	192.11[b]	105.22	112.40[b]	24.52	19.29
M.S.D		4.04	3.81	3.13	2.95	0.98	0.48
N Dose (kg ha^{-1})							
0		188.42	196.25	102.47	112.48	24.26	19.29
30		189.72	198.25	106.10	115.93	24.48	19.49
60		189.80	192.30	105.97	110.43	24.98	19.28
90		185.40	195.78	104.57	116.78	25.82	19.57
F Test	I	1.21	14.89**	0.34	4.46*	2.33	1.00
	D	1.10	1.827	1.23	4.37	2.11	0.39
	I x D	0.57	0.049	3.69*	0.67	1.44	0.74
General Average		188.34	195.64	104.78	113.90	24.89	19.41
V.C. (%)		2.94	2.65	4.10	3.53	5.43	3.36

** and * - meaningful at 1 and 5% of probability, respectively; Averages followed by distinct letter in the columns differ by Turkey test at 5% of probability; DMS - Minimum significant difference; V.C. - variation coefficient.

Table 4. Ear insertion height (cm) on season corn plants, according to *A. brasilense* foliar applying and nitrogen doses in topdressing (Selvíria, MS, Brazil, 2011/2012).

Inoculation	N doses (kg ha^{-1})				Regression equation	R^2
	0	30	60	90		
Presence	106.1[a]	105.5[a]	104.0[a]	101.6[a]	Not meaninful	-
Absence	98.8[b]	106.6[a]	107.9[a]	107.5[a]	$y = 99.04+0.298x-0.002x^2$	0.98*

*, significant at a 5% of probability by F test. Averages followed by distinct letter in the columns differ by Turkey test at 5% of probability.

brasilense supplied, sufficiently, the N demanded by the corn plants. According to Vacheron et al. (2013) plant growth promoting bacteria can fix and provide nitrogen to the plant, promoting growth. From 30 kg ha^{-1} of N may be available in the soil amount of nitrogen in the ammonium form sufficient to promote reduction of the bacteria activity, mainly, may have coincided with the V9 stage of development (elongation stage of stalk). According to Rudnick et al. (1997) and Hartmann (1988) the addition of nitrogen to the soil, especially in ammoniacal form decreases the activity of *A. brasilense* bacterium. Therefore, the presence of ammoniacal nitrogen in the soil at this stage of the plant, could have influenced the bacteria and their activity, interfering in the ear insertion height. According to Ritchie et al. (2003) in the V9 stage the plant stalk is in rapid elongation and one female inflorescence will develop from each of the nodes above the soil surface, except for the last six to eight nodes below tassel. The authors explain that the growth of female inflorescences of lower insertions in the stalk, occasionally stays slower and only one or two female inflorescences in upper position in the plant will develop into productive ears.

Still on Table 4, it is noticed that in inoculation absence, the insertion height adjusted to a quadratic equation with N doses increase in topdressing, which presented maximum point with estimated dose of 76 kg ha^{-1} of N.

On late season, the F test was significant at 5% for the ear insertion height (Table 3). This variable was positively influenced by the inoculation with *A. brasilense*, in a similar way to what happened to plant height, also on late season period. Therefore, we have the same explanation. It is noteworthy that higher plant height and ear insertion height can bring some implications. According to Sousa and Ferreira (2015), Carvalho et al. (2015) and Cabral et al. (2016) corn plants and ear insertion height higher have a greater tendency to tip over and stalk breakage, especially in regions of strong winds. However, in this study it was not observed such implication.

The corn stalk diameter did not respond to any of the

Table 5. Average values of ear length and ear diameter of season and late season corn according to the *A. brasilense* foliar inoculation and nitrogen applying in topdressing (Selvíria, MS, Brazil, 2011/12 and 2012/13).

Treatments		Ear length		Ear diameter	
		mm		mm	
		Sesaon	Late season	Season	Late season
Inoculation					
Presence		178.77	151.57 b	49.50	47.70
Absence		177.49	162.06 a	49.97	47.48
M.S.D		1.70	1.67	0.60	1.09
N Dose (kg ha^{-1})					
0		178.00	153.45	49.44	48.01
30		176.12	157.75	49.25	47.81
60		179.87	157.69	49.97	46.84
90		178.53	158.37	50.29	47.70
	I	2.46	10.72**	2.72	0.17
F Test	D	3.62*	0.50	2.71	0.97
	I x D	4.55**	0.11	1.48	2.96
General Average		178.13	156.80	49.74	47.59
V.C. (%)		1.30	5.78	1.65	3.12

** and *, significant at 1 and 5% of probability, respectively; averages followed by distinct letter in the columns differ by Turkey test at 5% of probability; M.S.D. - Minimum significant difference; V.C., variation coefficient. (1) y = 32.74+0.026x (R^2= 0.60**); (2) y = 26.66+0.025x (R^2= 0.94**); (3)y = 67.44+0.22x-0.0019x² (R^2= 0.98*); (4)y = 54.63+0.0567x (R^2= 0.92**).

Table 6. Ear Length (mm) on season corn according to the foliar appying of *A. brasilense* and nitrogen doses in topdressing (Selvíria, MS, Brazil, 2011/12).

Inoculation	N doses (kg ha^{-1})				Regression Equation	R^2
	0	30	60	90		
Presence	177.62	178.12^a	182.10^a	177.25	y= 177.01+0.14x-0.001x²	0.50*
Absence	178.37	174.12^b	177.65^b	179.82	y= 177.92-0.13x+0.002x^2	0.76**

** and *, significant at 1 and 5% of probability, respectively. Averages followed by distinct letter in the columns differ by Turkey test at 5% of probability.

treatments (Table 3). The same way, Fernandes et al. (2005), Meira et al. (2009), Goes et al. (2014) and Gazola et al. (2014) did not observe stalk diameter response with the increase in N doses. Dotto et al. (2010), Kappes et al. (2013), Souza (2014) and Marini et al. (2015) working with diazotrophic bacteria inoculation, did not observe influence on the corn stalk diameter.

According to the significance of 1% of the F test, it is verified that there was interaction between inoculation and N doses for ear length evaluation on season corn (Table 5).

According to the deployment shown on Table 6, it is noticed that only in doses of 30 and 60 kg ha^{-1} of N the inoculation presence provided superior ear length. Probably, until the dose of 60 kg ha^{-1} of N there was significant reduction of ammoniacal nitrogen in the soil in the course of time, allowing the bacteria resume their activity on the main moment in which the plant determines the ear length (V12). According to Rudnick et al. (1997) and Hartmann (1988) the addition of nitrogen to the soil, especially in the ammoniacal form decreases the activity of *A. brasilense* bacteria. In the course of time, after nitrogen fertilization in topdressing (V6), the ammoniacal nitrogen passes to the nitrate form by the action of Nitrosomonas and Nitrobacter bacteria. Cantarella (2007) states that in soils with aerobic conditions and high temperatures, the ammoniacal nitrogen are oxidized to nitrate form in approximately 15 to 30 days. This process decreases the amount of ammoniacal nitrogen in the soil that would be a limiting factor for the bacterium. Possibly, at this stage there was a reduction of the amount of ammoniacal nitrogen and allowed the *Azospirillum* bacteria resume their activity, resulting in increased ear length.

At the inoculation presence, the ear length adjusted to a quadratic model with N doses increase, showing longer length with the dose of 70 kg ha-1, but at the inoculation absence, there was the opposite, in other words, the ear length adjusted to a quadratic model, however, showing minimum point with the dose of 33 kg ha^{-1}. Dotto et al.

Table 7. Average values of thousand grain weight and grain productivity of season and late season corn according to the *A. brasilense* foliar inoculation and nitrogen applying in topdressing (Selvíria, MS, Brazil, 2011/12 and 2012/13).

Treatments		Thousand grain weight		Grain productivity		
		g		kg ha^{-1}		
		Season	Late season	Season	Late season	Average
Inoculation						
Presence		314.00	319.34	6,751[a]	6,098[a]	6,425[a]
Absence		314.62	311.54	5,883[b]	5,405[b]	5,644[b]
M.S.D		8.53	4.50	573.91	420.17	332.20
N doses (kg ha^{-1})						
0		306.19	316.65	6,273	5,847	6,060
30		312.95	311.59	6,353	5,917	6,135
60		319.44	312.02	6,172	5,558	5,865
90		318.65	321.51	6,470	5,685	6,078
F Test	I	0.02	13.00**	8.07**	11.77**	23.89**
	D	2.20	4.63**	0.17	0.64	0.54
	I x D	0.26	8.26**	0.70	0.31	1.25
General average		314.31	315.44	6.317	5,752	6,034
V.C. (%)		3.72	1.94	13.69	9.94	7.49

** and *, significant at 1 and 5% of probability, respectively. Averages followed by distinct letter in the columns differ by Turkey test at 5% of probability; M.S.D. – Minimum significant difference; VC, variation coefficient.

(2010) report that a higher contribution of inoculation associated to nitrogen fertilization is generally noticed. Nevertheless, there are many papers performed in Brazil in regions with similar climate to the present study (Kappes et al., 2013, Cunha et al., 2014) and regions with different climate (Cavallet et al., 2000; Repke et al., 2013; Marini et al., 2015) showing absence of significant results for the interaction between seed inoculation with *A. brasilense* and N doses for the ear length. According to Basi (2013) it is essential that field experiments be performed to evaluate the effects of inoculation with *A. brasilense* in order to obtain more results of this technology in corn crop in different conditions of year, climate and soil. This information reinforces the importance of the study of other forms of inoculation, since researches with foliar inoculation in different crops are scarce. Morais et al. (2016) reported that the use seed inoculation becomes impractical in the field, because the seeds generally marketed have already been treated with phytosanitary products, and the need to treat seeds again with bacteria is not attractive to farmers.

On late season, the F test showed significance of 1% for the inoculation on the ear length (Table 5). The inoculation presence provided decrease of 6.5% on ear length, in relation to the inoculation absent treatment. Differently, Cavallet et al. (2000) and Kappes et al. (2013), obtained increase of 6 and 3.7%, respectively, on the ear length.

The ear diameter was not influenced by the inoculation and the N doses, as seen by the F test that was not significant (Table 5). Similarly, Cunha et al. (2014) and Marini et al. (2015), using inoculation and Kappes et al. (2013), using inoculation and N doses applying in topdressing, did not find differences from the treatments about the ear diameter of corn.

The thousand grain weight on season was not influenced by the treatments, as verified by non-significant F test. But on late season period, the F test was significant at 1% for the interaction between inoculation and N doses (Table 7). According to the deployment (Table 8), it is noticed that on the doses of 0 and 30 kg ha^{-1} of N the inoculation presence provided increase of 7% and 4% on the thousand grain weight, respectively. Biari et al. (2008) verified increase of 30% on the hundred grain weight, from the inoculation treatment with *A. brasilense* associated to the applying of 50 kg ha^{-1} N in topdressing in comparison to the control treatment.

When the inoculation was performed (Table 8), the thousand grain weight adjusted to a quadratic model, presenting minimum point with the dose of 57 kg ha^{-1} of N. At inoculation absence, the thousand grain weight adjusted to a positive linear model with increase of N doses.

Costa et al. (2015) verified that the thousand grain weight responded in a positive linear way with N doses increase, at the inoculant presence on the seed, foliar and also on the inoculant absence. Rodrigues et al. (2014) observed that the hundred grain weight of corn responded in a negative linear way, with the N doses increase, when the seeds were inoculated with the strain

Table 8. Thousand grain weight (g) on late season corn, according to the foliar applying of *A. brasilense* and nitrogen doses in topdressing (Selvíria, MS, Brazil, 2012/2013).

Inoculation	N doses (kg ha-1)				Regression Equation	R^2
	0	30	60	90		
Presence	327.6[a]	318.0[a]	313.8[a]	318.0[a]	$y = 327.76-0.457x+0.004x^2$	0.99*
Absence	305.7[b]	305.2[b]	310.3[a]	325.0[a]	$y = 302.08+0.2104x$	0.77**

** and *, significant at 1 and 5% of probability, respectively. Averages followed by distinct letter in the columns differ by Turkey test at 5% of probability.

AbV_5 of Azospirillum.

In relation to the corn productivity, the F test was significant at 1% for inoculation in both crops and in the average (Table 7). The presence of foliar inoculation with *A. brasilense* provided higher values, whereas the average of the two periods of cultivation, there was increase of 14% in productivity. Several authors reported increase in corn grain productivity with the use of inoculation in seeds with *A. brasilense*. Cavallet et al. (2000) reported increase of 17% in productivity, Hungria et al. (2010) 27%, Bartchechen et al. (2010) 15%, Lana et al. (2012) 11%, Braccini et al. (2012) 26%, Kappes et al. (2013) 9%, Souza (2014) 6% and Mazzuchelli et al. (2014) reported increase of 22% in productivity. Works that evaluate foliar inoculation with *A. brasilense* in corn crops are scarce. Costa et al. (2015), working with inoculation in the seed, foliar (stage V4) and control, associated to foliar applying of N doses (0, 25, 50, 75 and 100%, with the dose of 100% corresponding to 50 kg ha^{-1} of N), verified that there was increase of 36% in corn grains productivity when the seed inoculation was made in relation to the control. The authors also verified increase of 22% in productivity with foliar applying without foliar N applying. According to Okon and Labandera-Gonzalez (1994) the productivity increase in response to the inoculation is usually about 5 to 30%.

In general, it is noticed that the crops were little responsive to the doses of N. This can be attributed to the fact that corn was sown under soybean residues, which showed average yield of 3,600 kg ha^{-1}. According to Duarte et al. (2013) the nitrogen present in soybean residues can be used by the late season corn. The authors estimate that for the corn grown in succession, are availed about 15 kg of N for each ton of soybean, or 54 kg ha^{-1} of N when produces 3.6 ton ha^{-1} of soybean.

When observing the previous assessments, it is noticed that for corn grown on season, what probably influenced the higher productivity in the presence of inoculation was the final plant population, expressing the higher potential to overcome the productivity of the treatment without inoculation. According to Von Pinho et al. (2008), there is linear relation between grain productivity and plants density. The authors found that for each increase of 1,000 plants ha^{-1} in plant population, there was increase in grains productivity.

For the late season corn, the increase on plant height and the thousand weight grains with the treatment in inoculation presence, influenced to culminate in higher productivity. Higher plants tend to be more productive because suffer less stress during the development and accumulate greater amounts of reservation in the stalk (Silva et al., 2006). According to Braccini et al. (2012) the corn inoculation with *A. brasilense* promotes increase in plant height and in corn grains productivity, when compared to the control group.

Conclusions

The foliar inoculation with the *A. brasilense* bacterium proved to be advantageous for the corn crop, and therefore, an option for the farmer. Because of the large volume of products used in seed (fungicide, insecticide, plant growth regulators) and the lack of information on the degree of interference of these products on the bacteria, it becomes pertinent the study of other inoculation forms on crops. Other studies that verify the influence of *A. brasilense* bacteria in foliar application on the physiology of the shoot and root of crops should be encouraged.

Conflict of Interests

The authors have not declared any conflict of interests.

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In vitro susceptibility of *Corynespora cassiicola* isolate from Brazil fields to fungicide

Wheverton Castro Cabral, Hercules Diniz Campos, Lilian S. Abreu S. Costa* and Gustavo André Simon

Laboratory of Nematology, Department of Plant Pathology - University of Rio Verde (UniRV), Góias, Brazil.

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Corynespora cassiicola **which cause the target spot in soybeans can lead to significant reductions in grain yield. Chemical control mechanisms recommended for disease control was performed with low efficacy in the field due to loss of the pathogen sensitivity to fungicides. This study evaluated the effect of fungicides in inhibiting *C. cassiicola* using *in vitro* test. Four isolates from different regions of Rio Verde - GO were used. The experimental design was completely randomized with nine treatments and five doses (0.0, 0.1, 1.0, 10 and 100 mg). The fungicides, in the various concentrations, were added in PDA medium and poured into Petri dishes, 80 mm in diameter. Then 5 mm discs, containing fungy mycelia, were transferred to the center of the plate and incubated in growth chamber at 25°C with photoperiod of 12 h. The mycelial growth in colony diameter was measured every 24 h. The inhibition percentage of each fungicide on various isolates of fungi was determined, by observing area under the curve of mycelial progress (AUCMP) and by determining the mycelial growth speed rate (MGSR) was determined. All treatments showed a decrease in SRMG with increased applied dose, the fungicide fluazinam had the best performance, with 100% mycelial growth inhibition at all dose tested and in both areas in which the isolate was obtained. The choice of product and dose to be applied directly will be helpful in the chemical control programs ensuring higher yields at the end of the crop cycle.**

Key words: Target spot, *Corynespora cassiicola*, *in vitro,* test.

INTRODUCTION

The fungus, *Corynespora cassiicola* (Berk. & MA Curtis) CT Wei, causal agent of the target spot, is associated with wide range of host species (Silva et al., 1995). In Brazil, the target spot has existed in soybeans since 1976 (Almeida et al., 1976), and as a result of higher susceptible seeding, its incidence has increased in recent seasons, being found in almost all soybean production regions in Brazil (Godoy et al., 2012). In soybean, the losses in yield is up to 20 - 50% (Silva et al., 2008). Control strategies recommended for the disease is the use of resistant cultivars, seed treatment, the rotation/succession of culture with corn and grass species and chemical control (Almeida et al., 2005; Silva et al., 2008).

Fungicides described for complex late season diseases (CLSD) are the same recommended for target spot

*Corresponding author. E-mail: lilianufla@yahoo.com.br.

Table 1. Fungicides and doses used in the experiment to evaluate the sensitivity of *Corynespora cassicola* isolates.

Fungicides (active ingredient)	Concentration gi.a. L^{-1} ou Kg^{-1}	Chemical group
Picoxystrobin + Cyproconazole	200 + 80	Strobilurin + Triazol
Pyraclostrobin + Epoxiconazole	133 + 50	Strobilurin + Triazol
Azoxystrobin + Cyproconazole	200 + 80	Strobilurin + Triazol
Pyraclostrobin + Fluxapyroxad	333 + 167	Strobilurin + Carboxamide
Trifloxystrobin + Prothioconazole	150 + 175	Strobilurin + Triazolinthione
Procymidone	500	Dicaboximida
Fluazinam	500	Fenilpiridinilamina
Carbendazim	500	Benzimidazol
Methyl thiophanate	500	Thiophanate
Control treatment (without fungicide)	--	---

control in the shoot of soybean culture, being: azoxystrobin, azoxystrobin + cyproconazole, carbendazim, difenoconazole, flutriafol, pyraclostrobin + epoxiconazole, tebuconazole, methyl thiophanate, methyl thiophanate + flutriafol, trifloxystrobin + cyproconazole, trifloxystrobin + propiconazole (Embrapa, 2007). However, there are concerns about chemical control options, as fungicides from benzimidazole, triazole and strobilurin groups recommended for the control of this disease have presented low efficacy in the field (Godoy et al., 2012).

After these reports on the difficulty in the chemical control of the disease in recent harvests in the Midwest region of Brasil, some studies have shown a variability between populations of *C. cassiicola* and consequently the reduction or loss of the pathogen sensitivity to fungicides (Avozani et al., 2014; Teramoto et al., 2012; Soares et al., 2012). This response has occurred when successive applications of the same product are done in association with improper application conditions (eradicant applications, subdoses and inadequate technology) (Reis et al., 2010).

However, studies that characterize isolates from different regions are scarce and little is known about the variability of the same, making it an obstacle for genetic improvement programs and also to evaluate the efficacy of chemical control due to possible variability of these pathogens.

Considering the difficulties in the control strategy of fungi that cause the target spot and the need for studies on sensitive populations to fungicides, this study aimed at evaluating the sensitivity of *C. cassiicola* isolated from experimental areas of the city of Rio Verde.

MATERIALS AND METHODS

Assay

The experiment was carried out in Phytopathology Laboratory at the University of Rio Verde – UniRV – 2014/2015. The experimental design was a completely randomization with six replicates, using nine fungicides in four doses of active ingredient (AI) [100 ppm (20 mg), 10 ppm (2 mg), 1 ppm (0.2 mg) and 0.1 ppm (0.02 mg)] obtained from the stock solution (Table 1). Four isolates of *C. cassiicola* from different locations in the city of Rio Verde, where field trials (efficacy test to products) had already been done were used in sensitivity tests (Table 2). For each treatment, a control was added without fungicides application.

Isolation and *in vitro* test

For isolation, the trefoils of three plants were selected in the plots with disease symptoms. The material was taken to the Phytopathology Laboratory and the fragments plant tissues were disinfected in a solution of sodium hypochlorite 1% by three minutes. Later, the fragments were washed with distilled water to remove excess. Then, the peace of fragments were distributed into acrylic boxes gerbox (11 x 11 x 3.5 cm) containing a nylon foam and two overlapping sheets of filter paper, moistened with sterile distilled water and kept in chamber growth at 25°C ± 2:12 photoperiod. After fungal growth, the colonies visually recognised were transferred to another dish containing medium of potato dextrose agar (PDA), later kept in chamber growth at 25ºC ± 2:12 photoperiod.

For the *in vitro* tests, the different doses of fungicides were prepared by dissolving the commercial fungicide formulation in sterile deionized water (SDW) until use. They were then further diluted to obtain the desired concentration and poured into plastic Petri dishes (80 mm diameter) and added at the time of PDA culture medium preparation and after been poured into Petri dishes of 80 mm.

The day after culture medium preparation, 6 mm-diameter mycelial plugs of each isolate, taken from seven-day-old colonies, were placed on the center of each dish. The plates were sealed with PVC plastic film and incubated in a growth chamber at 25 ± 2°C and 12 h photoperiod provided by three fluorescent 40 W lamps placed at 50 cm above the plates. When the colony in the control treatment reached the edge of the plates, the diameter of all colonies was measured with a digital calliper as described by Avozani et al. (2014).

Evaluations

The first evaluation took place after 48 h of the experiment. The diameter of each colony was measured in two directions

Table 2. Sites of *Corynespora cassicola isolates*, in the Rio Verde city, used in the *in vitro* sensibility tests.

Characterisation	Sites	Altitude (m)	Coordinates
Isolated A	Agricultural Research Center– CPA	731	S: 17°47'05.0" O: 50°59'47.0"
Isolated B	Rio Doce Farm	751	S: 17°36'10.0" O: 51°32'54.0"
Isolated C	Laje Farm	712	S: 17°40'23.0" O: 50°49'46.0"
Isolated D	São Tomaz Rio do Peixe Farm	689	S: 18°02'30.0" O: 51°02'19.0"

(represented the total growth percentage), at 48 h intervals from the time of inoculation up to the end of the experiment. After measurements, the percentages of inhibition in fungal growth were determined in each treatment, calculating the mycelial growth speed rate (MGSR), used to calculate the inhibition of mycelial growth. This performed MGSR was calculated based on equation MGSR= Σ[(D-Da)/N] (Dias et al., 2005). Where: D = current average diameter of the colony; Da = the average diameter of the colony in the previous day; N = number of hours or days after inoculation.

A completely randomized experimental design using four replicates was adopted. A Petri dish was used as an experimental unit. Data on fungal colony diameter were transformed into growth percentage. The inhibitory concentration (IC_{50}) able to inhibit 50% of mycelial growth for evaluated fungicides and each isolate was calculated from the generated equation.

Classification of isolates based on fungicides sensitivity used was performed according to the criteria proposed by Edginton et al. (1971), in which chemical compounds with IC_{50} less than 1 mg/L was considered highly fungitoxic, with IC_{50} between 1 and 50 mg/L are moderately fungitoxic and IC_{50} higher than 50 mg/L are not fungitoxic. A useful tool to quantify the shift in sensitivity to a fungicide in a fungus is the sensitivity reduction factor (SRF) (Kunz et al., 1998), which is calculated by dividing the IC50 of the fungal strain suspected of having reduced/lost its sensitivity by the IC50 of the sensitive strain. SRF value of 1 means no change in sensitivity, while values > 1 indicate the shift for sensitivity reduction (Reis et al., 2010; Russel, 2004).

Data analysis

All the assays were repeated twice using a completely randomised experimental design with four replicates per treatment. Data were subjected to Shapiro-Wilkand Bartlett tests (significance level, P>0.05) for normality and homoscedasticity, respectively. Distribution of isolates (% inhibition colonisation) was subjected to one-way ANOVA, and means were compared using Scott-Knott tests (P< 0.05) (Scott and Knott, 1974). The regression model was fit to the quantitative variables as log transformation using the Sigma Plot 11.0 program.

RESULTS

Evaluation of mycelial growth speed rate (MGSR) of *C. cassicola* isolates by different fungicides doses

After the calculation of the MGSR and in accordance with the regression analysis for each variable, it was observed that generally all fungicides produced decrease in growth of fungal mycelia with increasing dose. However, the picoxystrobin + cyproconazole treatments (Figure 1), pyraclostrobin + epoxyconazole (Figure 2), azoxystrobin + cyproconazole (Figure 3), pyraclostrobin + epoxiconazole + fluxapyroxad (Figure 4) and procymidone (Figure 5) showed significant reduction ($p < 0.05$) in mycelial growth with increased rates of fungicides for all isolates.

For the treatment containing the fungicide trifloxystrobin + prothioconazole (Figure 6) according to regression analysis to MGSR, the isolates from CPA, Rio Doce Farm and São Tomaz Rio do Peixe Farm showed a significant reduction in mycelial growth of *C. cassicola*. However, for the isolate from Laje farm, there was no dose effect in reducing growth in the studied treatment.

Treatment containing the fungicide carbendazim (Figure 7) and methyl thiophanate (Figure 8) showed a significant reduction in mycelial growth of *C. cassicola* in isolates from CPA, Laje Farm and São Tomaz Rio do Peixe Farm observed by regression analysis of MGSR. However, isolates from Rio Doce Farm showed no dose effect in reducing the growth.

Inhibition percentage of *C. cassicola* isolate by different doses of fungicides

In the inhibition evaluations, a partial or total inhibition of *C. cassicola* was observed. The control (0.0 mg) had mycelial growth of 100% in all evaluated replications. For isolates from CPA, there was 100% of inhibition at doses of 100 mg in all the treatments, except for treatment with methyl thiophanate where inhibition was 68.54% (Table 3). The treatment containing trifloxystrobin + prothioconazole inhibited 100% of the growth of *C. cassicola* at doses of 10 and 100 mg from São Tomaz Rio do Peixe Farm. On the same property, it was noted that the pyraclostrobin + epoxiconazole + fluxapyroxad, trifloxystrobin + prothioconazole, procymidone, fluazinam and carbendazim treatments, inhibited 100% of mycelial growth of *C. cassicola* in doses of 10 and 100 mg, with a

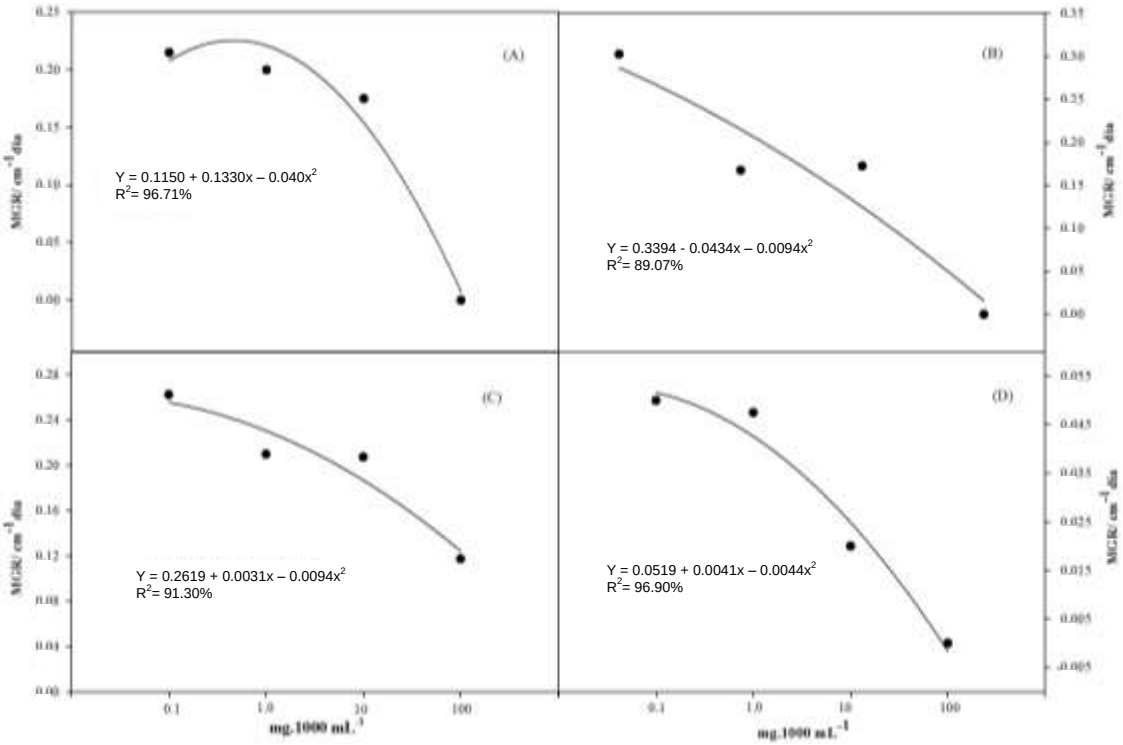

Figure 1. Mycelial growth rate (MGR - cm/day) of isolates: A (São Tomaz); B (CPA); C (Rio Doce Farm); D (Laje Farm) after treatment with fungicide picoxystrobin + cyproconazole, for used doses.

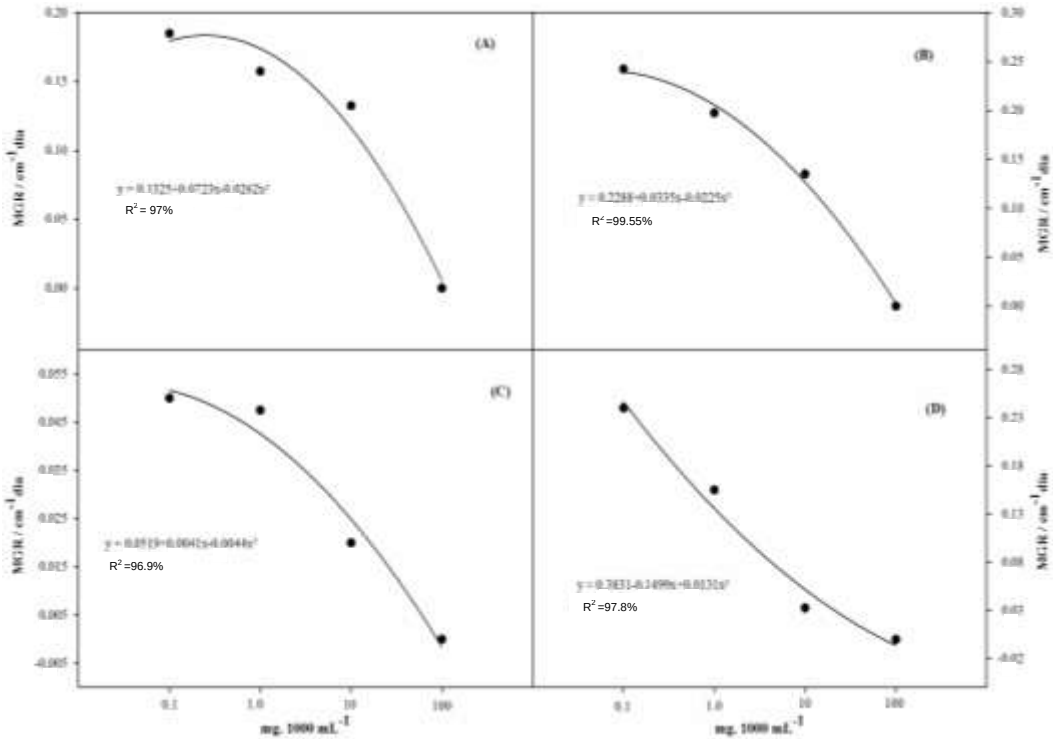

Figure 2. Mycelial growth rate (MGR - cm/day) of isolates: A (CPA); B (Rio Doce Farm); C (Laje Farm); D (São Tomaz Farm) after treatment with fungicide pyraclostrobin + epoxyconazole, in function of used doses.

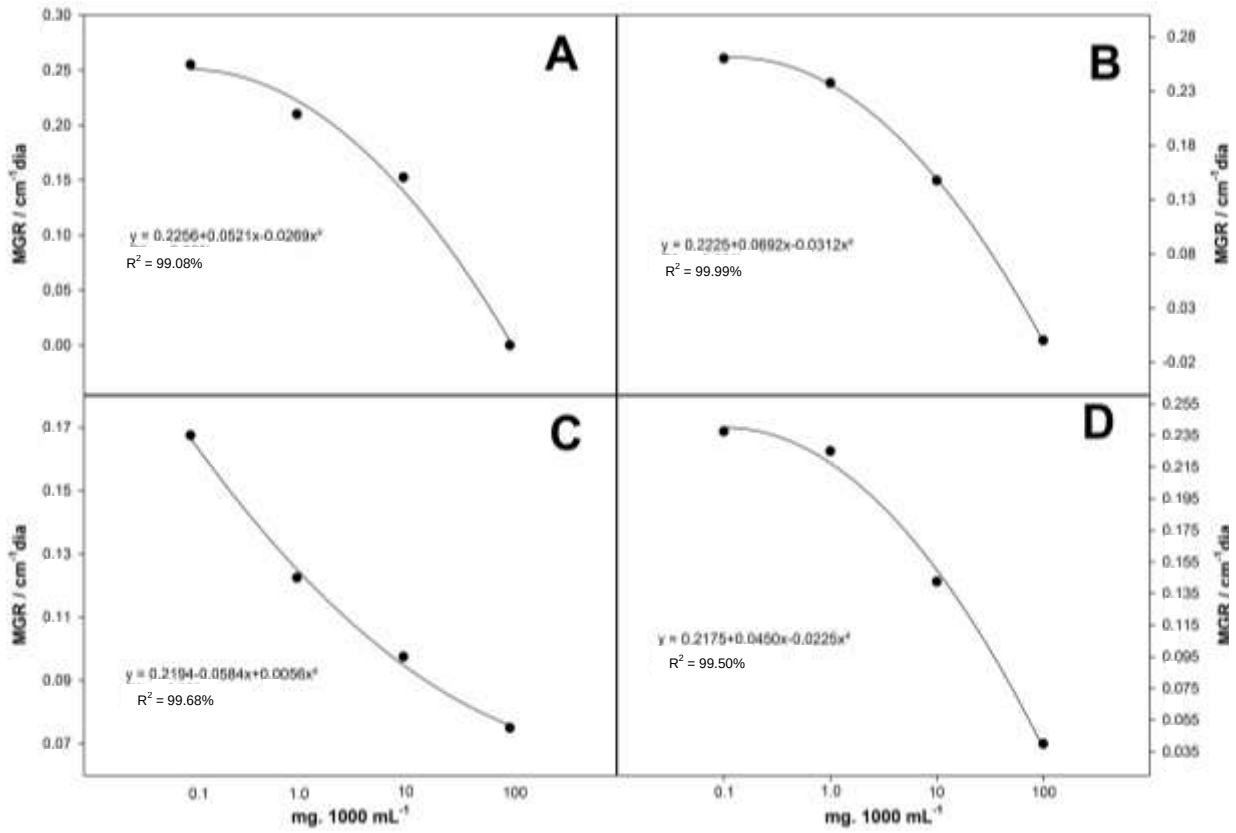

Figure 3. Mycelial growth rate (MGR - cm/day) of isolates: A (CPA); B (Rio Doce Farm); C (Laje Farm); D (São Tomaz Farm) after treatment with the fungicide azoxystrobin + cyproconazole, in function of used doses.

significant difference when compared with the doses of 1.0 and 0.1 mg. For azoxystrobin + cyproconazole treatments and methyl thiophanate in the dose of 100 mg, 79.22 and 55.52% of inhibition respectively were observed, which were lower percentages than other treatments that reached 100% of inhibition when 100 mg of active ingredient was used (Table 3).

In assessing the isolates from Rio Doce Farm, treatments that stood out with 100% of mycelial growth inhibition of *C. cassicola* were fluazinam and methyl thiophanate in four doses: 0.1, 1.0, 10 and 100 mg. On the other hand, picoxystrobin + cyproconazole and procymidone treatments achieved the maximum inhibition of 66.72 and 80.97%, respectively (Table 3). The treatment containing fluazinam had the best result in the mycelial growth inhibition, similar in the four doses (0.1, 1.0, 10 and 100 mg), inhibiting 100% of the growth in all isolates of the study areas (Table 3).

Evaluation of the inhibitory concentration of the *C. cassicola* isolates

Low concentrations of the fungicide picoxystrobin + cyproconazole reduced growth of isolates from CPA and Sao Tomaz farm at IC_{50} fungal growth, showing significant difference considering the other isolates. The IC_{50} for picoxystrobin + cyproconazole fungicide for isolate from Rio Doce and Laje Farms was not significant.

Treatment with pyraclostrobin + epoxiconazole in both study areas with their isolates was significant at the level of 0.01%, so the IC_{50} had significant effect on this active ingredient, being classified as highly fungitoxic (Table 4). In most cases, azoxystrobin + cyproconazole when compared with the other treatments applied in the Laje Farm, showed no significant effect. In the other areas of study, with the exception of Laje Farm, the IC_{50} demonstrated that azoxystrobin + cyproconazole has fungicidal action. Pyraclostrobin + epoxiconazole + fluxapyroxad had similar effect on other treatments for its areas, where the IC_{50} indicated high fungitoxic action of the active ingredient used.

The trifloxystrobin + prothioconazole treatment showed significant effect on the four study areas, where the IC_{50} showed high fungicidal activity of its active ingredient. Procymidone (Table 4) also showed IC_{50} with high fungicidal action in all the studied areas. Treatment with fluazinam at IC_{50} showed no significant effects. The carbendazim treatment showed no significant difference for the isolates from Rio Doce and Laje Farms.

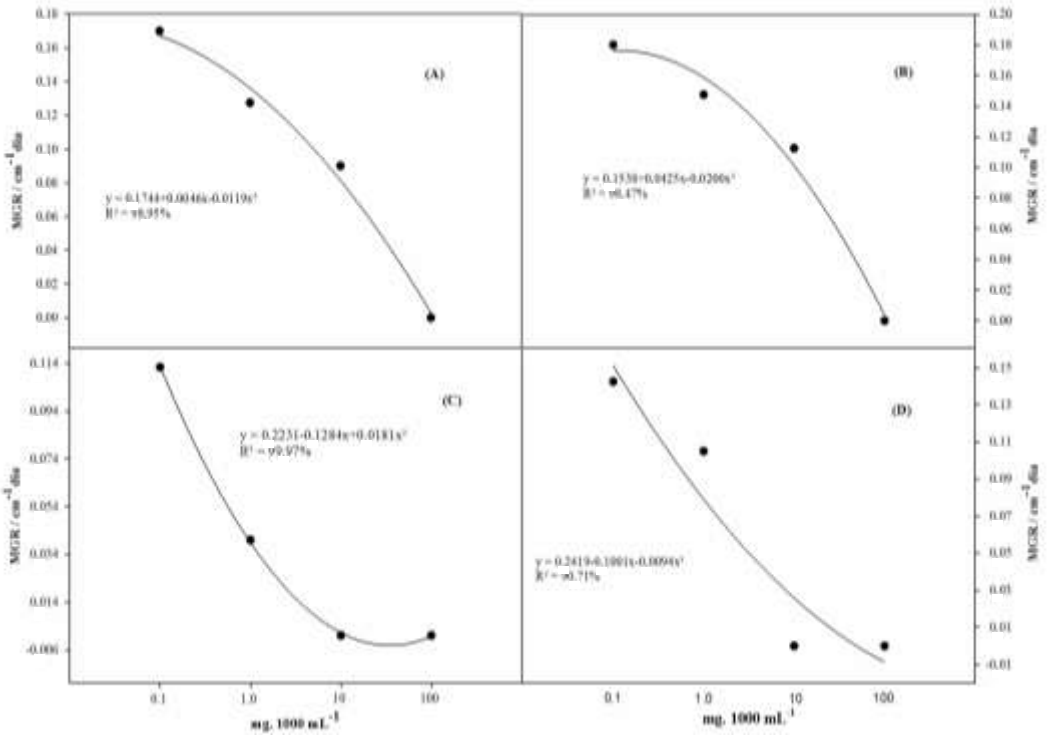

Figure 4. Mycelial growth rate (MGR- cm/day) of isolates: A (CPA); B (Rio Doce Farm); C (Laje Farm); D (São Tomaz Farm) after treatment with the fungicide fluxapyroxad + pyraclostrobin + epoxyconazole, in function of the used doses.

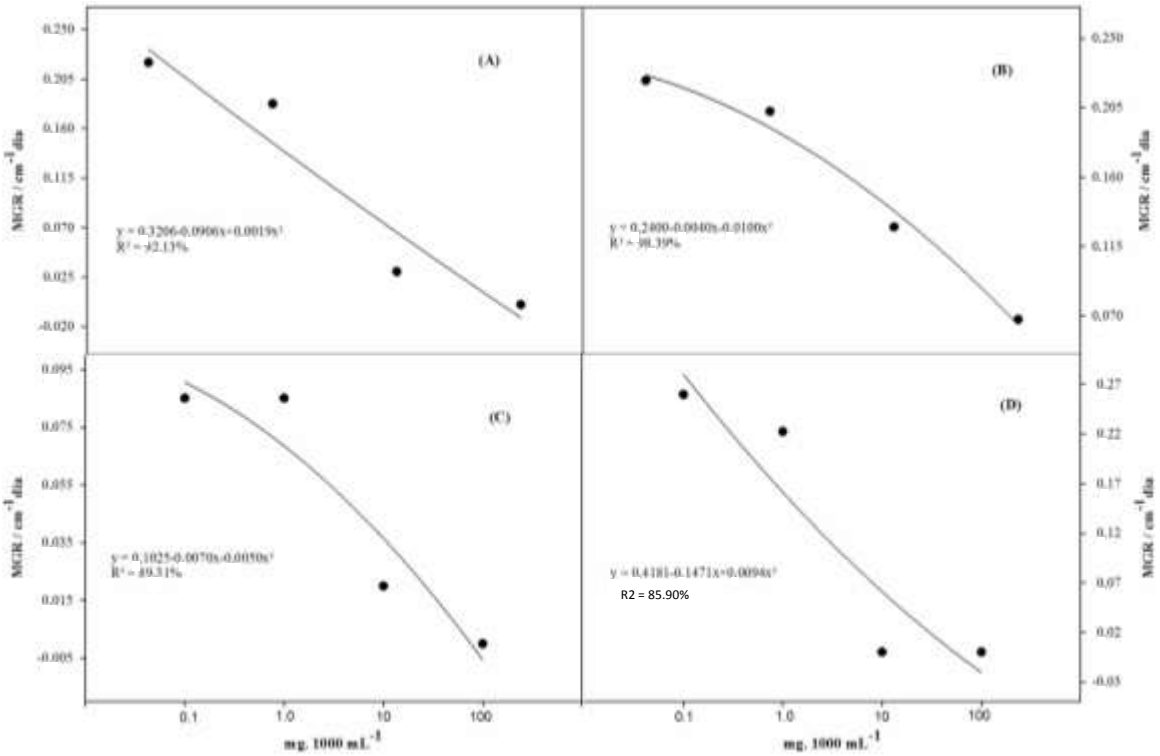

Figure 5. Mycelial growth rate (MGR- cm/day) of isolates: A (CPA); B (Rio Doce Farm); C (Laje Farm); D (São Tomaz Farm) after treatment with the fungicideprocymidone, in the function of used doses.

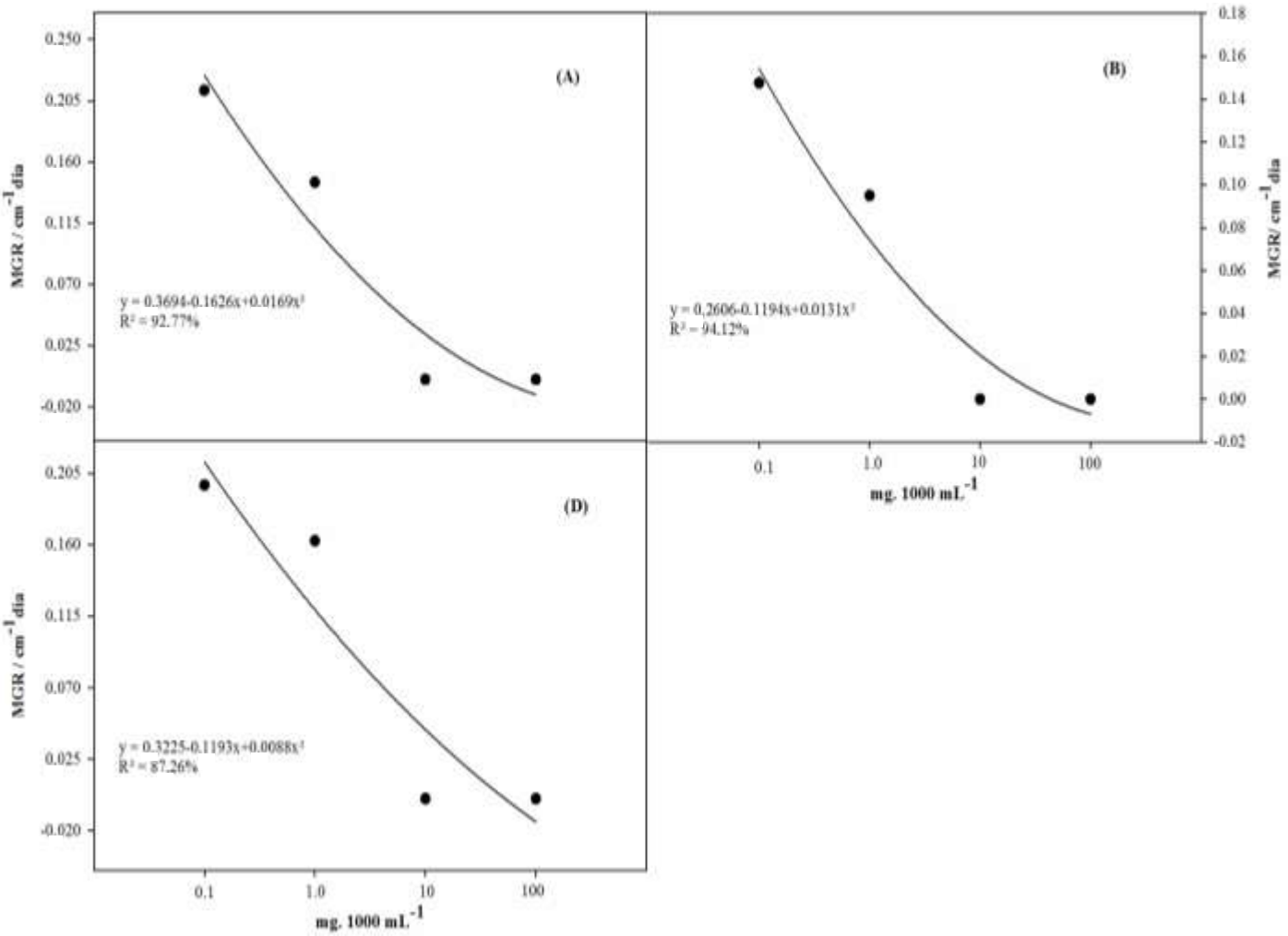

Figure 6. Mycelial growth rate (MGR- cm/day) of isolates: A (CPA); B (Rio Doce Farm); and D (São Tomaz Farm) after treatment with the fungicide trifloxystrobin + prothioconazole, in the function of used doses.

Methyl thiophanate demonstrated significance level of 0.01% for isolates from CPA and Laje Farm, as for isolates from Rio Doce and São Tomaz farms, there was no significant difference.

The fungicide pyraclostrobin + epoxiconazole was highly toxic for isolates from CPA and Laje Farm, however, to isolates from São Tomaz and Rio Doce farms it was moderately toxic. The fungicide azoxystrobin + cyproconazole was highly toxic to isolates from CPA and moderately toxic for isolated from São Tomaz and Rio Doce farms. However, to isolate from Laje Farm, the cyproconazole + azoxystrobin fungicide was not toxic. The pyraclostrobin + epoxiconazole + fluxapyroxad fungicide was highly toxic for all isolates tested. The trifloxystrobin + prothioconazole fungicide was moderately toxic for isolates from São Tomaz Farm and, the other isolates were highly fungitoxic. Procymidone was moderately toxic to isolate from São Tomaz Farmand to the others, it was highly toxic.

Fluazinam was highly toxic to all isolates used. Carbendazim was also highly toxic to isolates from CPA and São Tomaz Farm. For the isolate from Laje Farm, the fungicide carbendazim was moderately toxic and showed no antifungal effect on isolate from Rio Doce Farm. For the fungicide, methyl thiophanate was slightly toxic to isolates from CPA and Laje Farm, were slightly toxic and showed no fungitoxicity for all other isolates.

DISCUSSION

The differences in the behavior of the isolates from different areas indicate the possible change of genetic variability of these isolates causing low sensitivity to

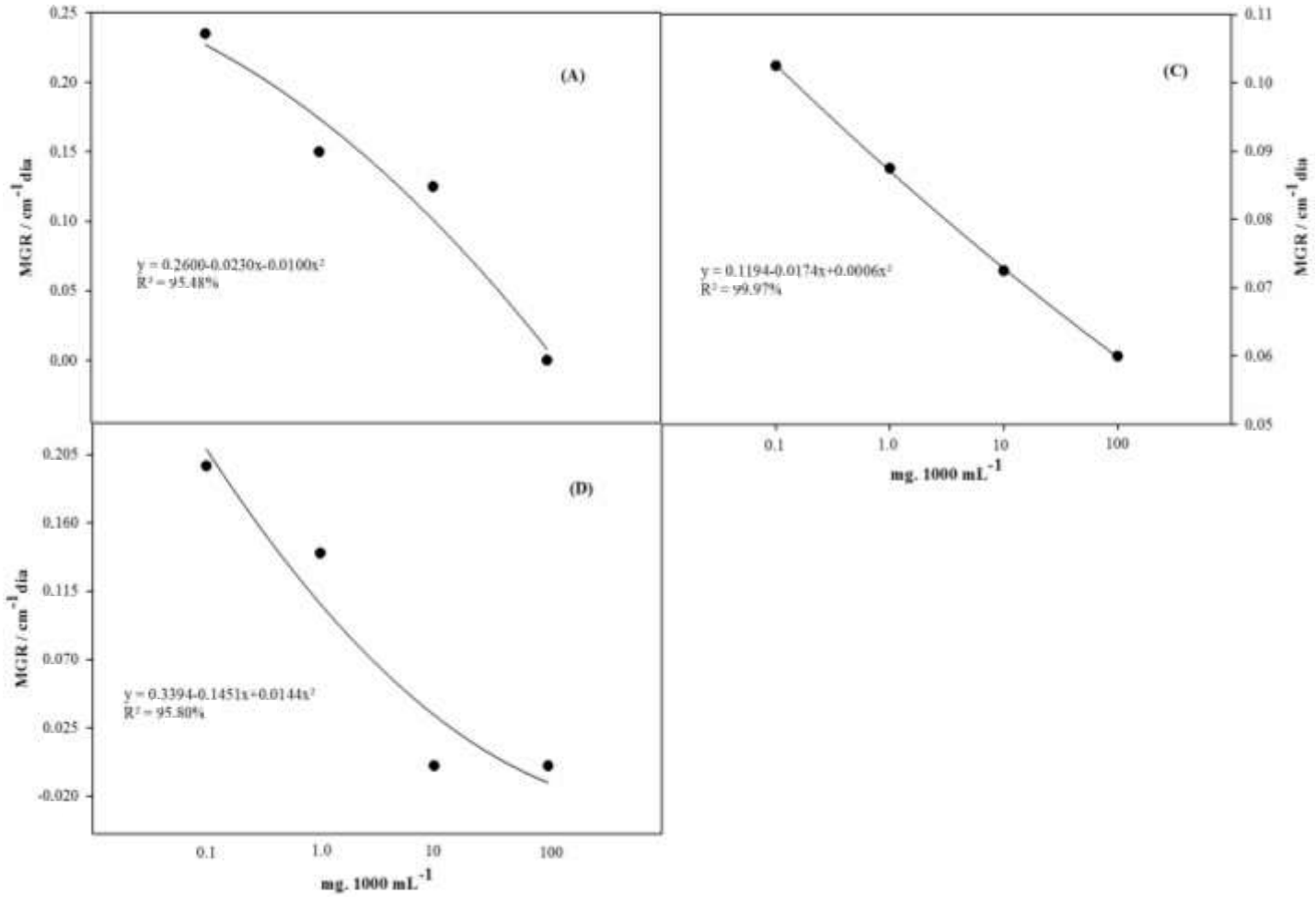

Figure 7. Mycelial growth rate (MGR - cm/day) of isolates: A (CPA); C (Laje Farm) and D (São Tomaz Farm), after treatment with the fungicide carbendazim, in the function of used doses.

fungicides. It is known that the abuse of systemic molecules to control pathogens causes reduction in the sensitivity to products (Reis et al., 2010). In some studies, the fungicide carbendazim had low efficiency in controlling the target spot, which could have been as a result of the resistance to this chemical group on the pathogen (Teramoto et al., 2013; Avozani et al., 2014). However, in this work, carbendazim fungicide did not appear to be inefficient in its toxicity. On the other hand, there was a highlight for methyl thiophanate considering its percentage inhibition of mycelial growth, which was less efficient in almost all locations.

The fungicides belonging to the chemical group of benzimidazoles act on fungi by inhibiting α and β tubulin specific proteins (Coutinho et al., 2006). The affinity of benzimidazole with tubulin is the main factor that determines the fungicidal activity in organisms. The higher the affinity, the more sensitive the organism to the fungicide.

Probably due to various selection factors, a mutation occurred to β-tubulin protein gene leading to formation of β-tubulin protein that has reduced binding affinity with benzimidazole leading to a new generation of resistant population (Brent, 1995; Hewitt, 1998). Therefore, the high selection pressure caused by intensive use of fungicides such as benzimidazoles, may result in the selection of resistant fungus at a short period of time (Parreira et al., 2009), explaining the difference of inhibition displayed by methyl thiophanate.

According to Deising et al. (2008), the resistance acquired by the pathogen population to the product is directly proportional to applied doses, frequency of application, degree of coverage, persistence in culture or in soil and the size of the treated area. This justifies the lower results observed for azoxystrobin + cyproconazole treatment at the highest dose when compared with the other treatments (Table 3). In the evaluations performed in this research, the product Fluazinam showed 100% efficient in the percentage inhibition of mycelial growth in all doses and in both areas in which they were obtained.

In previous work carried out by Töfoli et al. (2003), the fungicide fluazinam was also responsible for higher levels

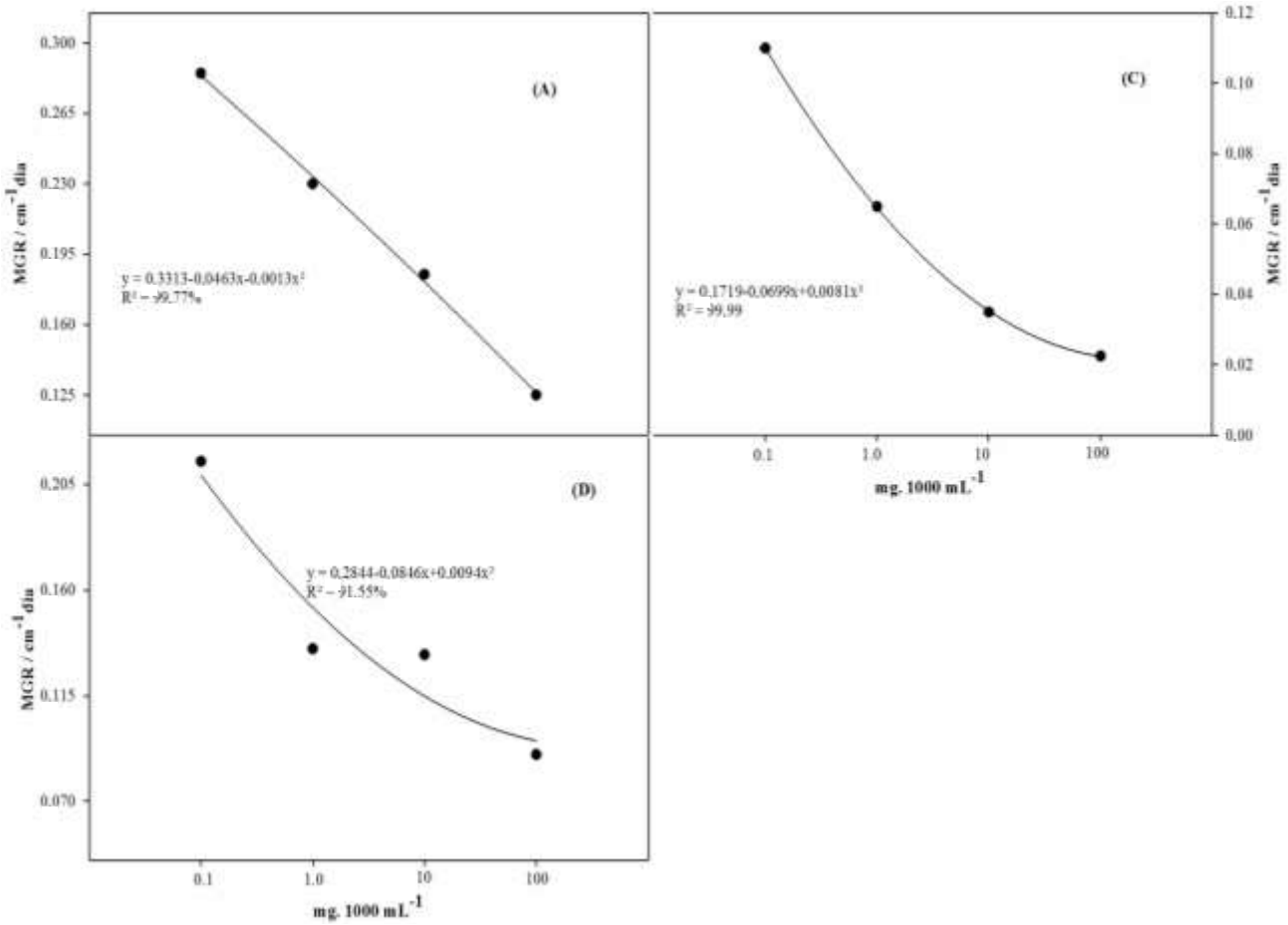

Figure 8. Mycelial growth rate (MGR-cm/day) of isolates: A (CPA); C (Laje Farm) and D (São Tomaz Farm), for the treatment containing the fungicide thiophanate methyl, in the function of used doses.

of mycelial growth inhibition in isolates of *Alternaria solani* and noted that the action of this fungicide showed complete inhibition of spore germination of *A. solanis* from doses of 1 µg.mL^{-1}. In other studies using the same product, fluazinam, Guimarães et al. (2008) found efficiency in the control of *Monosporascus cannonballus* at different doses.

The inhibitory concentration (IC_{50}) studies for different fungicides and specific to *C. cassiicola* in soybean are scarce, yet it is very useful in carrying out research and sensitivity monitoring, especially in areas where the control of this disease is not being efficient (Avozani et al., 2014). The isolates from the Laje Farm treated with azoxystrobin + cyproconazole showed no significant effect on the IC_{50} values but had high IC_{50} value.

This effect may be due to high-pressure selectivity for this area specifically, or the inappropriate use of the product in the past situations, which may, according to the obtained data, have selected individuals resistant to the products. The high value of IC_{50} (Table 4) clearly shows that this area of study (Laje Farm), the respective active component has low fungicide action. Since treatment with fluazinam at the IC_{50} showed no significant results, however, was highly fungitoxic for all used isolates. In this study, thecarbendazim treatment showed no significant difference for the isolate from Rio Doce and Laje farms, and for the Laje Farm, according to the IC_{50}, this fungicide can be classified as moderately fungitoxic, according to the criteria proposed by Edgington et al. (1971). Furthermore, Avozani et al. (2014) found that the isolates of *C. cassiicola* showed less sensitivity to the carbendazim active ingredient and the cyproconazole active ingredient presented best valore of IC50. In studying the sensitivity of isolates submitted to the treatments, it was noted that the increased resistance of the isolates from the Laje Farm for some treatments, should necessitate the investigation of the previous management methods in this region that could have contribute to the multiplication of resistant populations.

Conclusion

Generally, fungicides used showed good control levels

Table 3. Inhibition percentage of *Corynespora cassicola* from Rio Verde towns, after different fungicides doses.

Active ingredient	Sampling places	Inhibition (%)				CV
		0.1 mg	1.0 mg	10 mg	100 mg	
Picoxistrobina + Ciproconazol	CPA	23.72^{c}	58.05^{b}	57.32^{b}	100.00^{a}	9.04
Piraclostrobina + Epoxiconazol		60.77^{b}	53.09^{c}	66.50^{b}	100.00^{a}	
Azoxistrobina + Ciproconazol		36.09^{d}	46.78^{c}	61.58^{b}	100.00^{a}	
Piraclostrobina + Epoxiconazol + Fluxapyroxad		58.00^{b}	67.44^{c}	77.20^{b}	100.00^{a}	
Trifloxistrobina + Protioconazol		46.08^{c}	62.89^{b}	100.00^{a}	100.00^{a}	
Procimidona		44.73^{d}	54.54^{c}	92.55^{b}	100.00^{a}	
Fluazinam		100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	
Carbendazim		40.61^{c}	62.90^{b}	67.71^{b}	100.00^{a}	
Tiofanato Metílico		28.61^{b}	41.25^{c}	53.49^{b}	68.54^{a}	
Picoxistrobina + Ciproconazol	Fazenda Laje	8.77^{c}	15.66^{b}	25.37^{b}	100.00^{a}	20.08
Piraclostrobina + Epoxiconazol		16.84^{d}	33.33^{c}	79.78^{b}	100.00^{a}	
Azoxistrobina + Ciproconazol		14.44^{c}	19.33^{c}	40.34^{b}	79.21^{a}	
Piraclostrobina + Epoxiconazol + Fluxapyroxad		43.69^{b}	45.66^{b}	100.00^{a}	100.00^{a}	
Trifloxistrobina + Protioconazol		31.37^{b}	37.38^{b}	100.00^{a}	100.00^{a}	
Procimidona		10.13^{c}	21.70^{b}	100.00^{a}	100.00^{a}	
Fluazinam		100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	
Carbendazim		25.31^{c}	45.73^{b}	100.00^{a}	100.00^{a}	
Tiofanato Metílico		24.57^{c}	33.39^{b}	46.97^{a}	55.52^{a}	
Picoxistrobina + Ciproconazol	São Tomaz Rio do Peixe	8.78^{c}	15.66^{b}	23.37^{b}	100.00^{a}	20.08
Piraclostrobina + Epoxiconazol		16.84^{d}	33.33^{c}	79.78^{b}	100.00^{a}	
Azoxistrobina + Ciproconazol		14.45^{c}	19.33^{c}	40.34^{b}	79.22^{a}	
Piraclostrobina + Epoxiconazol + Fluxapyroxad		43.70^{b}	45.66^{b}	100.00^{a}	100.00^{a}	
Trifloxistrobina + Protioconazol		31.38^{b}	37.38^{b}	100.00^{a}	100.00^{a}	
Procimidona		10.13^{c}	21.70^{b}	100.00^{a}	100.00^{a}	
Fluazinam		100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	
Carbendazim		25.31^{c}	45.73^{b}	100.00^{a}	100.00^{a}	
Tiofanato Metílico		24.57^{c}	33.39^{b}	46.97^{a}	55.52^{a}	
Picoxistrobina + Ciproconazol	Fazenda Rio Doce	27.13^{c}	41.35^{b}	42.26^{b}	66.72^{a}	8.10
Piraclostrobina + Epoxiconazol		32.75^{d}	45.15^{c}	61.92^{b}	100.00^{a}	
Azoxistrobina + Ciproconazol		27.97^{c}	33.42^{c}	59.08^{b}	100.00^{a}	
Piraclostrobina + Epoxiconazol + Fluxapyroxad		50.57^{d}	59.10^{c}	68.14^{b}	100.00^{a}	
Trifloxistrobina + Protioconazol		59.08^{c}	73.28^{b}	100.00^{a}	100.00^{a}	

Table 3. Cont'd.

Procimidona	37.28[c]	43.48[c]	63.82[b]	80.97[a]
Fluazinam	100.00[a]	100.00[a]	100.00[a]	100.00[a]
Carbendazim	69.03[b]	100.00[a]	100.00[a]	100.00[a]
Tiofanato Metílico	100.00[a]	100.00[a]	100.00[a]	100.00[a]

Analysis by one way ANOVA. Means followed by same letter in the column are not significantly different according to the Scott and Knott's test at 5% probability.

Table 4. Inhibitory concentration at 50% and the sensitivity reduction factor (SRF) from different isolates of *Corynespora cassicola* for fungicides.

Sampling places – product	Equation*	Inhibition (%)			
		R^2	*P*	IC_{50}**	SRF
Picoxistrobina+ciproconazol					
A- CPA	Y= 9.91 Ln(x) + 48.36	88.89	< 0.01	1.18	1.18
B- Fazenda Rio Doce	Y= 5.20 Ln(x) + 38.38	88.37	***n.s.	9.34	9.34
C- Fazenda Laje	Y= 4.71 Ln(x) + 72.20	92.51	***n.s.	0.009	0.009
D- Fazenda São Tomaz	Y= 12.31 Ln(x) + 23.25	74.98	< 0.01	8.78	8.78
Piraclostrobina+epoxiconazol					
A- CPA	Y= 6.36 Ln(x) + 62.76	83.56	< 0.01	0.13	0.13
B- Fazenda Rio Doce	Y= 9.49 Ln(x) + 49.03	93.02	< 0.01	1.11	1.11
C- Fazenda Laje	Y= 8.24 Ln(x) + 72.09	79.90	< 0.01	0.07	0.07
D- Fazenda São Tomaz	Y= 12.31 Ln(x) + 42.69	96.45	<0.01	1.81	1.81
Azoxistrobina+ciproconazol					
A- CPA	Y= 8.97 Ln(x) + 50.78	90.99	< 0.01	0.92	-
B- Fazenda Rio Doce	Y= 10.50 Ln(x) + 49.03	90.25	< 0.01	1.10	-
C- Fazenda Laje	Y= 7.33 Ln(x) + 20.22	99.48	***n.s.	58.13	-
D- Fazenda São Tomaz	Y= 9.35 Ln(x) + 27.57	88.92	< 0.01	11.01	-

distinguishing between places where the experiments were carried out. All treatments caused an increase in productivity as compared to the control treatment. The fluazinam fungicide was better among the other fungicides with 100% of mycelial growth inhibition in all doses and in all areas in which the isolate was obtained so it is considered highly fungitoxic. The low sensitivity of these pathogens to some molecules can guide the development of management strategies reducing the loss in yield and quality of crops around the world.

Table 4. Cont'd.

Piraclostrobina+epoxiconazol+fluxapyroxad					
A- CPA	Y= 5.89 Ln(x) + 68.87	94.59	< 0.01	0.04	0.04
B- Fazenda Rio Doce	Y= 6.83 Ln(x) + 61.59	88.49	< 0.01	0.18	0.18
C- Fazenda Laje	Y= 10.44 Ln(x) + 63.31	85.95	< 0.01	0.28	0.28
D- Fazenda São Tomaz	Y= 9.69 Ln(x) + 61.18	81.38	< 0.01	0.31	0.31
Trifloxistrobina+protioconazol					
A- CPA	Y= 8.64 Ln(x) + 67.30	89.36	< 0.01	0.13	0.13
B- Fazenda Rio Doce	Y= 6.49 Ln(x) + 75.62	89.76	< 0.01	0.02	0.02
C- Fazenda Laje	Y= 6.06 Ln(x) + 81.40	60.00	< 0.01	0.006	0.006
D- Fazenda São Tomaz	Y= 11.66 Ln(x) + 53.76	83.35	< 0.01	1.38	1.38
Procimidona					
A- CPA	Y= 8.85 Ln(x) + 62.77	92.26	< 0.01	0.24	0.24
B- Fazenda Rio Doce	Y= 6.57 Ln(x) + 48.82	96.22	< 0.01	1.97	1.97
C- Fazenda Laje	Y= 10.05 Ln(x) + 54.53	91.69	< 0.01	0.64	0.64
D- Fazenda São Tomaz	Y= 15.11 Ln(x) + 40.56	84.79	< 0.01	1.87	1.87
Carbendazim					
A- CPA	Y= 7.95 Ln(x) + 58.65	93.00	< 0.01	0.34	0.34
B- Fazenda Rio Doce	Y= 100.00	-	***n.s.	-	-
C- Fazenda Laje	Y= 3.22 Ln(x) + 42.46	93.07	***n.s.	10.40	10.40
D- Fazenda São Tomaz	Y= 12.09 Ln(x) + 53.84	88.72	< 0.01	0.73	0.73
Tiofanato metílico					
A- CPA	Y= 5.73 Ln(x) + 41.37	99.78	< 0.01	4.51	4.51
B- Fazenda Rio Doce	Y= 100.00	-	***n.s.	-	-
C- Fazenda Laje	Y= 7.96 Ln(x) + 49.46	97.45	< 0.01	1.07	1.07
D- Fazenda São Tomaz	Y= 3.44 Ln(x) + 36.15	55.01	***n.s.	56.04	56.04

*y = Percentage of mycelial growth inhibition, x = concentration of the fungicide; ** calculated by the equation concentration (mg / L); *** n.s. = non significant.

Conflict of Interests

The authors have not declared any conflict of interests.

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Production and nutritional characteristics of pearl millet and Paiaguas palisadegrass under different forage systems and sowing periods in the offseason

Raoni Ribeiro Guedes Fonseca Costa, Kátia Aparecida de Pinho Costa*, Charles Barbosa Santos, Eduardo da Costa Severiano, Patrícia Soares Epifanio, Jessika Torres da Silva, Daniel Augusto Alves Teixeira and Valdevino Rodrigues da Silva

Federal Institute of Education, Science and Technology Goiano, Rio Verde, Campus (Instituto Federal de Educação, Ciência e Tecnologia Goiano, Campus Rio Verde), Rod. Sul Goiana Km 01, Cx. P. 66. CEP 75.901-970, Rio Verde –GO, Brazil.

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The intercropping of annual crops with perennial grasses is a production system that is frequently adopted in the Midwest region of Brazil due to its economic viability resulting from the use of the same area for agriculture and livestock. Most agriculture-livestock integration studies have evaluated the use of forage of the genus *Urochloa* in intercropped systems with corn, sorghum and sunflower. Consequently, there is a lack of information regarding pearl millet cultivation when grown simultaneously with tropical forages. Thus, the objective of this study was to evaluate the agronomic characteristics of pearl millet *Pennisetum glaucum* (L.) R. Br as well as the production and nutritional characteristics of Paiaguas palisadegrass (*Urochloa brizantha* cv. Paiaguas) under different forage systems and sowing periods in the offseason. The experiment was conducted at the Federal Institute of Goiás, Rio Verde campus. The experimental design was a randomized complete block with a 5 × 2 factorial arrangement and three replications. There were two sowing periods (February and March) and five forage systems: monocropped pearl millet; monocropped Paiaguas palisadegrass; pearl millet intercropped in rows with Paiaguas palisadegrass; pearl millet intercropped between rows of Paiaguas palisadegrass and pearl millet oversown and intercropped with Paiaguas palisadegrass. The results indicated that the Paiaguas palisadegrass did not affect the pearl millet grain yield, indicating that the intercropping of pearl millet and Paiaguas palisadegrass in the offseason is a promising cultivation technique for the production of grains during the offseason in Southeastern Goiás. However, the second sowing period provided better grain yields and a higher number of sacks per hectare. With respect to forage yield, the Paiaguas palisadegrass sown in oversown pearl millet was impaired by the intercropping and produced low forage yield. With respect to forage quality, the intercropped sowing system did not affect the nutritional characteristics of the Paiaguas palisadegrass.

Key words: *Urochloa brizantha*, agriculture-livestock integration, *Pennisetum glaucum* (L.) R. Br.

INTRDUCTION

Intercropping annual crops with tropical forages has been increasingly adopted by farmers in the Cerrado (Pacheco et al., 2008), because many studies showed the feasibility of intercropping annual crops with various forage species

when planted simultaneously (Petter et al., 2011).

In this kind of production system, the producer has the possibility of three ways of use of an area in a single offseason after harvesting summer soybean: the cultivation of an annual grain crop, the use of forage for grazing (livestock) and the production of straw for a no-till system. This system allows greater crop diversification, minimizes risks of crop losses and provides more options for adopting crop rotation and sequences under conservation agriculture system (Horvathy et al., 2012).

Despite the various benefits of intercropping, its agronomic efficiency depends on environmental conditions (Barducci et al., 2009). Additionally, it is important to consider that the establishment of intercrops with forage implies competition between the different crops, especially when sowing occurs simultaneously. The intercropping of annual crops and tropical forage species is possible because of temporal and spatial lags in their growth and biomass accumulation. Among the forage grasses used, those of the genus *Urochloa* stand out (Ikeda et al., 2007; Pariz et al., 2010; Machado and Valle, 2011; Ribeiro et al., 2015). The advantage of using *Urochloa* species in an intercropped system is related to their abundant root systems, which contribute to water infiltration, soil aggregation and aeration (Kluthcouski et al., 2004).

These grasses show good adaptation, tolerance and resistance to abiotic factors and produce high dry matter yield with good nutritional value that is capable to meet animal needs, especially in the dry season (Brighenti et al., 2008). Most research focused on intercropping corn (Maia et al., 2014), sorghum (Horvathy et al., 2014) and sunflower (Souza et al., 2015) with forage grasses. Limited research has been conducted on the use of pearl millet (*Pennisetum glaucum* (L) R. BR) intercropped with grasses of the genus *Urochloa*.

Due to its adaptation to Cerrado region, pearl millet has received attention in recent years, especially with the release of early, high-yield genotypes from genetic improvement programs. Consequently, pearl millet is no longer considered a simple species for cover or straw production in no-till systems (Dan et al., 2009) and has become a high-value crop for forage production, for grazing (Leão et al., 2012), for silage (Costa et al., 2012) and for grain (Costa et al., 2015).

As intercropping of pearl millet with *Urochloa* spp. is largely unexplored particularily, under offseason conditions, there is a need for more information with regard to optimal sowing period, planting systems and sustainable ways of production. The objective of this study was to evaluate the agronomic characteristics of pearl millet, as well as the production and nutritional characteristics of Paiaguas palisadegrass under different intercropping methods and sowing periods in the offseason.

MATERIALS AND METHODS

The experiment was conducted in the field (17°48' S; 50°55' W; and 748 m altitude), in the municipality of Rio Verde, Goiás, Brazil, in 2014 offseason on a Dystroferric Red Latosol (Embrapa, 2013). Before planting, soil samples were collected from the 0 to 20 cm layer to assess physical and chemical characteristics of the experimental plots. Overall, the following values were obtained: 600, 140, and 260 g kg^{-1} of clay, silt and sand, respectively; pH ($CaCl_2$): 6.02; Ca: 3.50 $cmol_c$ dm^{-3}; Mg: 1.43 $cmol_c$ dm^{-3}; Al: 0.05 $cmol_c$ dm^{-3}; Al+H: 5.9 $cmol_c$ dm^{-3}; K: 0.35 $cmol_c$ dm^{-3}; CEC: 11.14 $cmol_c$ dm^{-3}; P: 2.29 mg dm^{-3}; Cu: 3.5 mg dm^{-3}; Zn: 5.1 mg dm^{-3}; Fe: 34.1 mg dm^{-3}; OM.: 37.06 g dm^{-3}.

The area was prepared by desiccating weeds with an application of Transorb herbicide (glifosato 480 g L^{-1} at a spray volume of 150 L ha^{-1}). Harrowing was performed 30 days after the desiccation, with a disk harrow, to eliminate weeds that escaped herbicide action, followed by subsoiling and additional harrowing. One week before implementing the experiment, harrowing was undertaken again, and the field was sown in furrows using a seeder with an inter-row spacing of 0.50 m. The furrows for sowing the Paiaguas palisadegrass, in the inter-rows, and the oversowing of pearl millet were manually dug using hoes.

The experiment was based on a randomized blocks in a 5 × 2 factorial design, with three replicates. There were five forage systems: monocropped pearl millet; monocropped Paiaguas palisadegrass; pearl millet intercropped in rows with Paiaguas palisadegrass; pearl millet intercropped between rows of Paiaguas palisadegrass and pearl millet oversown and intercropped with Paiaguas palisadegrass, and two sowing periods (February and March). The pearl millet genotype used was ADR 8010 (medium-sized and dual purpose).

Sowing was carried out on February 12 and March 4, along with 240 kg ha^{-1} of P_2O_5 and 20 kg ha^{-1} of Fritted Trace Element-FTE BR 12 (9% Zn; 1.8% B; 0.8% Cu; 2% Mn; 3.5% Fe and 0.1% Mo). Monocropped and intercropped pearl millet was sown at a depth of 3 cm. The Paiaguas palisadegrass was sown in rows at a depth of 6 cm. When intercropped, the Paiaguas palisadegrass was sown at a distance of 0.25 m from the pearl millet rows, and in the oversown system, it was sown in the inter-row (0.25 m distance) 15 days after pearl millet seeding. Fourteen seeds of pearl millet per linear meter and 5 kg of pure viable seeds per hectare of the forage species were used. The plots in all forage systems consisted of eight rows 3.0 m long. The usable area was obtained by only considering the four central rows and eliminating 0.5 m from each row end.

At 30 and 50 days after emergence (DAE), 60 kg ha^{-1} N in the form of urea and 40 kg ha^{-1} K_2O in the form of potassium chloride were applied by casting. Hand weeding was performed weekly up to 50 DAE to control post-emergence weeds. Fall armyworms (*Spodoptera frugiperda*) were controlled using insecticide applications of Losbam 18 ml (1 L ha^{-1}) and Nomolt 1 ml (50 ml ha^{-1}) at 40 and 50 DAE, and there were two fungicide applications (37 and 44 DAE) with Priori Extra (azoxystrobin + cyproconazole) at 0.5 L ha^{-1}. During the experiment, daily rainfall and mean monthly temperature data were monitored (Figure 1).

The following agronomic characteristics of the monocropped and intercropped pearl millet were measured: plant height, stem diameter and panicle size at 30, 60 and 90 DAE. Harvesting of pearl

*Corresponding author. E-mail: katia.costa@ifgoiano.edu.br.

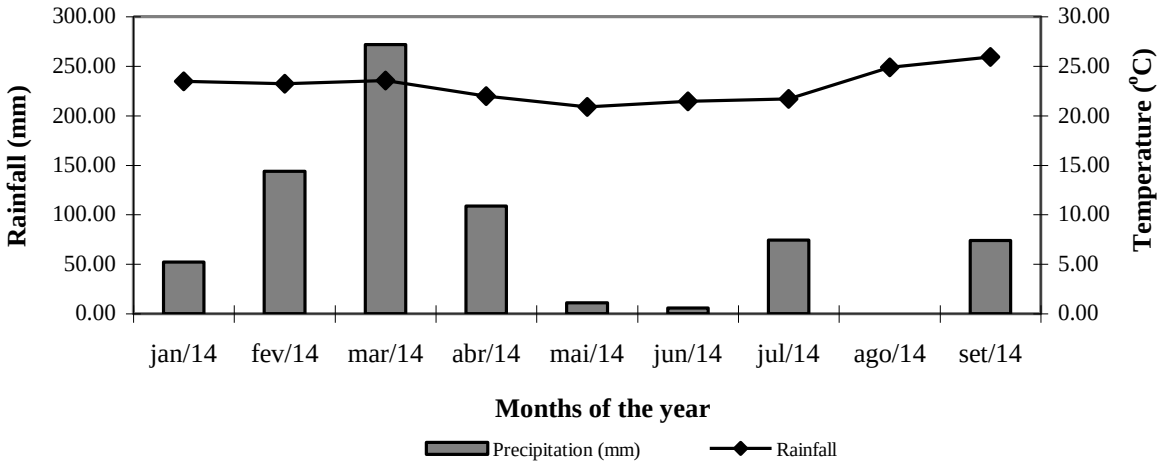

Figure 1. Rainfall and mean temperature recorded from January, 2014 to September, 2014, in Rio Verde-GO, Brazil.

millet was performed manually 115 and 118 DAE, for the first and second sowing dates, respectively, when the plants reached physiological maturity stage. At harvest, the grain yield (grain weight, corrected to 13% moisture), thousand grains weight (in grams, corrected to 13% moisture) and number of sacks per hectare were assessed in each of the usable plot areas.

The Paiaguas palisadegrass plant height was assessed (cm) using a graduated measuring tape, the number of tillers per linear meter were counted and the dry matter yield was measured until the onset of the rainy season (September). The forage was evaluated on successive cuts (0.20 m from the ground), based on samples of 1 m^2 that were randomly collected from each plot.

The first cut occurred at the time of pearl millet harvest on 06/04/14 and 06/24/14 for the first and second sowing periods, respectively. The second cut was conducted 78 days later, on 08/22/14 (first period) and 09/04/14 (second period), due to the low development of the forage grass under low rainfall, that is, the dry season. After both assessment cuts, a standard cut of all plants in the experimental area was carried out, at the same height used for the evaluated plants, and the resulting residue was removed from the area.

The collected material was packed in plastic bags and weighed for the assessment of total dry matter production. Then, the material was transported to the laboratory where a representative (500 g) sub-sample is taken from each plot and dried in a forced-air oven at 55°C. Subsequently, the samples were ground in a Wiley mill, using a 1-mm diameter sieve, and stored in plastic containers for further analysis.

Nutritional analyses were performed to determine the dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) using the methods reported by Silva and Queiroz (2002).

The *in vitro* dry matter digestibility (IVDMD) was assessed using the method described by Tilley and Terry (1963) and was adapted to the artificial rumen developed by ANKON® using the "Daisy incubator" device from Ankom Technology (*in vitro* true digestibility-IVTD). The rumen fluid was collected using two rumen-fistulated male cattle with a mean weight of 550 kg. The animals were maintained on *Urochloa brizantha* cv. Marandu pasture.

Data were subjected to analysis of variance and the means were compared using Tukey's test, with a significance level of 5%. Statistical analyses were performed using SISVAR 4.6 statistical software (Ferreira, 2011).

RESULTS AND DISCUSSION

The pearl millet height at 30, 60 and 90 DAE was not influenced (P>0.05) by forage system or by the interaction between forage system and sowing period. Therefore, it can be inferred that the Paiaguas palisadegrass plants did not influence the pearl millet development.

However, there was a significant effect (P<0.05) of sowing period on pearl millet height. The first sowing period resulted in lower plant height at 30 DAE over all forage systems. This result may be related to the uneven distribution of rainfall in February (Figure 1), with frequent dry spells observed at the beginning of emergence, which impaired the initial plant development. At 60 and 90 DAE, only the monocropped pearl millet plant height was influenced, with greater height measured for the first sowing period (Table 1). Notably, pearl millet is a plant that is sensitive to short days because it blooms in photoperiods lower than 12 h (Leão et al., 2012) and thus later sowing accelerates vegetative stage and advances blooming, which may have caused the lower plant height of monocropped pearl millet during the second sowing period.

Coimbra and Nakagawa (2006), who assessed the effect of sowing and cutting regimes on biomass and grain yield of forage millet, reported a mean height of 89.3 cm when the millet was sown in April and 210.9 cm when the millet was sown in September.

When assessing the stem diameter at 30 and 60 DAE,

Table 1. Plant height of monocropped pearl millet and pearl millet intercropped with Paiaguas palisadegrass under different forage systems at 30, 60 and 90 DAE.

Forage systems	Sowing periods	
	First	Second
	Plant height at 30 DAE (cm)	
Monocropped pearl millet	43.46[b]	76.33[a]
Row pearl millet × Paiaguas palisadegrass	42.60[b]	68.40[a]
Inter-row pearl millet × Paiaguas palisadegrass	42.20[b]	69.00[a]
Oversown pearl millet × Paiaguas palisadegrass	44.33[b]	75.53[a]
CV (%)	15.72	
	Plant height at 60 DAE (cm)	
Monocropped pearl millet	204.0[a]	185.6[b]
Row pearl millet × Paiaguas palisadegrass	186.3[a]	175.3[a]
Inter-row pearl millet × Paiaguas palisadegrass	195.0[a]	187.6[a]
Oversown pearl millet × Paiaguas palisadegrass	193.3[a]	189.3[a]
CV (%)	4.07	
	Plant height at 90 DAE (cm)	
Monocropped pearl millet	215.0[a]	198.0[b]
Row pearl millet × Paiaguas palisadegrass	192.3[a]	184.3[a]
Inter-row pearl millet × Paiaguas palisadegrass	206.3[a]	193.6[a]
Oversown pearl millet × Paiaguas palisadegrass	207.3[a]	194.6[a]
CV (%)	4.82	

Means followed by different letters within a row (sowing periods) differ according to Tukey's test at 5% probability level.

there was no significant effect (P>0.05) of the forage systems. However, at 90 DAE for both sowing periods, the lowest stem diameter was obtained for pearl millet intercropped in rows with Paiaguas palisadegrass. These results are attributed to the increased competition between the plants for water, light, nutrients and physical space, as the sowing of both species was carried out in the same row. At 90 days, low rainfall occured (Figure 1) and further increased plant competition for water.

In contrast, for the inter-row intercropping and oversown systems, there was no negative influence on the development of the pearl millet due to competition with the *Urochloa* plants, indicating the potential of these sowing systems. This is certainly due to both species being grasses, featuring the same C4 photosynthetic metabolism and thus efficiently using the available light (Taiz and Zaiger, 2010), in addition to having highly efficient root systems with respect to soil water and nutrient use.

When comparing the sowing dates, Table 2 shows that sowing in the first period resulted in a smaller stem diameter at 30 DAE compared with the second period, due to the lower rainfall in early stages. The opposite trend was observed at 60 DAE, when a larger stem diameter was obtained for the first sowing date. The main factor contributing to the greater stem diameter during the first period was certainly related to a better water balance during the post-sowing period (Figure 1), which favored plant development, and also to the plasticity of the species in using the best environmental conditions, coupled with the longer vegetative stage thanks to favorable photoperiod.

The panicle size at 60 DAE for the first sowing date was not affected (P>0.05) by forage system. However, at 90 DAE, there was an effect (P<0.05) of the forage system, where the lowest value was obtained in oversown pearl millet intercropped with Paiaguas palisadegrass (Table 3). Regarding to the second sowing date, only monocropped pearl millet differed (P<0.05) from the intercropped system in rows, with higher values for the panicle size at 60 and 90 DAE. This result is due to the competition of plants in this sowing system, as observed in the stem diameter assessment at 90 days.

Durães et al. (2003) reported that environmental factors affect plant growth rate and development. Temperature, in particular, influences the amount of grains at the time of

Table 2. Stem diameter of monocropped pearl millet and pearl millet intercropped with Paiaguas palisadegrass under different forage systems at 30, 60 and 90 DAE.

Forage systems	Sowing periods	
	First	Second
	Stem diameter at 30 DAE (mm)	
Monocropped pearl millet	0.20^{Ab}	0.90^{Aa}
Row pearl millet × Paiaguas palisadegrass	0.20^{Ab}	0.73^{Aa}
Inter-row earl millet × Paiaguas palisadegrass	0.20^{Ab}	0.83^{Aa}
Oversown pearl millet × Paiaguas palisadegrass	0.20^{Ab}	0.90^{Aa}
CV (%)	15.18	
	Stem diameter at 60 DAE (mm)	
Monocropped pearl millet	1.33^{Aa}	0.113^{Ab}
Row pearl millet × Paiaguas palisadegrass	1.23^{Aa}	0.096^{Ab}
Inter-row pearl millet × Paiaguas palisadegrass	1.26^{Aa}	0.100^{Ab}
Oversown pearl millet × Paiaguas palisadegrass	1.23^{Aa}	0.106^{Ab}
CV (%)	8.77	
	Stem diameter at 90 DAE (mm)	
Monocropped pearl millet	1.50^{Aa}	1.46^{Aa}
Row pearl millet × Paiaguas palisadegrass	1.03^{Bb}	1.10^{Ba}
Inter-row pearl millet × Paiaguas palisadegrass	1.30^{Aa}	1.53^{Aa}
Oversown pearl millet × Paiaguas palisadegrass	1.16^{ABb}	1.46A^{a}
CV (%)	11.08	

Means followed by different letters within a column (forage systems) and row (sowing periods) differ according to Tukey's test at 5% probability level.

Table 3. Panicle size of monocropped pearl millet and pearl millet intercropped with Paiaguas palisadegrass under different forage systems at 60 and 90 DAE.

Forage systems	Sowing periods	
	First	Second
	Panicle size at 60 DAE (cm)	
Monocropped pearl millet	25.10^{Aa}	25.86^{Aa}
Row pearl millet × Paiaguas palisadegrass	25.43^{Aa}	23.18^{Bb}
Inter-row earl millet × Paiaguas palisadegrass	25.13^{Aa}	24.92^{ABa}
Oversown pearl millet × Paiaguas palisadegrass	23.63^{Aa}	24.07^{ABa}
CV (%)	4.39	
	Panicle size at 90 DAE (cm)	
Monocropped pearl millet	26.60^{Aa}	28.06^{Aa}
Row pearl millet × Paiaguas palisadegrass	27.33^{Aa}	24.13^{Bb}
Inter-row earl millet × Paiaguas palisadegrass	27.00^{Aa}	27.00^{ABa}
Oversown pearl millet × Paiaguas palisadegrass	23.90^{Ba}	25.76^{ABa}
CV (%)	5.09	

Means followed by different letters within a column (forage systems) and row (sowing periods) differ according to Tukey's test at 5% probability level.

Table 4. Grain yield, thousand grain weight and sacks per hectare of monocropped pearl millet and pearl millet intercropped with Paiaguas palisadegrass under different forage systems.

Forage systems	Sowing periods	
	First	Second
	Grain yield (kg ha^{-1})	
Monocropped pearl millet	2008^{Ab}	2614^{Aa}
Row pearl millet × Paiaguas palisadegrass	2264^{Ab}	2592^{Aa}
Inter-row pearl millet × Paiaguas palisadegrass	2338^{Ab}	2974^{Aa}
Oversown pearl millet × Paiaguas palisadegrass	2041^{Ab}	2360^{Aa}
CV (%)	21.29	
	Thousand grain weight (g)	
Monocropped pearl millet	9.61^{Aa}	8.33^{Aa}
Row pearl millet × Paiaguas palisadegrass	9.61^{Aa}	7.66^{Aa}
Inter-row pearl millet × Paiaguas palisadegrass	9.58^{Aa}	8.56^{Aa}
Oversown pearl millet × Paiaguas palisadegrass	9.21^{Aa}	7.65^{Aa}
CV (%)	13.48	
	Sacks per hectare	
Monocropped pearl millet	37.39^{Ab}	43.57^{Aa}
Row pearl millet × Paiaguas palisadegrass	39.41^{Ab}	43.21^{Aa}
Inter-row pearl millet × Paiaguas palisadegrass	38.97^{Ab}	49.58^{Aa}
Oversown pearl millet × Paiaguas palisadegrass	39.34^{Aa}	36.01^{Aa}
CV (%)	19.68	

Means followed by different letters within a column (forage systems) and row (sowing periods) differ according to Tukey's test at 5% probability level.

harvest. Under low temperatures, the amount of grains is probably reduced by the direct effect of spikelet death, spikelet sterility or male sterility, combined with the photoperiod. Temperature is also a major factor in productivity because the delay in sowing causes an acceleration of the crop cycle, thereby reducing the vegetative stage and advancing blooming.

Costa et al. (2005), who compared the yield and biomass of different cultivars of millet, observed panicle lengths of 32.2 cm (BRS 1501), 35.5 cm (SounaII) and 59.9 cm (ENA2), which were higher values than those observed in the present study.

When comparing sowing periods, Table 3 shows that only the pearl millet intercropped in rows with Paiaguas palisadegrass was affected, with a smaller panicle size for the second sowing period. According to Madhusudhana and Govila (2001), the mean panicle length has a direct effect on grain yield.

The grain yield, thousand grain weight and number of sacks per hectare of pearl millet were significantly similar (P>0.05) for allforage systems and for both evaluation periods (Table 4). This proves the feasibility of the intercropped systems because the Paiaguas palisadegrass did not hinder pearl millet development with respect to final grain yield.

However, regarding sowing time, average grain yield obtained in the second sowing period was higher over all forage systems. A similar result was observed for the number of sacks per ha^{-1} except for the isolated case where oversown pearl millet was intercropped with Paiaguas palisadegrass; in this system, the sacks per ha^{-1} did not differ between the evaluated periods. Moreover, sowing date had no significant effect on thousand grain weight.

The average pearl millet grain yield was similar to that obtained by Costa et al. (2005), who evaluated millet genotypes sown in two periods. These authors reported a millet grain yield of 2.456 kg ha^{-1} for ENA 2 cultivar.

Geraldo et al. (2000) reported thousand grain weight values of 6.8 g in cultivar BN2, 6.8 g in IAPAR, 12.0 g in HKP3 and 12.1 g in Guerguera. These values were lower than that found in the present study for cultivar ADR 8010. The difference in results may be due to the different hybrids used in the studies, e.g., ADR 8010 is a hybrid derived from genetic improvement that has a high yield potential.

Based on these results, it can be stated that even under intercropping conditions, where there is greater competition for light, water and nutrients, the intercropped pearl millet was not affected by the presence of Paiaguas palisadegrass for either sowing period, showing the interest of an agriculture-livestock integration system for

Table 5. Plant height (cm) and number of tillers of monocropped Paiaguas palisadegrass and Paiaguas palisadegrass intercropped under different forage systems and sowing periods.

Forage systems	Sowing periods	
	First	Second
	Plant height - First cut	
Monocropped Paiaguas palisadegrass	91.66^{Aa}	73.00^{Ab}
Row pearl millet × Paiaguas palisadegrass	85.33^{ABa}	66.33^{ABb}
Inter-row pearl millet × Paiaguas palisadegrass	73.33^{ABa}	55.00^{Bb}
Oversown pearl millet × Paiaguas palisadegrass	53.33^{Ca}	39.33^{Cb}
CV (%)	7.83	
	Plant height - Second cut	
Monocropped Paiaguas palisadegrass	63.66^{Aa}	58.00^{Aa}
Row pearl millet × Paiaguas palisadegrass	47.33^{Aa}	41.00^{Aa}
Inter-row pearl millet × Paiaguas palisadegrass	52.00^{Aa}	49.00^{Aa}
Oversown pearl millet × Paiaguas palisadegrass	22.33^{Ba}	22.66^{Ba}
CV (%)	23.31	
	Number of tillers - First cut	
Monocropped Paiaguas palisadegrass	320.66^{Ab}	478.00^{Aa}
Row pearl millet × Paiaguas palisadegrass	298.33^{Aa}	285.33^{Ba}
Inter-row pearl millet × Paiaguas palisadegrass	327.33^{Aa}	271.00^{Ba}
Oversown pearl millet × Paiaguas palisadegrass	230.33^{Ba}	209.33^{Ca}
CV (%)	20.20	
	Number of tillers - Second cut	
Monocropped Paiaguas palisadegrass	263.33^{Aa}	272.33^{Aa}
Row pearl millet × Paiaguas palisadegrass	187.66^{Ba}	168.00^{Ba}
Inter-row pearl millet × Paiaguas palisadegrass	205.33^{Ba}	177.00^{Ba}
Oversown pearl millet × Paiaguas palisadegrass	159.00^{Ca}	145.00^{Ca}
CV (%)	9.13	

Means followed by different letters within a column (forage systems) and row (sowing periods) differ according to Tukey's test at 5% probability level.

the production of grains in the offseason.

U. brizantha cv. BRS Paiaguas

The height of Paiaguas palisadegrass was affected (P<0.05) by the interaction between forage system and sowing period (Table 5). It was observed that over both periods, the height of the first cut of the monocropped Paiaguas palisadegrass and where this species was intercropped (in rows with pearl millet and in the inter-rows between pearl millet) was higher than that measured in the oversown system.

Notably, oversowing method dramatically affected forage development. This is due to the fact that the grass was established 15 days after pearl millet seeding sown, which resulted in higher interspecific competition between the plants as the millet was already at the 2 stage when the Paiaguas palisadegrass was sown. This caused shading in the early stages of the grass growth and consequently reduced the quantity and quality of radiation intercepted by the lower stratum of the canopy affecting Paiaguas palisadegrass growth.

Similar plant height results were obtained by Seidel et al. (2014), who found that the mean height of *U. brizantha* cv. MG4, when simultaneously planted, was 88 and 78.75 cm in a row and inter-row sowing system, compared with heights of 33.25 and 35.75 cm when the grass was sown 25 days after the sowing of corn, equivalent to decreases of 62.22 and 54.61%, respectively.

Regarding to sowing periods, Table 5 shows that for the first cut, the second sowing date resulted in lower heights in all forage systems. This was due to the uneven distribution of rainfall in February (Figure 1) when a drought occurred during early emergence and impaired the initial plant development.

When evaluating the plant height of the second cut (Table 5), it was observed that the lowest heights in both periods were measured when Paiaguas palisadegrass was intercropped with oversown pearl millet. Even after pearl millet harvest, Paiaguas palisadegrass did not exhibit the same development as that reported for other forage systems. In this context, it can be inferred that this sowing method is an ineffective cultivation technique for cattle pasture during the offseason.

In contrast, the similar plant height measured during the second cut of monocropped Paiaguas palisadegrass compared with row and inter-row intercropping indicates that the pearl millet did not negatively affect the development of Paiaguas palisadegrass, as there was no competition for resources. The upright growth of both forage crops contributed to the favorable outcome of the intercropping.

The sowing period did not influence ($P>0.05$) plant height measured at the time of the second cut in any of the forage systems. Note that Paiaguas palisadegrass and pearl millet have high water use efficiency but under low water availability (Duraes et al., 2003), the presence of pearl millet can lead to increased competition and strongly interfere with the development of the palisadegrass, as observed in the present study. The absence of a sowing period effect at the time of the second cut reinforces this statement, as in the absence of pearl millet, the forage exhibited the same performance at the second cut as that observed at the first cut, indicating that the shadow produced by the plants might reduce forage. Both species have the same C4 photosynthetic metabolism and are therefore highly dependent on light to achieve their optimum photosynthetic rate. In addition, these species have a highly efficient root system with respect to water and soil nutrient use and are therefore considered highly competitive (Taiz and Zaiger, 2010).

The intercropping of Paiaguas palisadegrass with oversown pearl millet negatively affected ($P<0.05$) the number of tillers (Table 5) in the first cut in the first sowing period. The low amount of available light for Paiaguas palisadegrass, when shadowed by pearl millet, hampered the emergence and development of new side buds that give rise to new tillers, thus reducing their numbers (Soares et al., 2009). However, for the row and inter-row intercropped systems, the results were similar to the monocropped Paiaguas palisadegrass. The highest number of tillers was measured in monocropped Paiaguas palisadegrass for the second sowing period.

Seidel et al. (2014) reported a lower number of tillers for *U. brizantha* cv. MG4 than that observed in the present study, that is, 62.75 and 68.25 in the row and inter-row system under simultaneous sowing with corn and 163.67 and 149 in the row and inter-row oversown system (25 DAE), respectively, verifying that in contrast to the observations in the present study, shading by corn induced a higher tillering rate. In the present study, reduced tillering in the overseeded system is due to lower quantity and quality of light intercepted by the canopy. Tillering is induced by the perception of blue light by the phytochromes located in the basal and axillary buds and also the pattern red: far red. And thus the low intensity and luminous quality reduced induction of tillering.

Considering sowing periods within forage systems, only the monocropped Paiaguas palisadegrass exhibited a significantly ($P<0.05$), higher number of tillers with the second period sowing. This result corroborates the higher plant height in the first sowing period. The size of the leaf blade is a major factor in the production of new tillers, as stated earlier regarding the quality of light reaching the lower strata, which can be reduced due to increased light interception by the canopy, thereby delaying the development of axillary buds in tillers (Soares et al., 2009). This explains the higher number of tillers observed in the second sowing period, during which a lower plant height was measured.

The dry matter yield was affected ($P<0.05$) by forage systems, in which oversown system exhibited the lowest average yield; The dry matter yield (was 70.0 and 59.0% lower for the first sowing period and 50 and 56.8% lower for the second sowing period for the first and second cuts, respectively, indicating that planting under this system did not provide a satisfactory dry mass yield (Table 6).

The dry matter yields registered in the present study were similar to those given by Leonel et al. (2009), that is, 7.568 kg ha^{-1} in exclusive marandu grass cultivation. Pariz et al. (2010) reported yields of 4.128 and 4.168 kg ha^{-1} for *Urochloa* intercropped with corn in rows and by casting. Machado and Valle (2011), evaluating the agronomic performance of *Urochloa* grass genotypes in succession to soybean for three years (2007, 2008 and 2009), found that Paiaguas palisadegrass (B6 lineage) yielded 4.541, 5.299 and 6.116 kg ha^{-1} of dry matter, respectively.

A negative effect of oversowing on the dry matter yield of the MG4 grass was reported by Seidel et al. (2014), who found decreases of 81.7 and 62.5% when the forage was sown 25 days after the corn with row and inter-row systems, respectively,.

The sowing periods did not affect ($P>0.05$) the dry matter yield of the forage systems in both cuts (Table 6). The second cut provided the lowest yield, due to low regrowth in the absence of rainfall with decreasing temperatures (Figure 1).

For the two cuts, the leaf:stem ratio differed significantly ($P<0.05$) between forage systems, with monocropped Paiaguas palisadegrass, showing the lowest ratio compared to intercropped systems. This result is probably due to increased growth and development of the grass under a monocropped system, which resulted in a higher

Table 6. Dry matter yield (kg ha^{-1}) and leaf:stem ratio of monocropped Paiaguas palisadegrass and Paiaguas palisadegrass intercropped under different forage systems and sowing periods.

Forage systems	Sowing periods	
	First	First
	DM - First cut	
Monocropped Paiaguas palisadegrass	7.408^{Aa}	6.320^{Aa}
Row pearl millet × Paiaguas palisadegrass	5.488^{Aa}	4.332^{Aa}
Inter-row pearl millet × Paiaguas palisadegrass	5.379^{Aa}	4.265^{Aa}
Oversown pearl millet × Paiaguas palisadegrass	2.169^{Ba}	2.590^{Ba}
CV (%)	21.67	
	DM - Second cut	
Monocropped Paiaguas palisadegrass	2.574^{Aa}	2.450^{Aa}
Row pearl millet × Paiaguas palisadegrass	2.183^{Aa}	2.281^{Aa}
Inter-row pearl millet × Paiaguas palisadegrass	2.038^{Aa}	2.619^{Aa}
Oversown pearl millet × Paiaguas palisadegrass	1.272^{Ba}	1.058^{Ba}
CV (%)	14.8	
	Leaf:stem ratio - First cut	
Monocropped Paiaguas palisadegrass	0.940^{Ba}	0.894^{Ba}
Row pearl millet × Paiaguas palisadegrass	1.392^{Aa}	1.261^{Aa}
Inter-row pearl millet × Paiaguas palisadegrass	1.299^{Aa}	1.268^{Aa}
Oversown pearl millet × Paiaguas palisadegrass	1.240^{Aa}	1.234^{Aa}
CV (%)	13.32	
	Leaf:stem ratio - Second cut	
Monocropped Paiaguas palisadegrass	1.020^{Ba}	1.010^{Ba}
Row pearl millet × Paiaguas palisadegrass	1.423^{Aa}	1.223^{Aa}
Inter-row pearl millet × Paiaguas palisadegrass	1.280^{Aa}	1.353^{Aa}
Oversown pearl millet × Paiaguas palisadegrass	1.276^{Aa}	1.230^{Aa}
CV (%)	7.68	

Means followed by different letters within a column (forage systems) and row (sowing periods) differ according to Tukey's test at 5% probability level.

leaf:stem ratio due to the elongation of the leaf blade. These results were more favorable than those observed by Leonel et al. (2009), who reported a leaf:stem ratio of approximately 1.0 when intercropping corn with marandu grass. However, the leaf:stem ratio was similar across all forage systems (P>0.05) for the two sowing periods.

Higher contents of NDF and ADF were measured in the first cut of the monocropped Paiaguas palisadegrass forage system of both sowing periods; these values differed (P<0.05) from those obtained in intercropped systems (Table 7). This may be associated to the higher leaf:stem ratio of intercropped systems, which resulted in a higher amounts of fibers.

The NDF and ADF contents were similar (P>0.05) in the second cut. It should be noted that pearl millet was no longer present at the time of the second cut; thus, there was more uniform growth of the forage, even in the periods (August and September) when rainfall was not stable.

The NDF and ADF results obtained in the present study were similar to those reported by Pariz et al. (2010), who evaluated the bromatological composition of *Urochloa* cultivars intercropped with corn, and found NDF contents ranging from 66.4 to 74.3% and from 70.3 to 78.1%, and ADF contents ranging from 40.0 to 43.1% and from 41.6 to 49.5% for mulato and marandu grasses, respectively.

When evaluating the sowing periods, Table 7 shows that the NDF contents of the first and second periods were similar (P>0.05) across all forage systems, and the same result was found for the ADF contents of the first

Table 7. Contents of NDF (%) and ADF (%) of monocropped Paiaguas palisadegrass and Paiaguas palisadegrass intercropped under different forage systems and sowing periods.

Forage systems	Sowing periods	
	First	First
	Contents of NDF - First cut	
Monocropped Paiaguas palisadegrass	74.12^{Aa}	74.04^{Aa}
Row pearl millet × Paiaguas palisadegrass	68.38^{Ba}	69.59^{Ba}
Inter-row pearl millet × Paiaguas palisadegrass	68.78^{Ba}	70.56^{Ba}
Oversown pearl millet × Paiaguas palisadegrass	68.07^{Ba}	68.68^{Ba}
CV (%)	4.20	
	Contents of NDF - Second cut	
Monocropped Paiaguas palisadegrass	67.15^{Aa}	65.57^{Aa}
Row pearl millet × Paiaguas palisadegrass	65.44^{Aa}	62.24^{Aa}
Inter-row pearl millet × Paiaguas palisadegrass	63.80^{Aa}	63.37^{Aa}
Oversown pearl millet × Paiaguas palisadegrass	66.36^{Aa}	63.18^{Aa}
CV (%)	4.25	
	Contents of ADF - First cut	
Monocropped Paiaguas palisadegrass	47.86^{Aa}	46.71^{Aa}
Row pearl millet × Paiaguas palisadegrass	44.19^{Ba}	40.76^{Ba}
Inter-row pearl millet × Paiaguas palisadegrass	42.99^{Ba}	41.91^{Ba}
Oversown pearl millet × Paiaguas palisadegrass	43.89^{Ba}	40.26^{Ba}
CV (%)	7.68	
	Contents of ADF - Second cut	
Monocropped Paiaguas palisadegrass	39.91^{Aa}	33.34^{Ab}
Row pearl millet × Paiaguas palisadegrass	39.55^{Aa}	35.16^{Ab}
Inter-row pearl millet × Paiaguas palisadegrass	37.87^{Aa}	35.22^{Ab}
Oversown pearl millet × Paiaguas palisadegrass	39.62^{Aa}	33.45^{Ab}
CV (%)	5.02	

Means followed by different letters within a column (forage systems) and row (sowing periods) differ according to Tukey's test at 5% probability level.

cut. However, the second sowing period resulted in lower ADF levels for the second cut, with an average value of 34.29%.

The NDF and ADF contents of the second cut were lower compared to the first cut due to the fact that cutting was carried out after a shorter growth cycle. In addition, after pearl millet harvest, there was resumption of growth of new tillers, which was also influenced by the onset of the rainy season (September), providing better quality forage. This proves that pearl millet intercropped with Paiaguas palisadegrass can be considered an excellent alternative for use in an agriculture-livestock integration system in the offseason when there is low forage yield and quality.

The evaluation of the CP contents of the first and second cuts showed significant (P>0.05) similarity between forage systems and sowing periods (P>0.05) (Table 8). The CP contents obtained in the present study were similar to those found by Maia et al. (2014), who evaluated the bromatological composition of forage grasses of the genus *Urochloa* in the offseason, after the harvest of corn in a crop-livestock integration system, and found mean CP contents ranging from 9.0 to 13.4%, in September and October, respectively. Machado and Valle (2011) found CP contents ranging from 11.9 to 15.5% for Paiaguas palisadegrass (B6 lineage).

Van Soest (1994) reported that cellulolytic rumen bacteria have satisfactory development if the CP content is equal to or above 7.0%. Therefore, it can be concluded that all CP contents obtained across all forage systems and sowing periods should meet the nutritional requirements. Thus, the procedure has proven to be

Table 8. Contents of CP (%) and IVDMD (%) of monocropped Paiaguas palisadegrass and Paiaguas palisadegrass intercropped under different forage systems and sowing periods.

Forage systems	Sowing periods	
	First	First
	Contents of CP - First cut	
Monocropped Paiaguas palisadegrass	12.44^{Aa}	12.57^{Aa}
Row pearl millet × Paiaguas palisadegrass	11.68^{Aa}	12.24^{Aa}
Inter-row pearl millet × Paiaguas palisadegrass	12.85^{Aa}	12.69^{Aa}
Oversown pearl millet × Paiaguas palisadegrass	11.88^{Aa}	12.52^{Aa}
CV (%)	5.83	
	Contents of CP - Second cut	
Monocropped Paiaguas palisadegrass	13.20^{Aa}	14.16^{Aa}
Row pearl millet × Paiaguas palisadegrass	14.29^{Aa}	13.57^{Aa}
Inter-row pearl millet × Paiaguas palisadegrass	13.55^{Aa}	14.68^{Aa}
Oversown pearl millet × Paiaguas palisadegrass	14.40^{Aa}	14.09^{Aa}
CV (%)	6.23	
	Contents of IVDMD - First cut	
Monocropped Paiaguas palisadegrass	47.41^{Ba}	48.45^{Ba}
Row pearl millet × Paiaguas palisadegrass	51.73^{Aa}	52.86^{Aa}
Inter-row pearl millet × Paiaguas palisadegrass	53.97^{Aa}	52.77^{Aa}
Oversown pearl millet × Paiaguas palisadegrass	52.25^{Aa}	56.44^{Aa}
CV (%)	8.54	
	Contents of IVDMD - Second cut	
Monocropped Paiaguas palisadegrass	54.66^{Aa}	49.83^{Aa}
Row pearl millet × Paiaguas palisadegrass	55.00^{Aa}	49.24^{Aa}
Inter-row pearl millet × Paiaguas palisadegrass	55.90^{Aa}	54.11^{Aa}
Oversown pearl millet × Paiaguas palisadegrass	48.53^{Aa}	51.98^{Aa}
CV (%)	8.71	

Means followed by different letters within a column (forage systems) and row (sowing periods) differ according to Tukey's test at 5% probability level.

relevant in terms of quality forage production in the offseason period – characterized by a lack of forage to meet animal production demand; the offseason period when there is usually low forage availability due to the seasonality of forage production.

The IVDMD was affected by the forage systems ($P<0.05$) in the first cut in both sowing periods, where the monocropped Paiaguas palisadegrass had a lower value compared to intercropped systems (Table 8). This result may be associated with a higher leaf:stem ratio in this monocropped system, which resulted in higher contents of NDF and ADF (Table 7). According to Fernandes et al. (2002), an increase in digestibility is associated with changes in the chemical composition, such as a decrease in NDF, ADF contents and hemicelluloses content, while providing readily digestible carbohydrates for rumen microorganisms.

Regarding the sowing periods, the IVDMD values were similar ($P>0.05$), confirming that sowing period had no significant effect on forage quality. Maia et al. (2014) found higher IVDMD values for genotypes of *Urochloa* intercropped with corn, ranging from 68.5 to 77.58%. The difference from values registered in our study is due to the time of assessment, as the corn was harvested in February in the case of Maia et al. (1014) trial, while in the present study, the pearl millet was harvested in July.

Conclusion

The intercropping of pearl millet with Paiaguas

palisadegrass in the offseason proved to be a promising technique for grain production in Southeastern Goiás, where the Paiaguas palisadegrass did not affect the pearl millet grain yield. However, delayed sowing period (March …th) provided better grain yield and a higher number of sacks per hectare.

Regarding forage yield, Paiaguas palisadegrass grown with oversown pearl millet is impaired by intercropping. With respect to the forage quality, the intercropped sowing system did not affect the nutritional characteristics.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Physical and physicochemical composition of mangaba fruits (*Hancornia speciosa* Gomes) at three maturity stages

Plácido G. R.[1]*, Silva R. M.[2], Cagnin C[2], Silva M. A. P.[1] and Caliari M.[3]

[1]Graduate Program in Animal Science, Instituto Federal Goiano - Rio Verde Campus, Rio Verde, Goias, Brazil.
[2]Food Engineering Course, Instituto Federal Goiano - Rio Verde Campus, Rio Verde, Goias, Brazil.
[3]Graduate Program in Food Science and Technology, Escola de Agronômia e Engenharia de Alimentos, Universidade Federal de Goiás, Goiânia, GO, Brazil.

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The mangaba (*Hancornia speciosa* Gomes) is a typical fruit of the Brazilian cerrado and caatinga. It has excellent nutritional composition but there are no studies reporting the fruit characteristics and its industrial use. The aim of this study was to analyze the physical and physicochemical characteristics of mangaba fruit in three different maturity stages. Fruit weight, volume, length, titratable acidity, vitamin C, soluble solids and color at 1/3 ripe, 2/3 ripe and ripe maturity stages were analyzed. Physical characteristics showed great potential for industrialization due to the size and weight of fruits. High vitamin C levels and low acidity indexes were obtained, which are of interest for use of fruits in jams, jellies and liquors. However, soluble solids content was lower for ripe fruits, which indicates low sugar contents. Thus, the fruit has potential for consumption in the natural form and for processing and can be incorporated into many food products.

Key words: Maturation, composition, economic potential.

INTRODUCTION

Mangaba tree (*Hancornia speciosa Gomes*) is a native species of the *Apocynaceae* family. The fruit species occurs spontaneously in the Mid-western, Northern, Northeastern and Southeastern Brazil, being especially appreciated by consumers and cottage industries for the production of sweets, liquor, wine, soft drinks and jellies (Ganga et al ., 2010; Silva et al., 2007).

The fruit exploitation is extractive and accounts for almost the entire demand of the domestic production (Freitas et al., 2010). Technical mangaba cultivation occurs in few Brazilian areas, mostly in Northeastern Brazil (Silva, 2004).

Mangaba has excellent physical features, aroma, flavor and nutritional qualities (Santos et al., 2009). The

*Corresponding author. E-mail: macaliari@ig.com.br.

Table 1. Physical and physicochemical characteristics of mangaba fruits at three maturity stages.

Parameter	Fruits 1/3 ripe	Fruits 2/3 ripe	Ripe fruits
Volume (cm^3)	18.20[b]	24.68[b]	41.74[a]
Length (mm)	31.37[b]	33.76[b]	40.76[a]
Mass (g)	18.70[c]	27.01[b]	41.91[a]
Vitamin C (mg de ascorbic acid /100g)	188.33[a]	184.57[a]	174.12[a]
Titratable Acidity (% total acid)	6.50[a]	4.50[a]	6.41[a]
Soluble solids (°Brix)	8.95[a]	8.78[ab]	7.94[b]
L	39.81[a]	48.42[b]	44.26[ab]
a*	-21.48[a]	-21.10[a]	-21.84[a]
b*	40.60[a]	44.95[a]	40.25[a]

fruit is fleshy with ellipsoid or rounded shape with creamy and juicy pulp and sweet and slightly acidic flavor. The color of the fruit is predominantly greenish or yellowish and may have red pigments (Santos et al., 2012).

There are few studies on the physical and physicochemical characteristics of mangaba fruits at different maturity stages. Therefore, this study aimed to study mangaba fruits and its physical and physicochemical characteristics at three maturity stages.

MATERIALS AND METHODS

Mangaba fruits from an area under homogeneous cultivation in the region of Caçu-GO. (18°33'S and 51°08'W) were manually harvested in October 2013 and brought to the Fruits and Vegetables Laboratory, Instituto Federal Goiano, Rio Verde Campus-GO. Fruits were selected by size, color and absence of mechanical injuries, sanitized in chlorinated water for three minutes (100 mg / L).

For physical, vitamin C, titratable acidity and soluble solids analyses, fruits were divided into three maturity stages. The average volume of the fruit was calculated using a 1000mL Vidrolab test tube by water displacement. Fruit diameter was analyzed with Digital Caliper Model Digimess. The average weight of fruits was carried out by weighing 50 fruits on an analytical scale.

Soluble solids content was determined using the Abbe Refractometer and expressed in °Brix (AOAC, 1992). Titratable acidity levels were determined by AOAC methodology (1992). Ascorbic acid content was determined by titration with potassium iodate (AOAC, 1992). External appearance of fruits was evaluated with respect to color instrumental parameters according to the CIELab L *, a *, b * system, and the results were expressed in L *, a * and b *, where L * (brightness) ranged from black (0) to white (100), a * values ranged from green (-60) to red (60), and b * values from blue (-60) to yellow (+60). The results were presented according to the Tukey test ($p < 0.05$).

RESULTS

Volume, longitudinal diameter and mass of mangaba fruits analyzed showed significant differences from each other. According to Table 1, the physical characteristics of mangaba fruits showed higher values for ripe fruits. Freitas et al. (2012) studied ripe mangaba fruits and determined average mass of 20.97 g, which is lower than value determined for ripe fruits of this work, 41.91g. Mangaba clones analyzed by Souza et al. (2007) showed mass values between 42.07 and 21.74 g. The length of these clones ranged from 35.01 to 45.62 mm (Souza et al., 2007), whose values are close to those found for the three maturity stages of mangabas fruits studied here.

The physical characteristics of fruits showed acceptable values according to factors specific to species such as genetic characteristics, maturity stage, place of cultivation and harvest times (Soares et al., 2008).

The highest vitamin C levels were found for fruits 1/3 ripe. In mangaba clones, values ranged from 139.83 to 188.75 mg of ascorbic acid / 100 g pulp (Souza et al., 2007), which are close to those found in this work. Mangaba fruits kept under refrigeration showed lower values (132.60 to 166.49 mg $100g^{-1}$) (Campos et al., 2011). Carnelossi et al. (2004) observed higher values for ripe and fallen mangaba fruits, ranging from 252.7 to 274.7 mg 100 g^{-1}.

Differences in vitamin C levels may be related to the role of this vitamin as antioxidant due to oxidative reactions that occur during fruit ripening. Research with "citrus" revealed that there is a drop in the vitamin C content of fruits during fruit ripening (Malgarin et al., 2008).

Titratable acidity values ranged from 0.045 to 0.065% citric acid for mangaba fruits. The titratable acidity of newly harvested ripe mangaba fruits analyzed by Campos et al. (2011) was 0.554 g / 100 g of pulp, Carnelossi et al. (2004) determined 0.8%, and Soares et al. (2008) found values ranging from 0.38 to 0.78% citric acid, which are higher than values found in this study.

Differences in acidity levels may be associated with fruit breathing and hydrolysis rates of pectin present in the cell wall of fruits (Borges et al., 2000), which may vary according to the region where fruit is grown.

Soluble solids values ranged from 7.94 to 8.95 ° Brix, which are lower than those found by Cohen and Sano (2010) from 17.7 to 20.3 ° Brix. For mangaba fruits kept

Table 2. Pearson correlation among physical and chemical variables for mangaba fruits 1/3 ripe.

Variables	Volume	Length	Mass	Vitamin C	Titratable acidity	Soluble solids	L	a	b
Volume	-	0.65 ns	0.47 ns	0.45 ns	0.08 ns	0.41 ns	0.42 ns	-0.65 ns	0.59 ns
Length	-	-	0.40 ns	0.03 ns	-0.01 ns	0.35 ns	0.87 *	-0.54 ns	0.66 ns
Mass	-	-	-	0.84 *	0.76 ns	0.544 ns	0.02 ns	-0.96 *	0.90 *
Vitamin C	-	-	-	-	0.95 *	0.18 ns	-0.42 ns	-0.83 *	0.54 ns
Titratable acidity	-	-	-	-	-	-0.10 ns	-0.48 ns	-0.70 ns	0.32 ns
Soluble solids	-	-	-	-	-	-	0.34 ns	-0.51 ns	0.74 ns
L	-	-	-	-	-	-	-	-0.12 ns	0.40 ns
a	-	-	-	-	-	-	-	-	-0.89 *
B	-	-	-	-	-	-	-	-	-

Table 3. Pearson correlation among physical and chemical variables for mangaba fruits 2/3 ripe.

Variables	Volume	Length	Mass	Vitamin C	Titratable acidity	Soluble solids	L	a	b
Volume	-	0.66 ns	0.63 ns	-0.61 ns	0.33 ns	-0.34 ns	0.50 ns	0.68 ns	-0.02 ns
Length	-	-	0.41 ns	-0.36 ns	0.42 ns	0.19 ns	0.28 ns	0.05 ns	0.06 ns
Mass	-	-	-	-0.63 ns	0.28 ns	-0.12 ns	0.79 ns	0.65 ns	0.15 ns
Vitamin C	-	-	-	-	0.43 ns	-0.32 ns	-0.74 ns	-0.27 ns	-0.34 ns
Titratable acidity	-	-	-	-	-	-0.59 ns	-0.07 ns	0.42 ns	-0.29 ns
Soluble solids	-	-	-	-	-	-	0.04 ns	-0.73 ns	0.25 ns
L	-	-	-	-	-	-	-	0.30 ns	0.70 ns
a	-	-	-	-	-	-	-	-	-0.35 ns
b	-	-	-	-	-	-	-	-	-

at room temperature with yellow aspect and red pigments, values ranging from 11 to 15 ° Brix were found (Santos et al., 2009). However, Parente et al. (1985) found values between 7.5 and 13 ° Brix for mangaba fruits harvested in the Federal District.

Mangaba is a climacteric fruit, therefore, the SS content increases with maturation. This fact is related to the biosynthesis of soluble sugars or degradation of polysaccharides (Kays, 1997). The decrease in SS contents can be related to fruit selection and low values are related to the region that mangaba is grown, since this rate is related to the presence of sugars in chemical reactions.

Regarding the color of fruits at different maturity stages, only brightness showed statistical differences, parameters a * and b * remained statistically equal and brightness tends to increase during ripening. Jha et al. (2006) reported that the brightness value of fruit shell decreases during growth and increases during ripening when yellowing occurs.

Comparing variables analyzed in Table 2, positive correlation between mangaba mass and vitamin C, vitamin C and total acidity, length and brightness was found. In addition, negative correlation between vitamin C and color parameter a *, mangaba mass and color parameters a * and b * and between color variables a * and b * was also found. The vitamin C or ascorbic acid content was positively associated with total acidity, indicating that ascorbic acid may influence the acid flavor of fruits. The negative correlation between color parameters a * and b * is compatible, since there are changes in fruit pigments, i.e., there is a decrease in chlorophyll and increase in carotenoids.

Table 3 showed no significant correlations among variables analyzed. In Table 4, fruit volume is positively related to vitamin C content; fruit mass is negatively related with color parameter b * and parameter L is negatively related with parameter b *.

Conclusion

Mangaba fruits showed physical qualities similar to those found in literature and are consumed by industries and in the fresh form by consumers. It was observed that fruits showed relevant physicochemical characteristics, with high vitamin C contents and low acidity. The correlation between physical and chemical variables can estimate production parameters during the processes of mangaba selection. However, further studies are needed to analyze the SS content, which values were not in agreement with literature.

Table 3. Pearson correlation among physical and chemical variables for mangaba fruits 2/3 ripe.

Variables	Volume	Length	Mass	Vitamin C	Titratable acidity	Soluble solids	L	a	b
Volume	-	0.66 ns	0.63 ns	-0.61 ns	0.33 ns	-0.34 ns	0.50 ns	0.68 ns	-0.02 ns
Length	-	-	0.41 ns	-0.36 ns	0.42 ns	0.19 ns	0.28 ns	0.05 ns	0.06 ns
Mass	-	-	-	-0.63 ns	0.28 ns	-0.12 ns	0.79 ns	0.65 ns	0.15 ns
Vitamin C	-	-	-	-	0.43 ns	-0.32 ns	-0.74 ns	-0.27 ns	-0.34 ns
Titratable acidity	-	-	-	-	-	-0.59 ns	-0.07 ns	0.42 ns	-0.29 ns
Soluble solids	-	-	-	-	-	-	0.04 ns	-0.73 ns	0.25 ns
L	-	-	-	-	-	-	-	0.30 ns	0.70 ns
a	-	-	-	-	-	-	-	-	-0.35 ns
b	-	-	-	-	-	-	-	-	-

Table 4. Pearson correlation among physical and chemical variables for ripe mangaba fruits.

Variables	Volume	Length	Mass	Vitamin C	Titratable acidity	Soluble solids	L	a	b
Volume	-	0.65 ns	0.63 ns	0.96 *	-0.71 ns	0.31 ns	-0.20 ns	-0.44 ns	-0.48 ns
Length	-	-	0.40 ns	0.69 ns	-0.45 ns	0.44 ns	-0.43 ns	-0.64 ns	-0.32 ns
Mass	-	-	-	0.74 ns	-0.29 ns	-0.03 ns	-0.67 ns	0.20 ns	-0.88 *
Vitamin C	-	-	-	-	-0.68 ns	0.33 ns	-0.37 ns	-0.40 ns	-0.63 ns
Titratable acidity	-	-	-	-	-	0.17 ns	-0.31 ns	0.74 ns	0.01 ns
Soluble solids	-	-	-	-	-	-	-0.45 ns	-0.2 ns	-0.31 ns
L	-	-	-	-	-	-	-	-0.34 ns	0.86 *
a	-	-	-	-	-	-	-	-	-0.34 ns
b	-	-	-	-	-	-	-	-	-

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Influence of spatial arrangements on silvicultural characteristics of three *Eucalyptus* clones at integrated crop-livestock-forest system

André Dominghetti Ferreira[1,3]*, Ademar Pereira Serra[1], Valdemir Antônio Laura[1], Alexandre Cassiano Batistela Ortiz[2], Alexandre Romeiro de Araújo[1], Denise Renata Pedrinho[3] and Alex Mendonça de Carvalho[4]

[1]Empresa Brasileira de Pesquisa Agropecuária, Embrapa Gado de Corte, Campo Grande, MS, Brasil.
[2]Engenheiro Agrônomo, Campo Grande, MS, Brasil.
[3]Programa de Pós-graduação em Produção e Gestão Agroindustrial, Uniderp, Campo Grande, MS, Brasil.
[4]Universidade Federal de Lavras, Lavras, MG, Brasil.

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This research evaluated the influence of different spatial arrangements on the growth of three *Eucalyptus* clones as well as the characteristics that influence the quality of the timber. The experiment was carried out at Embrapa - Beef Cattle station, Campo Grande city, Mato Grosso do Sul State, Brazil. The design was in randomized blocks in a factorial scheme (3 × 3) with plots subdivided by time and four repetitions. Three clones of *Eucalyptus* were used (Urocam VM1, Grancam 1277 and Urograndis I144), and there were three spatial arrangements (single, double and triple row). At 20 and 32 months after planting, the variables, total plant height, diameter at breast height (DBH), volume of timber per tree, volume of timber per hectare, straightness and forking, and cylindricity were evaluated. The spatial arrangements influenced the behavior of the genetic material, and the greatest tree heights were observed in the triple row arrangements. The single row arrangement provided greater gains in DBH. The Grancam clone stood out from the others in the characteristics of straightness and forking, independent of the spatial arrangement and time of evaluation. It was concluded that the volume of timber per tree and the volume of timber per hectare were associated with the planting density, low density results in lower volume.

Key words: Agroforestry-pasture systems, spacing, timber quality.

INTRODUCTION

The search to increase the sustainability of agricultural production systems has stimulated interest in integrated production models, cultivating various species in the same area. To adopt integrated crop-livestock-forest

*Corresponding author. E-mail: andre.dominghetti@embrapa.br.

systems (ICLF), the different components of the system need to be installed optimally to maximize the yield of each component. The tree can be considered as the component that applies the greatest influence on the others, since it has a high capacity to compete for water, light and nutrients. Thus, it is fundamental to use the correct tree species and the right spacing arrangement in the area to be cultivated.

Previous research results justify greater usage of species from the *Eucalyptus* genus in ICLF systems, because of their high adaptability to various soil and climate conditions, vigorous growth, efficiency in the use of water and mineral resources, tolerance of low-fertility soils, and low to moderate rates of infestation by pests, diseases and weeds. They also produce quality timber for multiple uses, including the furniture industry (Del Quiqui et al., 2001; Silva et al., 2015). Oliveira et al. (2009) emphasized that the production of *Eucalyptus* timber for sawmills, rather than for pulp, demands a longer cutting cycle, specific silvicultural treatment and wide spacing, which alters the silvicultural management patterns that are used currently for most forest communities in Brazil, since the mainly product is pulp and paper.

The correct spacing and density of the tree component in the ICLS are of paramount importance, as they may be in single or double rows, or in in groups of more than two rows, with different spacing between plants, rows and stands. However, the number of trees per area and the spacing between them will be defined as a function of the objective of the system, considering the production of timber, width of the agricultural machinery of the property, establishment of crops with a short cycle, livestock intervention, and facilities for the pruning and harvest of timber (Balbino et al., 2011). Thus, the association of trees, pasture and agricultural crops should be in the appropriate dimensions to obtain the greatest yield of meat, grain and forest products (Montoya et al., 2000).

Studies on the responses of *Eucalyptus* trees in ICLFS are limited, given that most studies with the genus concentrate on spacing in pure forest communities, using 2 to 3 m between plants and between planting rows. Indeed, according to Oliveira et al. (2009) the spatial arrangement of trees and the maintenance of the same useful area per plant have an influence on growth characteristics and, consequently, on yield.

Thus, the objective of this research was evaluate the influence of different spatial arrangements for *Eucalyptus* trees planted in integrated crop-livestock-forest system on the growth and initial yield of three clones at Mato Grosso do Sul state, Brazil.

MATERIALS AND METHODS

Description of the experimental area

The integrated crop-livestock-forest systems were installed in January 2012 at the Embrapa Beef Cattle Research Center in Campo Grande, Mato Grosso do Sul State, the soil class of the area is a distroferric red latosol (LVdf), as described by Santos et al. (2013). The area is located between the geographical coordinates: 20°27'04" S and 54º42'57" W. The climatic pattern in the region is described, in accordance with Köppen (1948), as transition zone between Cfa and Aw wet tropical. The mean annual rainfall is 1560 mm, with a wet summer and a dry winter.

Establishing the experimental area

Soil acidity in the experimental area was corrected with a surface application of lime (3.5 t ha^{-1}), incorporated by means of a harrow with 18 discs and a width of 81 cm. Dolomitic limestone was used with calcium carbonate equivalent (CCE) of 75% (25% of calcium oxide and 11% of magnesium oxide). Due to the complexity of the integrated crop-livestock-forest system, a decision was taken to correct soil acidity to meet the needs of the most demanding crop, making the soil saturation volume (V%) reach 60 to fulfill the nutritional requirements of the soybean crop. Soybean (*Glycine max* cv. BRS 285) was seeded in November 2011 in a conventional tillage system, and rows were marked out in advance for the later preparation of trenches in which *Eucalyptus* would be planted with the different spatial arrangements described subsequently.

The planting of *Eucalyptus* clones was carried out after soybean was established. Preparation and planting took place in January 2012, with the planting trenches prepared using below-ground fertilizer, applying 200 g of the formula NPK 06-30-06 with 0.5% of zinc and 0.5% of boron per meter of trench. Cover fertilization of the *Eucalyptus* plants was carried out in two plots (3 and 9 months after planting), applying formula NPK 20-00-20 with 0.5% of boron and 0.5% of zinc, at a rate of 120 g $plant^{-1}$ in each dressing, as recommended by Gonçalves (1995).

For the planting, the tube surrounding each seedling was used to open a hole with the same dimensions as the root system of the seedling. The *Eucalyptus* seedlings measured on average 30 cm in height and were irrigated on the day of planting with 2 L of water per seedling. The spacing between the stands of *Eucalyptus* was 14 m, the gap was the space occupied by soybean crop (*Glycine max* cv. BRS 285) at the first year. After the harvested soybean, millet was sown as soil mulch for later planting in no-till system.

In November 2012, the spaces between the *Eucalyptus* stands were again used for the cultivation of soybean in the summer, under no-till planting. After the soybean had been harvested, the forage grass, *Brachiaria brizantha* cv. Marandu, was sown in March 2013. Animals were put out to pasture, after the forage grass had been established, in June 2013. The first grazing in the area took place when the *Eucalyptus* trees showed diameter at breast height (DBH) bigger than 6 cm, which allowed for lower branches to be removed to obtain better quality timber and so that the animals could then move into the area.

Experimental design and treatments

A randomized block design was used, in a factorial scheme (3 × 3), with plots subdivided in time and with four repetitions. Three *Eucalyptus* clones were used: Urocam VM1 (*Eucalyptus urophyla* × *E. camaldulensis*), Grancam 1277 (*E. grandis* × *E. camaldulensis*) and Urograndis I144 (*E. urophyla* × *E. grandis*) and three spatial arrangements (single, double and triple row). Each experimental plot had 10 plants in the single row arrangement, 20 in the double rows and 30 in the triple rows.

In the single row arrangement, the spacing was 14 m between stands and 2 m between trees in the row (14 m × 2 m), totaling 357 trees ha^{-1}. In the double row arrangements, spacing of 14 m between stands of trees were used, 3 m between rows within the

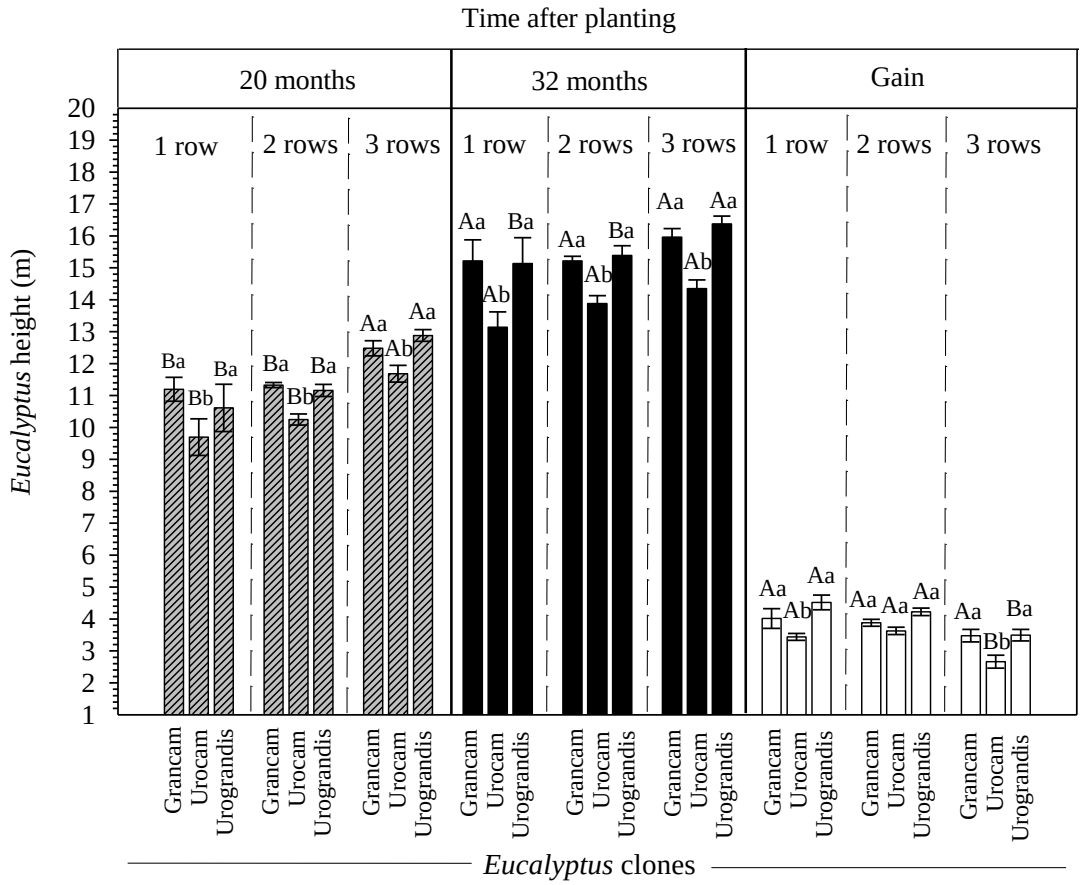

Figure 1. *Eucalyptus* height clones in three spatial arrangements assessed 20 and 32 months after planting and gain, between the measurement time. Different lowercase letters indicate significant difference (p≤0.05) among different clones within the same spatial arrangement, while different uppercase letters indicate significant difference (p≤0.05) among different arrangements, by ANOVA and Scott-Knott test of means. The error bars are standard errors.

stand and 2 m between trees in the row (3 m × 2 m) + 14 m, totaling 588 trees ha^{-1}. The triple rows were planted with a space of 14 m between stands, 3 m between rows within the stand and 2 m between trees in the row [(3 m + 3 m) × 2 m] + 14 m, totaling 750 plants ha^{-1}. The areas occupied by the tree component were 14.3, 29.4 and 40.0% in the single, double and triple row arrangements, respectively, in a total area of 3 ha.

Variables analyzed

Evaluations of total plant height, DBH, and grades for straightness and forking, and cylindricity, according to the scale proposed by Malinovski et al. (2006), were performed at 20 and 32 months after planting the trees. From the height and DBH data, the volume of timber per plant was calculated (using the form factor equal to 0.45) as was the volume of timber per hectare.

Statistical analysis

The data were submitted to analysis of variance and, when there were significant differences between means up to 5% significance, the means were compared by Scott Knott test with 5% probability, using SISVAR software (Ferreira, 2008).

RESULTS AND DISCUSSION

Height and DBH

There was a significant interaction between the spatial arrangement treatments and the *Eucalyptus* clones in both the tree heights and the height gains from 20 to 32 months after planting. Clone Urocam was inferior in tree height in all three spatial arrangements at both evaluation times (Figure 1).

All clones had greater height at 20 months after planting in the triple row arrangement compared with the single and double row arrangements (Figure 1). However, at 32 months after planting, only clone Urograndis had significantly greater height in triple rows than the other arrangements. The height gain between

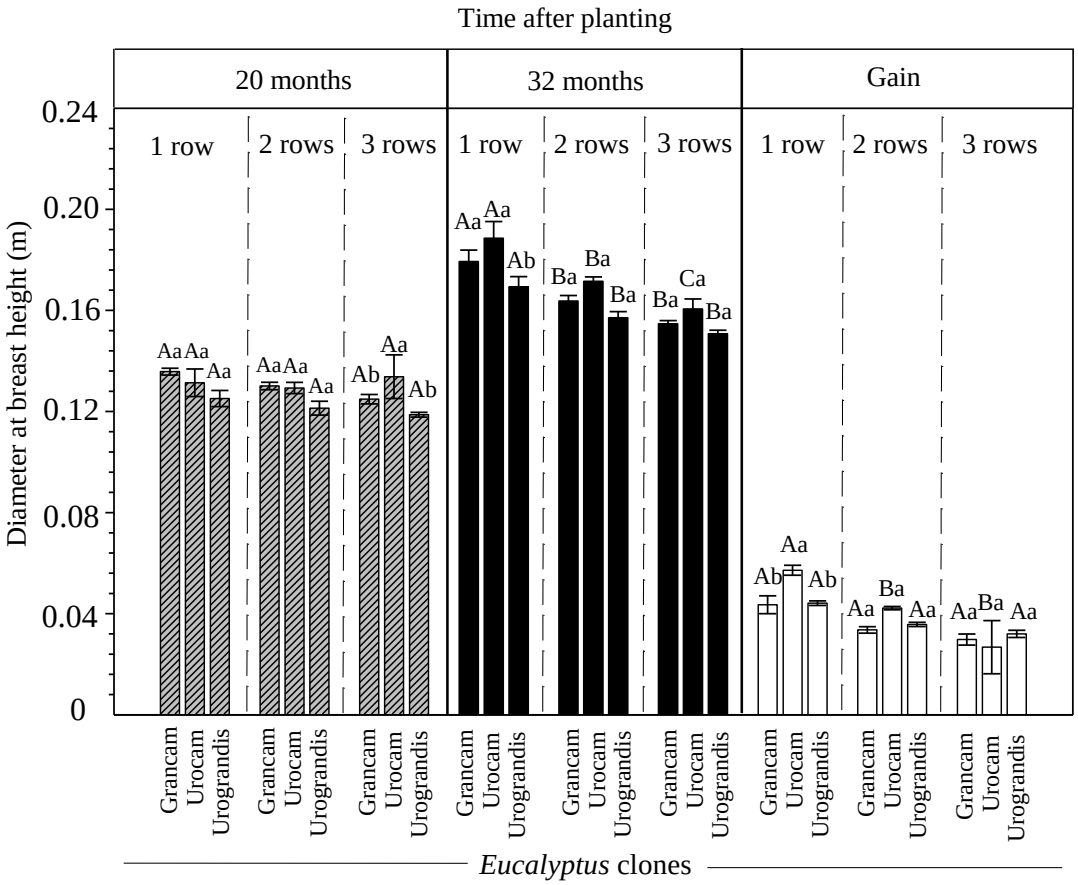

Figure 2. Diameters at breast height (m) of Eucalyptus clones in three spatial arrangements measured at 20 and 32 months after planting, and gain between the measurement time. Different lowercase letters indicate significant difference (p≤0.05) among different clones within the same spatial arrangement, while different uppercase letters indicate significant difference (p≤0.05) among different arrangements, by ANOVA and Scott-Knott test of means. The error bars are standard errors.

20 and 32 months of clones of Urocam and Urograndis was less in triple rows than in single rows and double rows, possibly due to greater competition for water, light and nutrients. The increase in tree height is linked to their genetic constitution, their tolerance of competition and their efficiency in using environmental resources (Binkley, 2004; Macedo et al., 2006; Magalhães et al., 2007; Boyden et al., 2008), explaining the influence of tree spatial arrangement on their increase in height. Although there are greater heights of plants in denser arrangements, these arrangements often show a reduction in mean height indices over time due to the greater concentration among trees in the competition for environmental resources (Bernardo, 1995; Kruschewsky et al., 2007). Silva (2005) highlighted that variations in the gains in *Eucalyptus* plant height are more accentuated after the third year. After this age, there are significant responses to the spacing used and, according to Garcia (2010), the increase in the useful area per plant provides smaller gains in height and greater gains in stem diameter. However, in the present work, the differences in the height of the clones as a function of the spatial arrangement were more marked up to 20 months after planting; after 32 months there was a reduction in the range of the values.

Clone Urocam possessed greater DBH than the other clones at 20 months after planting in triple rows (Figure 2). DBH did not differ significantly between clones in the other spatial arrangements. Clone Urograndis had lower DBH than the other clones after 32 months in the single row arrangement. DBH gains from 20 to 32 months did not differ significantly between clones within a spatial arrangement, except that clone Urocam had greater gain than the other clones in the single row arrangement.

The different spatial arrangements did not affect DBH of any of the clones at 20 months after planting. However, at 32 months after planting, highest DBH was found in the single row arrangement for all three of the clones (Figure 2). The greatest DBH gain from 20 to 32 months was in clone Urocam planted in the single row

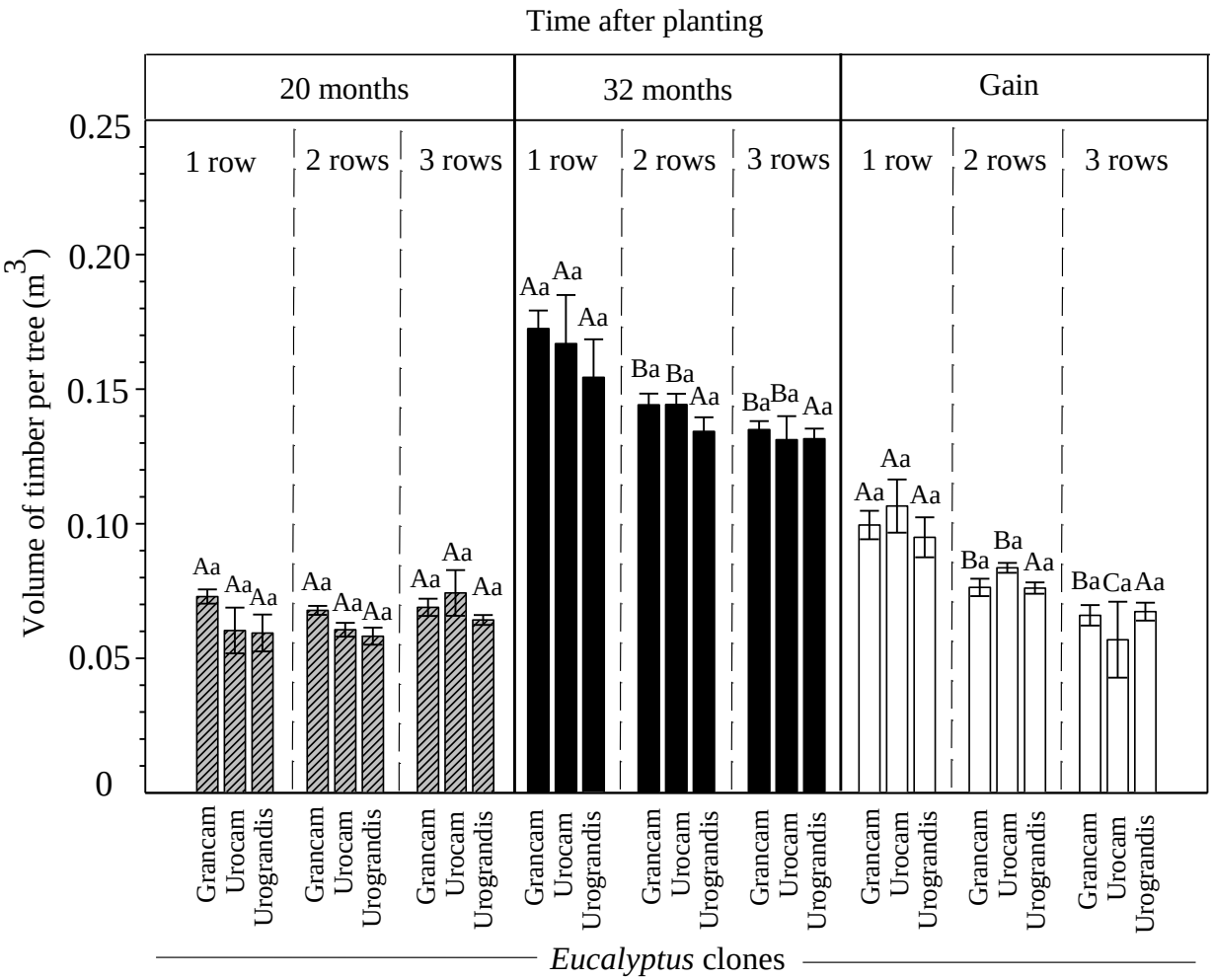

Figure 3. Volume of timber per tree (m^3) of *Eucalyptus* clones in three spatial arrangements assessed at 20 and 32 months after planting, and gains between the measurement time. Different lowercase letters indicate significant difference (p≤0.05) among different clones within the same spatial arrangement, while different uppercase letters indicate significant difference (p≤0.05) among different arrangements, by ANOVA and Scott-Knott test of means. The error bars are standard errors.

arrangement at 32 months age.

The DBH results were therefore linked to the spatial arrangement, noting that the single row arrangement, which provides the greatest useable area per plant, provided the highest DBH values. Often, the greater the area available for plant growth, the greater will be the stem diameter (Berger et al., 2002; Sanquetta et al., 2003; Lima, 2010). The increase in the useable area per plant results in greater availability of environmental resources such as water, light and nutrients.

Volume of timber per tree and volume per hectare

Timber volume per tree did not differ significantly (p>0.05) among clones (Figure 3). However, at 32 months after planting, clones Grancam and Urocam had higher volume per tree in a single row arrangement than in the other spatial arrangements. The gain in timber volume per tree from 20 to 32 months after planting reflected the volume data at 32 months, with the greatest gains for clones Grancam and Urocam obtained in the single row arrangement. Spatial arrangements that provide a larger useable area per plant often increase the basic density of timber (Berger et al., 2002), which is desirable in trees from integrated crop-livestock-forest systems because the timber produced in these systems is used preferably for sawn logs from sawmills.

The triple row arrangement provided the lowest tree volume for two of the clones, probably due to greater competition among plants for water, light and nutrients (Martins et al., 2009; Reiner et al., 2011). However, the reduction in planting space can increase timber production by area (Reiner et al., 2011).

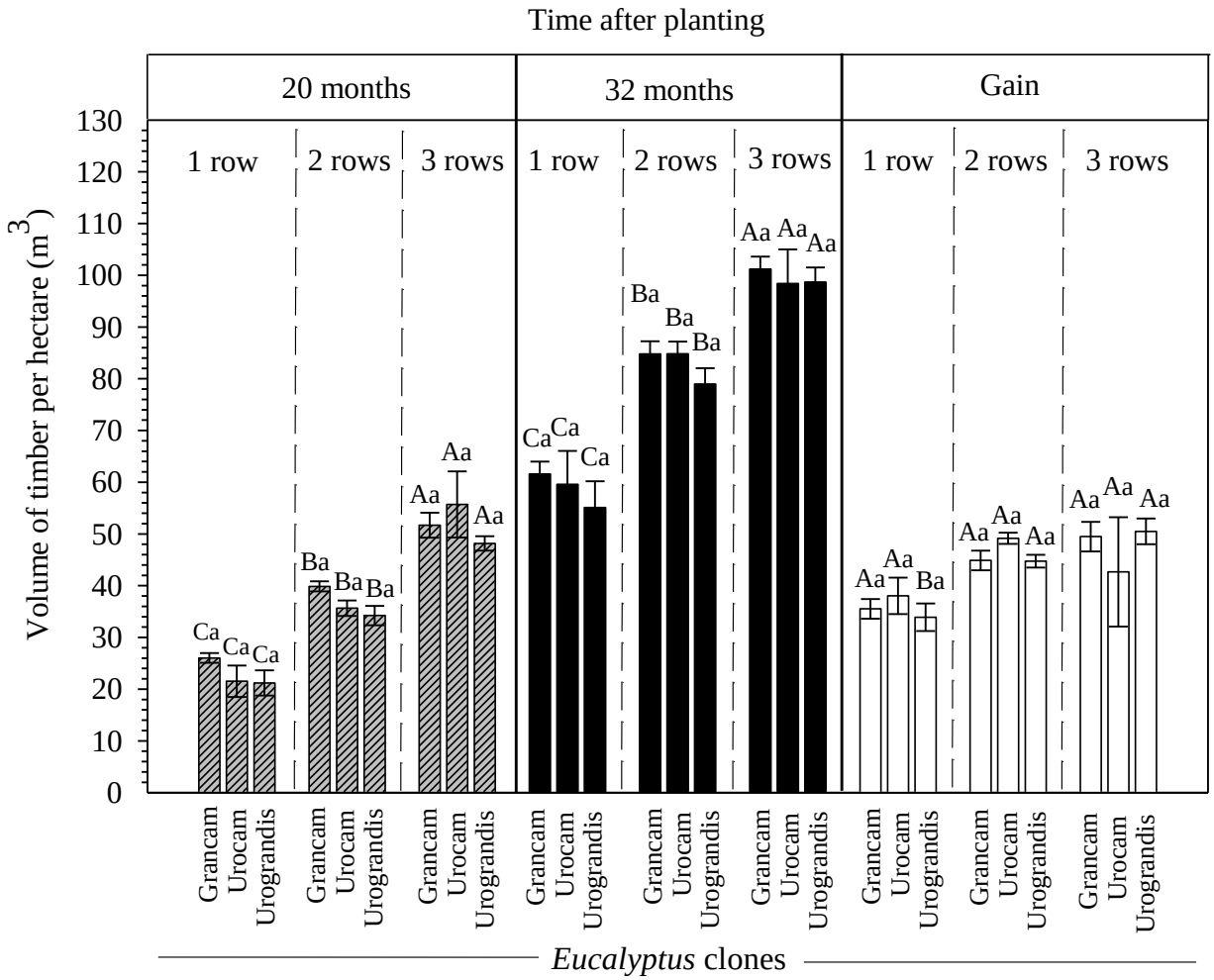

Figure 4. Volume of timber per hectare (m^3) of *Eucalyptus* clones in three spatial arrangements assessed 20 and 32 months after planting, and gains between the times of evaluation. Different lowercase letters indicate significant difference ($p \leq 0.05$) among different clones within the same spatial arrangement, while different uppercase letters indicate significant difference ($p \leq 0.05$) among different arrangements, by ANOVA and Scott-Knott test of means. The error bars are standard errors.

Timber volume per hectare at both 20 and 32 months after planting, as well as the gain between these times, did not differ significantly among the three clones (Figure 4). However, the triple row arrangement provided greater timber yield per hectare at both evaluation times in all clones, corroborating previous results (Leles et al., 2001; Gonçalves and Mello, 2004; Muller et al., 2005).

Although the DBH of trees in the single row arrangements was significantly greater than that of plants in triple rows, this was not enough to compensate for the decrease in the number of trees per hectare, as found previously by Schneider et al. (2000), Muller et al. (2005), Leite et al. (2006), Garcia (2010), Santos (2011) and Paulino (2012). This demonstrates that there is a strong relationship between the number of plants per area and the base area of the trees. However, the difference in volume of timber per hectare between less- and more-densely planted spatial arrangements tends to fall as the tree community ages. This is because of greater competition for water, light and nutrients among the individual trees in denser plantings, which reduces their growth rate. More-densely planted communities reach site capacity before wider-spaced plantings, generating a plateau in the dimensions of the timber products (Oliveira et al., 2009). Initial differences in yield dwindle as the more widely spaced plants consume the available natural resources, sometimes resulting in the same yield per hectare across all the arrangements (Berger et al., 2002).

Straightness and forking are two of the main parameters that determine timber quality for an individual tree (Mattos et al., 2003). Clone Grancam possessed superior trunk quality at both time points and in all three spatial arrangements, except that it had similar quality to clone Urograndis in the triple row arrangement at 20

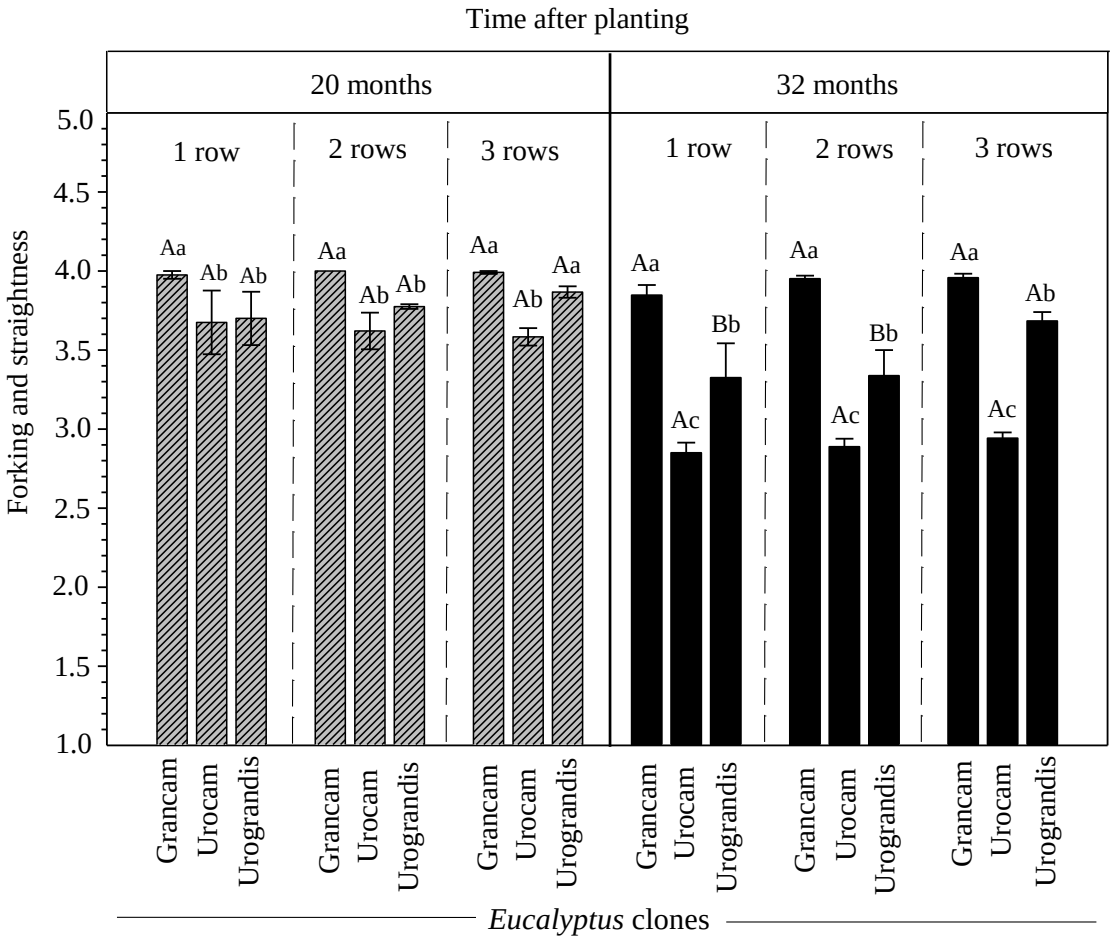

Figure 5. Forking and straightness of *Eucalyptus* clones in three spatial arrangements measured at 20 and 32 months after planting. Different lowercase letters indicate significant difference (p≤0.05) among different clones within the same spatial arrangement, while different uppercase letters indicate significant difference (p≤0.05) among different arrangements, by ANOVA and Scott-Knott test of means. The error bars are standard errors.

months after planting (Figure 5).

Spatial arrangements did not affect straightness and forking at 20 months but, at 32 months after planting, clone Urograndis had higher trunk grade in the triple row arrangement than the other arrangements (Figure 5). This may possibly have been influenced by the higher grades of trees in the central row of the stands, with trees in the outer rows forcing the central trees to grow straighter in shadier conditions.

Cylindricity of the trunk did not differ significantly at either evaluation time, either between clones or between spatial arrangements (Figure 6). However, the cylindricity of a tree trunk can be influenced by age, with older plants often having a more cylindrical trunk (Scolforo and Figueiredo, 1993). Less-dense spatial arrangements sometimes make it possible for trunks to develop with less cylindricity according to Grosser (1980), cited by Scanavaca and Garcia (2003). Nevertheless, in this work, no influence of the spatial arrangement on *Eucalyptus* trunk cylindricity was detected up to 32 months after planting.

Conclusions

Clones Grancam and Urograndis had greater tree height than Urocam when planted in single, double or triple row arrangements. Clone Grancam had the highest final grade of trunk straightness and forking, followed by clone Urograndis, in all three arrangements. Growth of the *Eucalyptus* clones was also influenced by the spatial arrangements. The triple row arrangement provided greater tree height only for clone Urograndis. However, the single row arrangement provided greater DBH for all three clones. The volume of timber per tree and the volume of timber per hectare were most closely

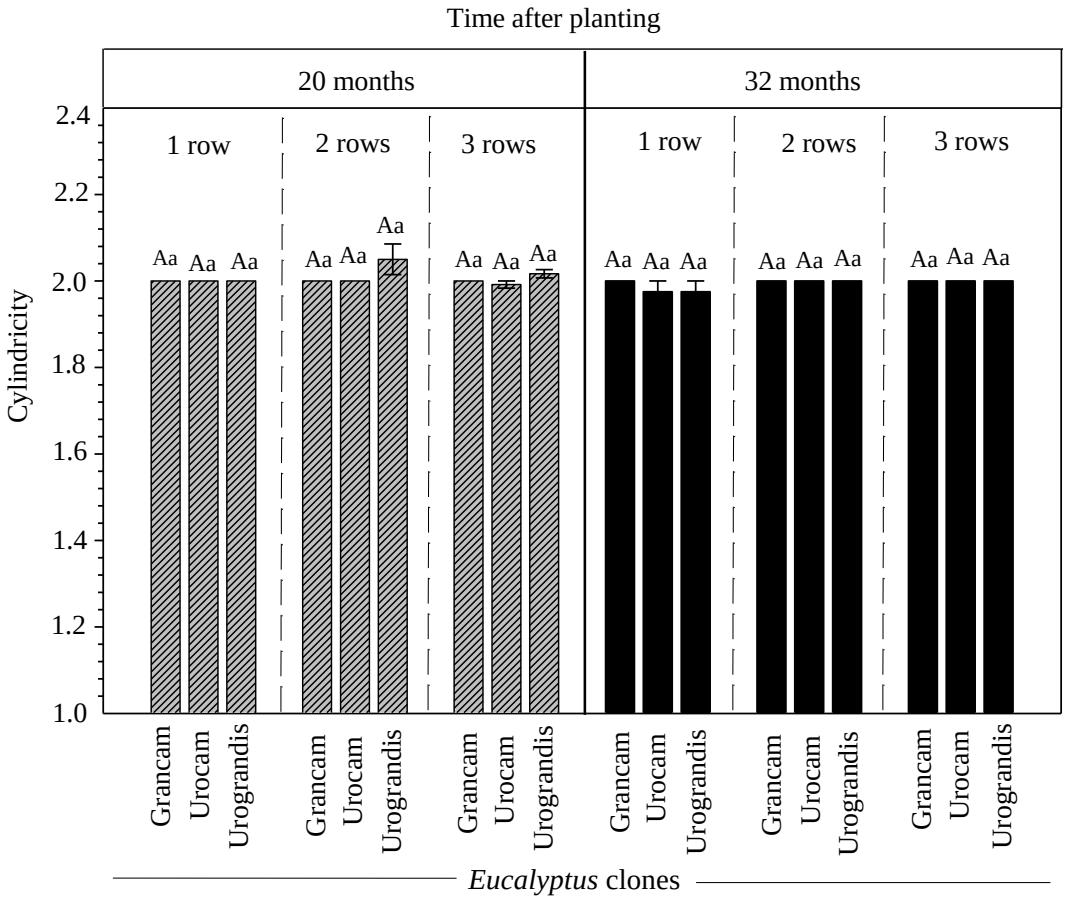

Figure 6. Cylindricity of *Eucalyptus* clones in three spatial arrangements assessed 20 and 32 months after planting. Different lowercase letters indicate significant difference (p≤0.05) among different clones within the same spatial arrangement, while different uppercase letters indicate significant difference (p≤0.05) among different arrangements, by ANOVA and Scott-Knott test of means. The error bars are standard errors.

associated with reduced planting density and increased planting density, respectively.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Sources of technical inefficiency of smallholder farmers in milk production in Ethiopia

Zewdie Adane, Kaleb Shiferaw and Berhanu Gebremedhin*

International Livestock Research Institute (ILRI), Ethiopia, P.O. Box 5689, Addis Ababa, Ethiopia.

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This paper estimates technical inefficiency in milk production of smallholder dairy farmers in the highlands of Ethiopia and identified factors associated with the observed inefficiency using a stochastic frontier production function approach. The analysis utilizes a cross-section data collected from 1,277 farm households. The result indicates a mean technical efficiency of 55%, suggesting a sizeable technical inefficiency in milk production. The result further shows that household wealth, education level and access to markets as well as institutions are the main drivers of technical efficiency in dairy production. Evidently, by improving smallholder access to market and institutions as well as investing on adult education can bring considerable gain in milk production.

Key words: Stochastic frontier production function, technical inefficiency, smallholders, milk production, Ethiopia.

INTRODUCTION

It has been well documented that rural poverty reduction is associated with growth in agricultural productivity (De Janvry and Sadoulet, 2010; Byerlee et al., 2005; World Bank, 2007). One way to increase productivity is by improving efficiency (Ferrell, 1957). The efficiency gains thus obtained could lead to resource savings that can be put into alternative uses (Bravo-Ureta and Rieger, 1991). The implication is that to bring about desirable changes in agriculture, it is important to consider the efficiency aspect.

Dairy plays an important role in the Ethiopian agricultural sector and the national economy (Tegegne et al., 2013). The sector is a source of livelihoods for a vast majority of the rural population in terms of consumption, income and employment. Recent estimates by the nation's Central Statistical Agency (CSA) indicate that there are about 55 million cattle, of which 44.6% are male and 55.4% are female (CSA 2014). The CSA survey further indicates that 2.8 billion liters of milk was produced in 2012/2013, out of which 42.3% was used for household consumption. This shows that dairy production is an important agricultural activity in the country and provides livelihood for significant proportion of smallholders.

According to FAO (2014), over the period 1993 to 2012, total annual milk production has been growing, but

*Corresponding author. E-mail: b.gebremedhin@cgiar.org. Tel: +251 116172405.

JEL codes: C18 Q12 Q13.

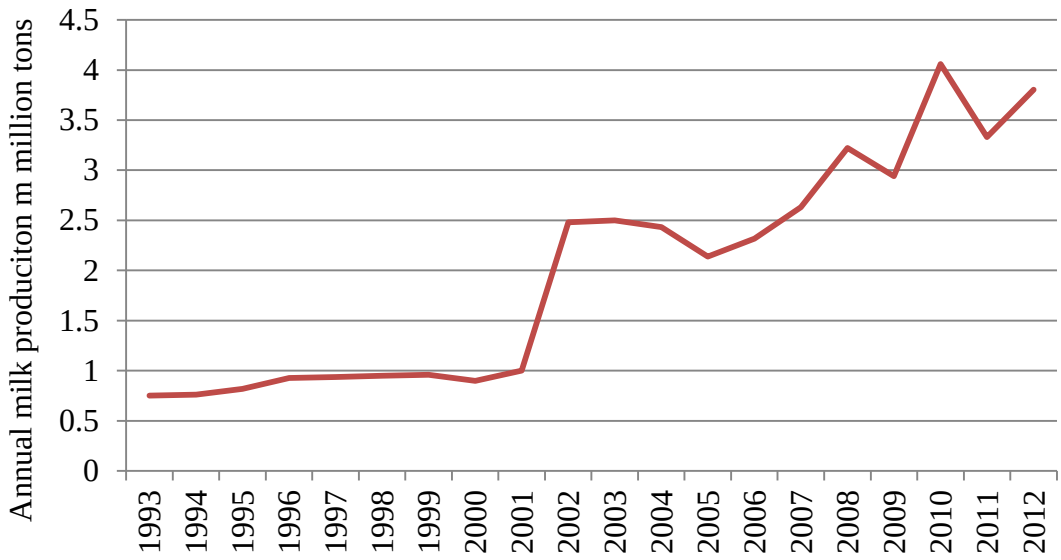

Figure 1. Trend in milk production in Ethiopia between 1993 and 2012. Source: FAOSTAT, 2014.

at a moderately slow rate (Figure 1). Mohamed et al. (2004) attributed the growth mainly to technological interventions and policy reforms. However, Nathaniel et al. (2014) argued that since dairy inputs and services provisions are still at infant stage and the expansion of improved dairy cows is limited in the country, the increase in milk production came mainly from increased number of cows rather than increased productivity. In fact, the national estimate shows that average milk yield per cow per day for indigenous breed is low, at about 1.37 L.

This calls for understanding of the efficiency level of the dairy sector and identifying factors associated with inefficiency. The result of such analysis is expected to better inform research, development and policy decisions and also help to prioritize interventions in the sector. Although, there exist several studies on efficiency analysis of Ethiopian agriculture (Alene and Hassan, 2006; Haji, 2006; Makombe et al., 2011; Nisrane et al., 2011), to the best of the author's knowledge, there exists no such study on milk production. This study, therefore, tried to contribute to the existing gap in knowledge on efficiency factors in dairy production in Ethiopia.

Approaches for measuring efficiency

There are at least three different types of efficiency measures in economic theory. These are technical efficiency, allocative efficiency and economic efficiency. Technical efficiency measures the success of a firm in applying the best practice so as to produce the maximum attainable output level from a given input set at a given level of technology while allocative efficiency measures a firm's success in choosing optimal set of inputs consistent with relative factor prices (Farrell, 1957). On the other hand, a firm's economic efficiency measures the overall efficiency which is defined as the product of technical and allocative efficiency (Bravo-Ureta and Rieger, 1991). This paper exclusively focuses on measuring technical efficiency in milk production in Ethiopia.

Much effort has been exerted to develop the best methodology for measuring efficiency. Following Farrell's (1957) seminal paper on efficiency measurement, a number of approaches have been proposed. The two most prominent and widely applied methods are the Stochastic Frontier Analysis (SFA) and the Data Envelopment Approach (DEA). The SFA has been independently developed by Aigner et al. (1977) and Meeusen and van den Broeck (1977). Charnes et al. (1978) then proposed the DEA as the main alternative to SFA. These methods have been compared for their strengths and weaknesses and were applied for investigating efficiency under different assumptions in various countries and sectors.

SFA is a parametric approach in the sense that it follows a defined production or cost function. The function in the model involves a composite error term that accounts both for the statistical noise in the data as well as the inefficiency in production (Erkoc, 2012). Therefore, any deviation from the efficient frontier (ideal output from a given input set) is attributed to both the stochastic disturbances such as errors in measurement, topography, weather and effects of unobserved and uncontrollable variables and to the individual-specific factors that affect the inefficiency (Coelli, 1995).

Once the individual inefficiency levels are estimated, the major factors causing the inefficiency can easily be identified from the inefficiency model. One of the drawbacks of this method is the imposition of restrictive

assumptions on the functional form of the production function and the distribution of random errors. Nonetheless, SFA has been widely applied for analyzing agricultural efficiency both in developed and developing countries. Greene (2008) provided a detailed and comprehensive discussion on different variants of SFA models.

DEA on the other hand tackles the same question with a non-parametric and non-stochastic method. DEA employs linear programming methodology to construct the efficient frontier based on available information on the firms' inputs and outputs in the data. Thus, it is free from functional form restriction and distributional assumptions which are rather important in SFA. The lack of assumptions on the underlying production technology makes DEA suitable to accommodate problems that may arise from such restrictions (Erkoc, 2012).

However, the use of linear programming in DEA which does not allow decomposing the stochastic noise from the inefficiency effect is one major deficiency of the approach. Those who are not on the efficient frontier are considered to be inefficient; and such deviations are attributed only to inefficiency. Furthermore, the fact that this method is non-parametric makes it vulnerable to measurement errors and outliers. As a result, it has been argued that DEA is less convenient for applications particularly in developing country agricultural setting where data quality is doubtful and such measurement errors are much pronounced (Erkoc, 2012; Coelli, 1995). A book length discussion on DEA can be found in Coelli et al. (2005).

MATERIALS AND METHODS

Model specification

There is always a trade-off as to whether to choose the stochastic frontier approach which is prone to misspecification bias or the DEA which suffers from measurement errors (Erkoc, 2012). However, a bulk of the literature suggests that as long as there is no severe misspecification problem, stochastic production frontier method is more suitable for efficiency analysis in a developing country agriculture setting where there are serious issues with data quality and accuracy (Coelli, 1995). Therefore, based on the dominant discourse in the efficiency debate, this study applies the stochastic frontier approach to assess the efficiency level and identify factors that lead to inefficiency of smallholder dairy producers.

The stochastic production frontier analysis begins with specifying a log-linear production function both in input and output as follows:

$$Y_i = \alpha + x_i^{'}\beta + \varepsilon_i \quad (1)$$

$$\varepsilon_i = v_i - u_i \quad (2)$$

Where Y_i represents the natural logarithm of observed output of the i^{th} household, x_i is a vector of the natural logarithms of N inputs for the i^{th} household and β is the vector of unknown technology parameters. The error term ε_i is composed of two components u_i and v_i . The first component u_i is a non-negative random variable measuring the inefficiency. The second error component, v_i, on the other hand, is a stochastic disturbance term assumed to be independently and identically distributed as $N(0, \sigma_v^2)$ over the observations. To form the density of Y_i in Equation 1, the joint density of ε_i needs to be computed. Following Greene (2008), this is given by:

$$f_{\varepsilon,u}(\varepsilon_i, u_i) = f_u(u_i) f_v(\varepsilon_i + u_i) \quad (3)$$

Integrating Equation 3 with respect to u_i then gives the marginal density of ε_i. This measures the contribution of observation i to the log-likelihood (ibid):

$$lnL_i(\alpha, \beta, \sigma_v^2, \sigma_u^2 | Y_i, X_i) = ln\, f_\varepsilon(Y_i - \alpha - \beta X_i\, \alpha, \beta |\, \sigma_v^2, \sigma_u^2) \quad (4)$$

In the literature, the inefficiency term u_i may take exponential (Meeusen and van den Broeck, 1977), half-normal (Aigner et al., 1977), truncated-normal (Stevenson 1980) as well as gamma (Greene 2003) distributions. Though half normal is the most commonly used specification in cross-section studies (Coelli, 1995; Bravo-Ureta and Pinheiro, 1993; Bauer, 1990) the assumption of zero mean for u_i is unnecessary restriction (Stevenson (1980). Thus, u_i in Equation 4 is assumed to have truncated-normal distribution of $U_i \sim N(\mu_i, \sigma_u^2)$, $u_i = |U_i|$. Furthermore, the model assumes heterogeneity in u_i and following Kumbhakar et al. (1991) and Huang and Liu (1994), exogenous variables that influence efficiency are introduced as follows:

$$\mu_i = z_i'\eta \quad (5)$$

Where $\mu_i$is variable mode of the truncated normal distribution, z_i is a vector of household specific explanatory variables that affect household level inefficiency and η is unknown vector of coefficients to be estimated. Then, the log-likelihood will have the following form (Greene 2008):

$$\ln L(\alpha, \beta, \sigma, \lambda, \eta) = -N\left[\ln\sigma + \tfrac{1}{2}\ln 2\pi + \ln\Phi(\mu_i/\sigma_u)\right] + \sum_{i=1}^{N}\left[-\tfrac{1}{2}\left(\tfrac{\varepsilon_i+\mu_i}{\sigma}\right)^2 + \ln\Phi\left(\tfrac{\mu_i}{\sigma\lambda} - \tfrac{\varepsilon_i\lambda}{\sigma}\right)\right]' \quad (6)$$

Where $\lambda = \sigma_u/\sigma_v$, $\sigma^2 = \sigma_u^2 + \sigma_v^2$, $\sigma_u = \lambda\sigma/\sqrt{1+\lambda^2}$ and $\varepsilon_i = Y_i - \alpha - x_i'\beta$

The log-likelihood function in Equation 6 can then be estimated using Stata (Belotti et al., 2013). Once the parameters are estimated, the technical efficiency (TE) of individual household is given as $TE_i = \exp(-u_i)$. Since u_i is not directly estimated from Equation 6, the method proposed by Jondrow et al. (1982) will be used to extract the estimate of $u_i$which is given by Kumbhakar and Lovell (2000) as:

$$E(u_i|\varepsilon_i) = \sigma_*\left[\frac{\tilde{\mu}_i}{\sigma_*} + \frac{\phi(\tilde{\mu}_i/\sigma_*)}{1 - \Phi(-\tilde{\mu}_i/\sigma_*)}\right] \quad (7)$$

Where $\tilde{\mu}_i = (-\varepsilon_i\sigma_u^2 + \mu\sigma_v^2)/\sigma^2$and $\sigma_* = \sigma_u\sigma_v/\sigma$. Technical efficiency of farms ranges from 1 to 0. The best practice farm gets a value close to 1 and the least efficient farm gets a value close to zero.

Empirical model

The empirical version of the stochastic frontier production model employed in this paper uses semi-log-linear Cobb-Douglas production function as the basis for the analysis.

Table 1. Description of the explanatory variables in the production frontier equation.

Variable	Variable description	Expected sign
$NCOW_i$	Total number of lactating cows of the i^{th} household during the 2012/13 production season	As the number of lactating cow increase evidently more milk can be produced (+).
$LABR_i$	Total number of labour available in the i^{th} household during the 2012/13 production season for herding, milking, feeding, etc., of dairy cows	Labour is a key input in dairy production. If a household has more labour available for herding, milking, feeding, etc., it is expected that the dairy cows can be better managed leading to higher milk production (+)
$GLAND_i$	Total grazing land available to the i^{th} household during the 2012/13 production season in hectares	As the size of grazing land increase it is expected that pasture grasses available will increase which further contribute to higher milk production (+).
$CROPRD_i$	Amount of crop residue of i^{th} household from own production available for livestock during the 2012/13 production season in kilograms	Crop residue from own production is another important input in the rural part of the country. Thus, it is expected that keeping other things constant a household with more crop residue will produce more milk. (+)
$PSUPP_i$	Total cost of purchased supplement for dairy cows of the i^{th} household during the 2012/13 production season in ETB	Supplements like concentrate feeds and industrial by-products are expected to increase milk production as they provide more nutrient to the cow (+)
$PFORAGE_i$	Total cost of purchased forage for dairy cows of the i^{th} household during the 2012/13 production season in ETB	In addition to the crop residue farmers sometimes purchase forage either to avail more feed to cows or to compensate for shortage of crop residue and pasture grasses. Thus, the effect on milk production can be either positive or negative (+/-).
$HELH_i$	Total health expenditure (drugs and expenses on vet services) the i^{th} household incurred for dairy cows during the 2012/13 production season in ETB	In the rural setting farmers visit veterinary clinics or buy vet drugs whenever animals are inflicted with disease. Thus, higher health expenditure could be associated with less milk production (-)
CCOW_i	Dummy variable that takes 1 if the household has crossbred cow and 0 otherwise	The sample households keep both local and crossbred dairy cows. This variable is used to account for yield differential due to genetic factors (+)
AEZ_i	Dummy variable that takes 1 if the agro-ecology zone is highland and 0 otherwise.	In Ethiopia, highlands are more favorable for dairy production than the lowlands partly due to feed, heat and water stresses (+)

$$\ln TOTM_i = \beta_0 + \beta_1 ln\, NCOW_i + \beta_2 lnLABR_i + \beta_3 ln\, GLAND_i + \beta_4 ln\, CROPRD_i + \beta_5 ln[max(PSUPP_i, 1 - V_1)] + \beta_6 ln[max(PFORAGE_i, 1 - V_2)] + \beta_7 ln\,[max(HELH_i, 1 - V_3)] + \beta_8 \mathrm{CCOW}_i + \beta_9 \mathrm{AEZ}_i + \varepsilon_i \quad (8)$$

Where; $TOTM_i$ = total annual milk production by the i^{th} household during the 2012/13 production season[1] in liters; V_i = one if the respective cost item is positive and zero otherwise; β_i are unknown coefficients to be estimated and ε_i is the compound error term as specified in Equation 2. The explanatory variables in Equation 8 and their expected signs are described in Table 1. The semi-log-linear specification is selected because it improves the normality of the error term and reduces the effect of outliers on the estimation outcomes, while we had to retain the binary explanatory variables as dummy variables (Wooldridge, 2002). To capture the possible effects of the exogenous variables that affect technical inefficiency, the following model is specified.

$$\mu_i = \eta_0 + \eta_1 HSEX_i + \eta_2 HAGE_i + \eta_3 HAGESQ_i + \eta_4 HEDUC_i + \eta_5 DWT_i + \eta_6 DDA_i + \eta_7 HWEAL_i + \omega_i \quad (9)$$

Where; η_i's are unknown coefficients of the inefficiency effect to be estimated corresponding to each exogenous variable described in Table 2 and ω_i is a stochastic error term that captures the effect of unaccounted household specific variables on technical inefficiency. Following Wang and Schmidt (2002), Equations 8 and 9 are estimated simultaneously.

[1] The 2012/13 production season in Ethiopia is the period that extends from 1 June 2012 to 31 May 2013.

Table 2. Description of the explanatory variables in the technical inefficiency model.

Variable name	Variable description	Expected sign
$HSEX_i$	Sex of the household head (1 Male, 0 Female)	The sex of the household head could have either positive or negative effect on the inefficiency (-/+)
$HAGE_i$	Age of the household head (in years)	It is expected that older farmers would have more experience on dairy production which would lead to less inefficiency (-)
$HAGESQ_i$	Age square of the household head	The relationship between inefficiency and age of the household head may not be linear. Age of the household head increase efficiency only until a certain point and beyond that point it decrease efficiency (+)
$HEDUC_i$	Highest education level of the household head. If the household head had no formal education this variable takes zero value	The more educated the household head the more likely that he/she can process information and apply trainings and advises of the extension system more effectively which could lead to low inefficiency (-)
DWT_i	Walking distance to district/woreda town from the household (in minutes)	Remote households with respect to major markets and administrative centers would have less access to market and institutions which could be associated with inefficiency (+)
DDA_i	Walking distance to Development Agent's (DA) office (in minutes)	As the distance to the DA office increase it is more likely that the household would get less extension service which would lead to higher inefficiency (+)
$HWEAL_i$	Total wealth of household i in ETB	We anticipate wealthy households to be less inefficient as they are more likely to adopt new technologies readily than poor households (-)

Data

The study is based mainly on a cross-sectional baseline data collected by the LIVES[2] project for the 2012/13 production year. The data was collected from February to April 2014 from randomly selected rural households in four regions of Ethiopia (Amhara, Oromia, SNNPR and Tigray). These four regions jointly constitute the largest share of the nation's crop and livestock productions and cover the major agro-ecologies of the country. From the randomly selected respondents, a total of 1,277 milk producers in a mixed crop-livestock agro-ecological setting have been considered for this analysis.

RESULTS

Descriptive result

The descriptive result show that out of the sampled households, only 11.1% (142) are female headed (Table 3). In terms of agro-ecology, about 22% of the sample households are located in lowland areas while the remaining 78% lives in the highlands where it is relatively favorable for milk production. About 93% (1,188) of the households own only local breed cows. This is consistent with the national estimate where the overwhelming majority of cow population is of the local breed.

On the other hand, on average, the sample households own less than two cows and produce about 322 L of milk during the target production year (Table 4). On average, a household has 2 household members who could readily be engaged in herding, feeding, milking and managing the dairy cows. In the Ethiopian rural setting, it is not uncommon to observe young people, mainly boys, to be involved in herding cows and the female do the milking. Ethiopian smallholder farmers mainly depend on green pasture measured in this paper in terms of size of grazing land per household and residue from own crop production to feed their animals (Tegegne et al., 2013). The implication is that total grazing land and crop residue from own production are the major inputs for dairy production. In this regard, the data shows that on average a household had about 0.15 hectare of grazing land for his/her dairy cows. The data further reveals that

[2] LIVES - Livestock and Irrigation Value Chains for Ethiopian Smallholders – is a project engaged in a research for development activity in order to support the development of commodity value chains in several livestock and irrigated crops in the four major regions (Amhara, Oromia, SNNPR and Tigray) of Ethiopia. It is financed by the Canadian Department of Foreign Affairs, Trade and Development (DFATD) and implemented by the International Livestock Research Institute (ILRI) in collaboration with the International Water Management Institute (IWMI) and Ethiopian partners.

Table 3. Summary of descriptive statistics of the dummy variables.

Variable	Category	Frequency	Percent	Cumulative
	Female	142	11.12	11.12
$HSEX$	Male	1135	88.88	100.00
	Total	1277	100.00	100.00
	Has no crossbred cow	1,188	93.03	93.03
$CCOW$	Has crossbred cow	89	6.97	100.00
	Total	1277	100.00	100.00
	Lowland	279	21.85	21.85
AEZ	Highland	998	78.15	100.00
	Total	1277	100.00	100.00

Table 4. Summary of descriptive statistics of the continuous variables.

	Obs	Mean	*Std. dev.*	Minimum	Maximum
$TOTM$	1277	321.9 453	427.4399	2.5	5040
$NCOW$	1277	1.403289	0.7539375	1	8
$LABR$	1277	1.618432	1.099241	0.2141328	14
$GLAND$	1277	0.1530393	0.2647411	0.0001766	3.8391
$CROPRD$	1277	1396.972	2563.348	3.2	30000
$PFORAGE$	1277	162.814	437.8954	0	4000
$PSUPP$	1277	129.1633	536.2894	0	8750
$HELH$	1277	36.77608	91.80133	0	1200
$HAGE$	1277	45.76899	12.0314	20	90
$HEDUC$	1277	2.510572	3.191032	0	15
DWT	1277	162.3602	116.9535	5	760
DDA	1277	30.81844	31.31202	0	240
$HWEAL$	1277	47108.56	63445.43	2080	584955

on average a household fed 1,396.9 kilograms of crop residue from own production to dairy cows during the production period.

In addition to own crop residue and green pasture, farmers also purchase forage and supplements for dairy cows. As can be seen from Table 3, during the production year farmers on average spent about 163 ETB [3] and 129 ETB on forage and supplements, respectively. Moreover, on average, farmers spent 36.8 ETB on animal health expenses during the year. This amount might seem insignificant but it should be noted that most health related services are provided by the government through the extension system free of cost or in highly subsidized manner.

The mean age of the head in the sample households is 46 years and the highest grade completed by the head is 2.5. The average wealth of a household is 47,108.6 ETB, and is highly skewed to the left. Apart from household characteristics, the geographic location with respect to institutions such as agricultural office and markets for inputs and outputs is also expected to have a bearing on the inefficiency in milk production. The data shows that 50% of the sample farmers lie within 162 and 30.8 walking minutes from the district town and development agent's office, respectively.

Econometric result

The maximum likelihood estimates of the stochastic production frontier function and the technical inefficiency model are presented in Table 5. All estimated coefficients in the production frontier have the expected signs with the exception of purchased forage. The number of cows owned during the production year, number of labor available for dairy production and management, purchased supplements such as concentrates and industrial by-products, ownership of crossbred cows and the agro-ecological zone have positive and significant effects on the amount of milk production.

[3] ETB Ethiopian Birr) is the legal currency of Ethiopia. 1ETB = 0.0496 USD as of October 30, 2014.

Table 5. Maximum likelihood estimates of the stochastic production frontier and inefficiency effects models.

Variables	Coef.	Std. Err.	z	P>|z|	[95% Conf. Interval]	
Frontier						
$lnNCOW$	0.9661175***	0.0515804	18.73	0.000	0..8650218	1.067213
$lnLABR$	0.065612*	0.0347109	1.89	0.059	-0.0024201	0.1336441
$lnGLAND$	0.0049814	0.0144493	0.34	0.730	-0.0233387	0.0333014
$lnCROPRD$	0.0247726	0.0165806	1.49	0.135	-0.0077248	0.05727
$lnPFORAGE$	-0.0057334	0.0073815	-0.78	0.437	-0.0202008	0.0087341
$lnPSUPP$	0.018285*	0.0094103	1.94	0.052	-0.0001588	0.0367289
$lnHELH$	-0.0095624	0.010551	-0.91	0.365	-0.030242	0.0111171
$CCOW$	1.19137***	0.0745044	15.99	0.000	1.045344	1.337396
AEZ	0.1239078***	0.0464481	2.67	0.008	0..0328712	0.2149444
Constant	5.430576***	0.1405915	38.63	0.000	5.155022	5.70613
Mu (inefficiency model)						
$HAGE$	-0.0106672	0.0490112	-0.22	0.828	-0.1067275	0.0853931
$HAGESQ$	-0.1520167	0.2872949	-0.53	0.597	-0.7151043	0.411071
$HSEX$	0.0001471	0.0004738	0.31	0.756	-0.0007815	0.0010756
$HEDUC$	-0.0815338*	0.0450849	-1.81	0.071	-0.1698985	0.006831
DWT	0.0019493**	0.0009811	1.99	0.047	0.0000265	0.0038722
DDA	0.0015506	0.0029306	0.53	0.597	-0.0041934	0.0072945
$lnHWEAL$	-0.5878623***	0.2364799	-2.49	0.013	-10.051354	-0.1243702
Constant	4.829882***	1.787545	2.70	0.007	1.326359	8.333405
σ_u	1.2998***	0.2320713	5.60	0.000		
σ_v	0.4312083***	0.0294664	14.63	0.000		
λ	3.014321***	0.2199617	13.70	0.000		
L. Likelihood	-1356.5460					
χ^2	835.19***					
N	1277					

*P <0.10; **P <0.05; ***P <0.01.

The five statistically significant variables determine the position of the efficient production frontier of milk production for the producers in the sample. Based on the estimated efficient frontier, the stochastic frontier methodology computes technical inefficiency levels depending on the distance of each farmer from the frontier.

The estimated coefficients of the inefficiency effect in Equation 9 are the main interest of this study. The signs of all coefficients in the inefficiency model are consistent with what is theoretically expected. The result in Table 5 indicates that coefficients associated with education, household wealth and distance to district town (proxy for access to input and output markets and institutions) were found to be statistically significant with expected signs. The log of household wealth was found to be highly significant at 1% level while distance to district town and education level of the household head were found to be significant at 5 and 10% levels, respectively. These results are consistent with the findings of Asres et al. (2013) who reported positive and significant effect of education, extension contact, farm size and off-farm income opportunities.

This study model did not detect statistically significant relationship between technical inefficiency and other household attributes such as age, sex and distance to DA post (proxy for access to extension services). Furthermore, the joint effect age and age square on technical inefficiency were found to be insignificant. However, the test of joint significance of all variables in the inefficiency model reveals that these variables are both relevant in explaining the efficiency levels of a households. The model estimates technical efficiency at household level. The result shows that on average, dairy producers are only 55% efficient when compared with the frontier (Table 6). The result further indicated that 95% of the households lie within 54 and 56% efficiency range.

The technical efficiency level found is higher than that reported by Asres et al. (2013), which reported an average technical efficiency of about 26%, based on data

Table 6. Estimate of technical efficiency.

	Obs	*Mean*	*Std. Err.*	*[95% Conf. Interval]*	
Mean efficiency	1277	0.5502247	0.005654	0.5391325	0.5613169

from three districts in North-Western Ethiopia. The same study found that only 19% of dairy producers in their study area had mean technical efficiency of more than 50%, showing significant room for improving dairy production by improving technical efficiency. In a developed country setting and a more commercialized dairy system in Pennsylvania, Wang (2001) found a mean technical efficiency of 85%, and that large farms were technically more efficient than small farms.

A number of tests were conducted to evaluate the specification of the model and reliability of results. The non-stochastic inefficiency hypothesis with a null hypothesis that the standard deviation of u_i equals zero is strongly rejected at 1% level of significance. The joint significance of the coefficient estimates for the variables in the inefficiency model have also been tested by the generalized likelihood ratio test. The null hypothesis that the coefficient estimates for the seven explanatory variable $\eta_1 = \eta_2 = \eta_3 = \eta_4 = \eta_5 = \eta_6 = \eta_7 = 0$, is rejected at the 1% level of significance. The test suggests that the combined effect of all the explanatory variables in the inefficiency model is significant although some variables are found to have individually statistically insignificant effects on technical inefficiency.

In general, the results of the above model specification test suggest that a conventional production function is not an adequate representation of the data and the inclusion of the inefficiency effect in the model is an improvement over the stochastic frontier which does not involve a model for technical inefficiency effect.

DISCUSSION

The result of the stochastic production frontier suggests that total number of lactating cows and ownership of improved cows in the herds have positive contributions to the amount of total annual milk production at household level. In addition, the agro-ecological zone in which the household resides determines the level of household milk production. Controlling for other factors, farmers who live in the highlands with more favorable rainfall and climatic conditions for dairy production produce more milk than those living in the low land areas. This could be because the heat and water stress in the dry and hot lowlands reduce milk output. This result suggests that highland and cooler areas may have better comparative advantage in milk production and that interventions may need to target these areas.

The availability of labor supply and purchased supplements are also found to be important factors for milk production at household level. This means that the higher the number of able workers per household available to manage the cows, the higher the milk output by the household. In addition, the more concentrate and other nutritious supplementary feed the household buys for the cows, the more milk output per household. This result suggests that feed and management in dairy production may be important consideration to increase milk production.

These results are consistent with other studies on dairy (Asrers et al., 2013; Lachaal et al., 2002; Kimenchu et al., 2014). The estimates of the frontier production function seem to suggest that input use and technology adoption (improved cows) primarily determine the level of milk production at household level. Furthermore, the results clearly show that external factors such as agro-ecology also determine the amount of milk output from a given input set.

More importantly, the technical inefficiency model provided important results that are relevant for research, development and policy decisions. The negative coefficients for education and wealth in the inefficiency model imply that the effects of both variables on milk production efficiency are positive. High education level is associated with low inefficiency. This could be because farmers with more years of schooling can better process information and use trainings and advice received through the extension services or other sources more effectively as compared to those who have lower education. Similarly, 'wealthier' households are more efficient as compared to their poorer counterparts. In addition, the result indicated that access to markets is a very important determinant of technical inefficiency. Those farmers who are further away from district towns are less efficient as compared to those who are relatively close, suggesting the importance of market incentives for dairy efficiency.

CONCLUSION AND IMPLICATIONS

The study used a cross section data collected from 1,277 rural farm households selected from the major four regions of the country to assess the level of technical efficiency and identify factors that are associated with the observed inefficiency in a stochastic production frontier framework. The result indicates that input use, adoption of improved technology and agro-ecology determine the amount of milk production at household level. Improving the availability of inputs and the efficiency of input markets are likely to increase milk production in the

highlands of Ethiopia. Moreover, milk production in the dairy sector can be increased by promoting improved dairy technologies including improved genetic resources. The result of the inefficiency effect model suggests that there is a room to significantly increase milk production per household by simply improving the technical efficiency. The mean efficiency of 55% implies that considerable gain in milk production is possible using the same amount of resources and technology. Education is an important variable for dairy efficiency. Our results imply that the education system should take into account the basic education needs of farmers whose literacy can be improved through formal and informal education. Targeted trainings and other capacity development activities may also be used to counter the negative effect of low literacy. Another short run remedy is to provide practical training on milk production and dairy management to farmers with no or low education. The current practical-oriented rural adult education programs seem to be appropriate interventions and move in the right direction, perhaps, not only for dairy but to improve agricultural efficiency in general. The need to improve infrastructure for increased access to major markets and institutions should also be a point of attention for policy.

Conflict of interests

The authors declare that no conflict of interest exists in relation to the content of the article.

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Isolation, identification and *in vitro* evaluation of *Bacillus* spp. in control of *Magnaporthe oryzae* comparing evaluation methods

Ivaneide de Oliveira Nascimento[1]*, Antônia Alice Costa Rodrigues[1], Flavio Henrique Moraes[2], Flávia Arruda de Sousa[1], Marta Cristina Filipe Corsi[3] and Aricléia de Moraes Catarino[1]

[1]Post-Graduation Program of Agroecology, Maranhão State of University, Campus São Luís, São Luís, Maranhão State, Brazil.
[2]Centro Universitário do Maranhão – UNICEUMA, São Luís, Maranhão State, Brazil.
[3]Embrapa Arroz e Feijão, Santo Antônio do Góias, Goiânia State, Brazil.

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Environmentally friendly technologies, such as the use of bacteria (e.g. *Bacillus* spp.) to control fungal diseases of rice (*Oryza sativa* L.), represent a promising alternative for the sustainability of agricultural ecosystems. The present work aimed to isolate, identify, and evaluate (*in vitro*) various *Bacillus* spp. from the rice phylloplane for their potential to control the rice fungus *Magnaporthe oryzae*. Samples were taken from the phylloplane of healthy young rice plants growing in commercial fields in ten municipalities of Maranhão (Brazil), and *Bacillus* spp. were subsequently isolated and molecularly identified. Both experiments utilized a randomized design, and data were submitted to analysis of variance as well as means comparison by the Tukey test. Twelve bacterial isolates were obtained and identified. Using a control isolate (B25), four *in vitro M. oryzae*-inhibition methods were compared, with the 'circle' method ultimately providing the highest mycelium growth inhibition. The most promising experimental isolates for biological control were then identified as *B. methylotrophicus* isolates B41, B31, and B22, which achieved 90.41, 69.47, and 67.55% inhibition, respectively. This study demonstrates the potential of Maranhão *Bacillus* spp. isolates for use as biological fungal-control agents.

Key words: biological control, pathogen, rice.

INTRODUCTION

Oryza sativa L., cultivated worldwide and contributing significantly to global energy intake, is subject to attack by various fungal diseases which affect crop yield and seed quality. Notable among these, the rice panicle and

*Corresponding author. E-mail: ivaneide_agro@yahoo.com.br.

leaf blast fungus *Magnaporthe oryzae* B. Couch is extensively distributed and has significant destructive power under the right conditions. In Brazilian agriculture (especially on highly-irrigated farmland), this fungus is one of the main factors affecting rice productivity and reproductive potential (Lobo, 2008).

Chemical antifungal agents are the most widely used countermeasures against crop disease, but the indiscriminate use of agricultural pesticides presents several environmental problems (e.g. contamination of food, soil, water, and animals; poisoning of farmers; and biological imbalance due to altered cycling of nutrients and organic matter (Bettiol and Ghini, 2001), as well as promoting pesticide resistance. Novel alternative agricultural disease control methods are required to maintain the sustainability of agricultural ecosystems. Sustainable agriculture requires strategies which increase food production without damaging the environment and public health, and which are also appropriate within the economic, ecological, social and political contexts of each region. The use of biological agents is one potential alternative which achieves these objectives. According to Filippi et al. (2012), biological agents produce chemical signals which elicit metabolic pathways involved in biomass accumulation and disease suppression, as well as increasing productivity through additional direct, indirect, and miscellaneous ecological antagonisms.

Natural plant resistance to pathogens depends on a defense system which acts in three possible ways:

(1) Constitutive resistance, which is inherited and manifests itself even in the absence of a specific pathogen aggressor;
(2) Local resistance activated at the site of aggression;
(3) Systemic acquired resistance, which protects the plant against systemic stresses (Campos, 2009).

Among the micro-organisms with high potential for the development of biocontrol agents - using *in vitro* production systems - are bacteria and fungi. In combating plant diseases, *Bacillus subtilis* and *Trichoderma* spp. are among the most widely-used bacteria and fungus respectively (Bettiol and Morandi, 2009). The genus *Bacillus* includes over 100 species (Euzeby, 2006). These bacteria are Gram positive, aerobic (facultatively anaerobic), spore-forming bacilli, sporulation provides a mechanisms of resistance to environmental changes, which result in an important aspect for the production of inoculants (Tejera et al., 2012). Various studies have employed this bacterial genus as a plant growth promoter, particularly *B. subtilis* and *B. licheniformis* (Lugtenberg and Kamilora, 2009).

The inhibition capacity of microorganisms on plant pathogens is due to different antagonistic mechanisms developed by them. Among which are the production of antimicrobial substances, competition for ecological niches, competition for nutrients, production of volatile antimicrobial compounds, detoxification, hyperparasitism, predation and parasitism, production of lytic enzymes, production of toxins, gene silencing, interference with phenomenon of Quorum Sensing, siderophore and hydrocyanic acid production (Romeiro, 2007). Some species of *Bacillus* can suppress fungal diseases, primarily by antagonistic action related to production of antifungal antibiotics such as iturina in *B. subtilis* (Araujo et al., 2005). In a study confronting *Bacillus* and *Pyricularia grisea* (Cooke) Sacc., Velusamy and Gnanamanickam (2008) observed the effect of these bacteria on biocontrol for the production of antibiotics. While Knaak et al. (2007) observed inhibition through the action of Cry 1 AB and Cry 1 AC proteins. In rice cultivation various *Bacillus* species in solution or microbiolized seeds have been tested against the *P. grisea*, *Rhizoctonia*. *Solani* Kühn, *B. oryzae* (Breda de Haan) Shoemaker, *Curvularia* sp., *Fusarium oxysporum* Schlecht, *F. solani* (Mart.) App. and Wollenw and *Gerlachia oryzae* (Hashioka and Yologi) W. Gams pathogens. In an experiment conducted by Remuska and Pria (2007), the bacterium *B. thuringiensis* proved to be very effective as an antagonist to *Sclerotium rolfsii* Sacc., *Monilinia fruticola* (G. Wint.) Honey, *Sclerotinia sclerotiorum* (Lib) de Bary and *F. solani*. The rice seeds microbiolization infected with *Pseudomonas fluorescens* (Flugge) Migula, *B. subtilis*, *Bacillus* sp. and *S. maltophilia* is considered an effective treatment in controlling of sheath blight caused by *R. solani* not only by the ability of these to reduce the disease, but also by the possibility of efficiency by using them associated with compounds that stimulate its activity (Ludwig and Moura, 2007).

The success of any biological control program relies on isolation and selection of pathogen-antagonistic microorganisms, which are able to exert their effects rapidly and at low cost (Mariano et al., 2000). Therefore, in order to contribute alternative biological agents for disease control, this study aimed to isolate, identify, select *Bacillus* spp. with the potential for use in the biological control of *M. oryzae* and propose the best method to verify maximum *M. oryzae* mycelial growth inhibition.

MATERIALS AND METHODS

Isolation and identification of rice plants Phylloplane antagonists

Healthy rice plants were collected from rice plantations in the state of Maranhão, Brazil (namely: Sao Luis, Arari, Vitoria do Mearim, Miranda, Davinopolis, and Grajau cities), which present climate, temperature, similar humidity and rainfall, with some changes. The climate of São Luís, Arari, Vitória do Mearim and Pindaré is humid (B1), Miranda and Davinópolis is sub-humid (C2) and Grajaú is sub-humid dry (C1). Most of these municipalities have temperature higher than 27 ºC; only Davinópolis is between 25 and 26°C. The moisture in São Luís is more than 82%, in Arari and Vitória do Mearim is between 79-82%, and in the cities of Pindaré, Miranda

and Grajaú, around 76-79%. Annual rainfall in São Luís varies from 2000 to 2400 mm; in the municipalities of Arari, Davinópolis and Grajaú, around 1200 to 1600 mm per year; in the cities of Vitória do Mearim, Pindaré and Miranda, around 1600 to 2000 mm per year (Geplan, 2002).

Plants were collected from rainfed rice cultivated in the vegetative phase with different ages and transported in paper bags to the laboratory of Plant Pathology the State University of Maranhão for frozen storage.

Isolation of B*acilli* from stored plants was performed according to the methods described by Mariano and Silveira (2005) and Bettiol (1995), with modifications. Briefly, discs were punched out of healthy leaves and washed in sterile distilled water (SDW). Washed leaf discs were placed in a test glass tube with 5 ml SDW, sonicated at 10 Hz for 10 min, and heated to 80 °C for 20 min in a water-bath. The resulting suspension was serially diluted (dilution factor 10^{-2}, or 1 in 100) in test tubes containing 4.5 mL of sterile distilled water (SDW). From the undiluted suspension and from each dilution (1 in 10, and 1 in 100), 0.1 ml was streaked onto Potato Dextrose Agar (PDA) medium solid in triplicate. After 48 h incubation under laboratory conditions (temperature of 25°C), bacterial colonies were inoculated into PDA solid medium and again plated by streaking. Colonies derived from resulting single colony-forming units (cfu) were transferred to test tubes containing Tryptone Soy Agar (TSA) solid medium.

Molecular identification of bacteria was carried out using 16S rRNA sequencing. Briefly, the gene encoding bacterial 16S ribosomal RNA was amplified by the Polymerase Chain Reaction (PCR), and subsequently sequenced. PCR was performed on a small number of bacterial cells directly sampled from each TSA culture using an autoclaved toothpick. The bacterial cells were deposited in wells already containing specific PCR reagents, and rapidly agitated with the tip of the toothpick. The PCR reaction consisted of 10 µL 5X PCR buffer, 1 µL each of forward and reverse primers (10 mM), 1 µL of dNTP (10 mM), 0.2 µL of GoTaq DNA polymerase (5 U / uL, Promega), and 36.8 µL autoclaved MilliQ water. The forward (5 '- AGAGTTTGATCCTGGCTCAG - 3') and reverse (5 '- ACGGTTACCTTGTTACGACTT - 3') primers were described by Weisburg et al. (1991). Amplification was performed in a thermocycler (model T100, BioRad), using the following program: initial denaturation at 94 °C for 4 min; 40 cycles of 94 °C for 30 s (step 1), 60 °C for 30 s (step 2), and 72 °C for 90 s (step 3); and final extension at 72 °C for 4 min.

Amplification was verified by electrophoresis in 0.8% agarose gel plus ethidium bromide (100 ng / ml), and imaged using a gel documentation system coupled to a UV trans-illuminator. Amplified products were purified by precipitation with polyethylene glycol (the second protocol described by Schmitz and Riesner (2006). Purified products were sequenced by the chain termination reaction method (Sanger et al., 1977). The reaction consisted of 5 µL PCR product, 1 ul Big Dye 3.1 reagent (Applied Biosystems), 1.5 µL dilution buffer, 0.3 µL each forward and reverse primers (10 M), and 2.2 µL autoclaved MilliQ water. The reaction was performed in a thermocycler, using the following program: initial denaturation at 95 °C for 1 min, followed by 25 cycles of 95 °C for 5 s (step 1) and 60 °C for 4 min (step 2).

Sequencing reaction products were precipitated by adding 40 ul of isopropanol (75 %), followed by centrifugation (12,000 g for 10 min). An additional 100 ul of isopropanol (75 %) was added, and centrifugation was repeated (12,000 g for 5 min). After discarding the supernatant, the pellet was dried and resuspended in 10 ul formamide before denaturation at 95 °C for 2 min. Sequencing was performed using a 3500xL capillary sequencer Genetic Analyzer (Applied Biosystems).

The determined sequences were searched against the type of species Ribosomal Database Project, Release 10 (http://rdp.cme.msu.edu) (Cole et al., 2009). The phylogenetic tree was constructed using MEGA 6.3 program.

Evaluation of *Bacillus* spp. for antagonistic potential against *M. oryzae*

To study the antagonistic effects of isolated *Bacillus* spp. on *M. oryzae*, the 12 identified *Bacillus* isolates were cultured in growth chamber at a temperature of 25 °C and photoperiod on PDA solid medium for 24-48 h, while *M. oryzae* - isolated from Embrapa Rice and Beans (Goiânia) - was cultured in PDA solid medium for 14 days.

Isolate B25 (*B. amyloliquefaciens*), selected during pre-experimental optimization, was used to comparatively evaluate four potential *in vitro* antagonism assay streaking methods: 'central stripe', 'the point', 'circle' and 'three stripes'.

Briefly, during the central stripe method, bacterial isolate colony material was transferred to the center of a Petri dish containing PDA. Subsequently, two 6 mm diameter discs (containing actively growing pathogen mycelium) were removed from the pathogen culture dish and deposited on either side of the central bacterial isolate, equidistant from it (Mariano and Silveira, 2005). In point method, retired, aseptically, a disc 6 mm diameter (containing actively growing pathogen mycelium), depositing it on the surface of the middle of the bioassay plate, near the edge. With the aid of platinum loop It peaked bacterial colony to the PDA medium in a diametrically opposite to that point where the mycelium disc is loaded (Mariano and Silveira, 2005). In contrast, during the circle method, a 6 mm diameter disc of pathogen-containing agar is transferred to the centre of a Petri dish containing BDA medium. Bacterial isolate is then inoculated onto the PDA in a circular pattern, with the circle diameter smaller than the dish diameter (about 5 cm) (Mariano and Silveira, 2005). In testing the above methods, a randomized experimental design with six replicates per method was employed. All methods employed aseptic technique, using a platinum loop for bacterial inoculum transfer. Assay evaluation (measurement of pathogen colony diameter) was carried out after seven days of incubation in growth chamber at 25 °C and photoperiod of 12 h.

The circle method, which yielded the best results (Table 2), was thus used to evaluated the antagonistic action of 11 *Bacillus* spp. isolates (B7, B7', B16, B35, B22, B22', B31, B41, B45, B25, and B46) against *M. oryzae*. This experiment employed a randomized design with four replicates per isolate, and pathogen colony diameter was evaluated after seven and 14 days of incubation. Colony diameter was measured in two perpendicular directions, using a millimeter ruler, and an average was calculated for each colony.

During both method selection and isolate evaluation, the control consisted of the pathogen alone. Data were analysed by means of Analysis of Variance (ANOVA) and means-comparison (Tukey test), using the Statistical Assistance Software (Assistat) version 7.7 beta (2015).

RESULTS AND DISCUSSION

Identification of bacterial antagonists extracted from rice leaves

From the collected rice plant material, 12 *Bacillus* spp. isolates were obtained and identified. The identification of these isolates based on sequence of the 16 S rDNA gene indicates that the bacteria B7, B7', B16 and B35 showed similarity of 66% to *B. thuringiensis* serovar tolworthi Pasteur Institute standard strain (AP014864). The isolates B12, B22, B22', B31, B40, B41, B45, B57, B58 and B62 showed 100% similarity to *B. methylotrophicus*

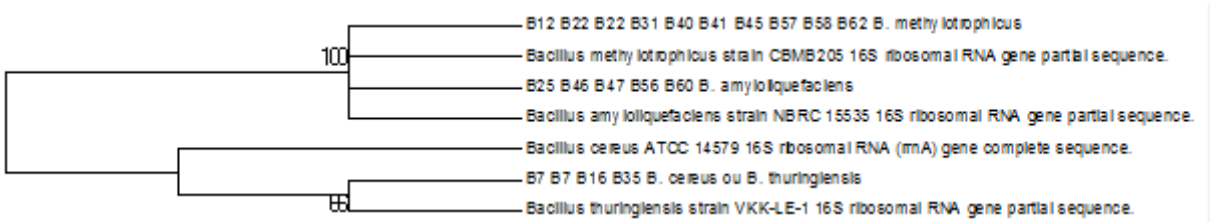

Figure 1. Phylogenetic tree constructed according to the Neighbor joining tree model with the 16 S rDNA sequences of strains of *Bacillus* species, using the MEGA 6.3 program. The bootstrap values shown are in the branching points.

Table 1. Identification and molecular origin of *Bacillus* spp. isolates obtained from healthy rice leaf samples, collected in Maranhão municipalities.

Strain	Source/variety	*Bacillus* species
B7	São Luís/ Arariba	*B. thuringiensis*
B7"	São Luís/ Arariba	*B. thuringiensis*
B12	Arari/ Tainha	*B. methylotrophicus*
B16	Miranda/ IAC-47	*B. thuringiensis*
B22	Arari/ 3 meses	*B. methylotrophicus*
B22"	Arari/ 3 meses	*B. methylotrophicus*
B25	Arari/ Primavera	*B. amyloliquefaciens*
B31	Vitória do Mearim/ Cica 7	*B. methylotrophicus*
B35	Pindaré/ Cabocla	*B. thuringiensis*
B41	Grajaú/ Cabocla	*B. methylotrophicus*
B45	Grajaú/ Cabocla	*B. methylotrophicus*
B46	Davinópolis/ Lageado	*B. amyloliquefaciens*

CBMB205 (NR_116240). The isolated B25, B46, B47, B56 and B60 showed 100% similarity to *B. amyloliquefaciens* NBRC15535 (NR_041455) (Figure 1).

These can be divided into three species: *B. thuringiensis, B. methylotrophicus*, and *B. amyloliquefaciens* (Table 1). The diversity of *Bacillus* spp. can be explained by the significant environmental differences between municipalities, different varieties of rice, and differing ages of the rice plants.

The most common specie, *B. methylotrophicus* (6 isolates), occurred in Arari, Vitória do Mearim and Grajaú in the first two municipalities, the temperature is higher than 27°C, have high humidity, ranging from 79 to 82% with rainfall around 1200 to 1600 mm and from 1600 to 2000 mm per year, in Grajaú observed temperature range of 26 and 27°C and relative humidity of 76 to 79%.

The second most frequent, *B. thuringiensis* (4 isolates) occurred in São Luís and Miranda, both places with temperature higher than 27°C, relative humidity greater than 82% and ranging 76-79%, respectively. What differentiates these two environments are variations in rainfall; in the second room this is less.

The specie of minor occurrence, *B. amyloliquefaciens* (2 isolates) is present in the municipalities of Arari and Davinópolis, the two places have rainfall 1200-1600 mm, the cities are different in relation to climate, because one is humid and the other is sub - humid, and in the second city the relative humidity is lower. It is observed that small variations in humidity, temperature and rainfall have influence in the occurrence of one or another specie of bacterium of the genus *Bacillus*.

In Arari ocorred greater diversity of species, where the climate is sub-humid, temperature above 27°C and rainfall between 1200-1600 mm per year. According to Romeiro (2007), any living organism, mainly bacteria, is able to perceive changes in the environment and the presence of other living beings in their proximity, this being crucial to their survival.

Microorganisms such as bacteria, yeasts, and filamentous fungi are commonly found on rice phylloplanes. The predominant type of microorganism is dependent on the phenological stage of the plant, with bacteria being dominant during early plant development, an increasing presence of yeasts, thereafter and finally and increasing presence of filamentous fungi. This microbial succession occurs as plant development increases the level of sugars present in the leaves (Michereff, 2001). Conversely, Filippi et al. (2012) report that plants also actively respond to a variety of environmental stimuli (e.g. gravity, light, temperature, physical stresses, water and nutrient availability, and chemical stimuli), including chemical stimuli produced by microorganisms associated with the soil and the plant itself. Corroborating findings from Bettiol and Morandi (2009) demonstrate that the phyllosphere bacterial populations change with plant age, local nutrient sources, and season. In addition, cultivation can also alter composition of the microbial community.

Evaluation of the antagonistic effect of *Bacillus* spp against *M. oryzae*

Selection of the *in vitro* antagonism assay method revealed a significant difference between the four methods tested. Two of these methods significantly outperformed the control: central stripe and circle, achieving 37.4 and 36.5% inhibition of *M. oryzae* mycelial growth, respectively (Table 2). These data demonstrate

Table 2. Comparative evaluation of four methods for *in vitro* assay of pathogen inhibition, using *B. amyloliquefaciens* as the antagonist and *M. oryzae* as the pathogen.

Method	Mycelial growth (cm)	Inhibition of mycelial growth (%)
control only (no antagonist)	2.14 [a]	0
'central point'	1.79 [ab]	16.4
'three stripes'	1.50 [bc]	29.9
'single stripe'	1.34 [c]	37.4
'circle'	1.36 [bc]	36.5

*Means followed by the same letter in the column do not differ statistically, by the Tukey test ($p<0.01$). CV (%) = 16.12.

Table 3. Mycelial growth inhibition evaluation *Magnaporthe oryzae* by *Bacillus* spp., using the Circle method.

Strain	Treatment	Colony diameter (cm)		% Inhibition	
		7 days	14 days	7 days	14 days
	Control	3.80 [abcB]	6.78 [aA]		
B25	*B. amyloliquefaciens*	2.54 [abcdA]	2.65 [cA]	33.16	60.91
B22	*B. methylotrophicus*	1.97 [abcdA]	2.20 [cdA]	48.16	67.55
B22'	*B. methylotrophicus*	2.52 [abcdA]	3.06 [cA]	33.68	54.86
B46	*B. amyloliquefaciens*	2.48 [abcdA]	2.68 [cA]	34.74	60.47
B45	*B. methylotrophicus*	4.00 [aB]	7.00 [aA]	- 5.26	-3.24
B7 e 7'	*B. thuringiensis*	2.98 [abcdA]	3.10 [cA]	21.58	54.27
B31	*B. methylotrophicus*	2.14 [abcd]e[A]	2.05 [cdA]	43.68	69.76
B35	*B. thuringiensis*	3.05 [abcdA]	3.15 [bcA]	17.11	53.54
B41	*B. methylotrophicus*	0.51 e[A]	0.65 [dA]	86.58	90.41
B16	*B. thuringiensis*	3.92 [abB]	5.08 [abA]	- 3.16	25.07

*Means followed by the same letter in the column do not differ statistically, by the Tukey test ($p<0.05$). CV (%) = 27.22.

the importance of choosing appropriate antagonist and pathogen cultivation and pairing methods for *in vitro* assays. Findings can also be influenced by the production of enzymes with activity against the fungal cell wall (Mavingui and Heulin, 1994). With regards to the circle method, the considerable inhibition of pathogen growth may be explained by the pathogen being surrounded by the antagonist, thereby blocking passage to the plate boundaries and forcing a confrontation. Lima et al. (2014), in an *in vitro* experiment employing the circle method with 10 antagonistic *Bacillus* spp. observed that at day 10 all isolates showed an inhibitory effect against the assessed pathogen (*F.oxysporum* f. sp. Lycopersici (Sacc.) Snyder and Hansen), by metabolite production.

During evaluation of the 11 *Bacillus* spp. for their potential to control *M. oryzae* mycelial growth, the circle method produced a significant effect ($p < 0.05$) on pathogen growth. At day seven, isolates B41, B22, and B31 (*B. methylotrophicus*) had achieved 86.58, 48.16, and 43.68% inhibition (respectively) compared to the control. At day 14, eight antagonists greater inhibited mycelial growth, with inhibition ranging from 53.54 to 90.41%. At the latter time point, isolates B41, B31, and B22 (*B. methylotrophicus*) had achieved 90.41, 69.47, and 67.55% inhibition (respectively) compared to the control (Table 3).

Inhibition levels observed during this experiment were thus higher than those reported previously by Remuska and Pria (2007), wherein the bacterium *B. thuringiensis* proved effective as an antagonist against *S. rolfsii* (a plant pathogen which causes fruit rot), *S. sclerotiorum* (a plant pathogen which causes stem rot), and *F. solani*., achieving 39.41, 37.97, 37.44, and 36.17% inhibition of mycelial growth, respectively.

Bacillus spp. may act through antibiosis, the production of toxic compounds capable of *in vitro* inhibition of mycelial growth of at least three fungi: *Alternaria alternata, Bipolaris oryzae* (Breda de Haan) Shoemaker, *Curvularia lunata* (Wakker) Boedijn Meyer, *Gerlachia oryzae* (Hashioka and Yologi) W. Gams, *Pyricularia oryzae* (Cooke) Sacc., *Rhizoctonia solani*, and *S. rolfsii* (Ludwig et al., 2009). In an *in vitro* bioassay, Vieira Jr. (2005) found that *B. cereus* (UFV-75) was capable of producing siderophores, volatile compounds, bacteriocins, and the enzyme chitinase. According to Lima et al. (2014), enzyme production by microorganisms may explain, at least in part, the biological control of plant

diseases by prokaryotic agents. Reinforcing this theory, Guerrero et al. (2011) state that plant commensal bacteria promote growth and have the ability to control plant pathogens through the production of inhibitory metabolites as well as induction of plant resistance to pathogens.

Regarding the mycelial growth inhibition results at seven and 14 days post-application of *Bacillus* isolates, higher inhibition by *B. methyltrophicus* was observed at day 14. These results and the Wiwattanatapee et al. (2004), who found that 20 days post-application of different isolates of *B. megaterium* de Bary, the inhibitory effect on rice-sheath burning had been lost and suggest that the inhibitory effect of *Bacillus* may increase to a maximum out of 14 and 20 days after the application, and then decrease.

Studies on greenhouse-grown rice plants have also demonstrated successful *Bacillus* spp. biological control of pathogens. Silva et al. (2014) found that each of nine *Bacillus* spp. isolates (especially isolates B41 and B35) reduced the extent of rice plant leaf damage caused by *C. lunata*. Ludwig et al. (2009) had previously found that treatment of rice plants with rhizobacteria reduced the relative frequency of stained grains, and that isolates FDs 416, FDs 418 (*Bacillus* spp.), and FDs 223 (*Pseudomonas fluorescens* Migula) provided significant levels of pathogen reduction (up to71.4, 57.1, and 50%, respectively). In scald-controls, isolates FS 416 and FS 418 achieved pathogen reduction of 63.2 and 60% respectively (Ludwig et al., 2009).

Several researchers, including Remuska and Pria (2007), Kupper et al. (2003), Ludwig and Moura (2007), Navon (2000), Ludwig et al. (2009), Silva et al. (2014), and Lima et al. (2014) have demonstrated the effectiveness of these bacteria, in corroboration with the results reported here.

Therefore, additional research in this area is warranted. Availability of a greater number of *Bacillus* spp. with the potential for biological control of pathogens, as well as an improved understanding of the antagonist-pathogen relationship, would contribute greatly to development of sustainable agricultural practices. Pursuing related novel lines of research (such as inoculation of antagonists into the greenhouse, evaluation of antagonist efficacy in inhibiting the formation of fungal aspersoria, investigation into production of volatile compounds, pairing of antagonists with other rice plant pathogens, and the influence of *in vitro* methods on defensive control efficiency) may also be helpful.

Conclusion

The rice phylloplane supports a diverse and dynamic *Bacillus* spp. Population; it was isolated and three species were identified: *B. thuringiensis, B. methylotrophicus*, and *B. amyloliquefaciens*. They were impacted by a variety of culture and environmental conditions. This study demonstrates the potential of Maranhão *Bacillus* spp. isolates for use as biological fungal-control agents and the most promising experimental isolates for biological control were identified as *B. methylotrophicus* isolates B41, B31, and B22. *In vitro* single stripe and circle methods are the best method to verify maximum *M. oryzae* mycelial growth inhibition.

Conflict of Interests

The authors have not declared any conflict of interests.

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Quantitative assessment of palm oil wastes generated by mills in Southern Benin

Tatiana Windekpè KOURA[1], Valentin KINDOMIHOU[1]*, Gustave DAGBENONBAKIN[2], Marc JANSSENS[3] and Brice SINSIN[1]

[1]Laboratory of Applied Ecology, Department of Natural Resources Management, Faculty of Agronomic Sciences (FSA), University of Abomey - Calavi (UAC), Benin Republic.
[2]National Institute for Agricultural Research of Benin Republic, Cotonou Benin Republic.
[3]Institute of Crop Science and Resource Conservation INRES, University of Bonn, Germany.

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While waste management is given more care for protecting the environment and human health, agro industrial wastes are still a concern, in developing countries. This study quantitatively assesses the palm oil wastes generated by mills and describes their management in Southern Benin. Twenty four out of 335 regional palm oil mills were randomly selected and assessed for waste quantities generated during the oil production season. From 1 ton (t) of full fruit bunches (FFB), each palm oil mill produces an average of 712.1 kg of fruits, 254.7 kg of empty fruit bunches (EFB), and 399.8 kg of palm kernel cake, 114.9 kg of fibre, 240.4 L of palm oil mills effluents (POME) and 152.3 L of crude palm oil. Numeric classification analyses resulted in four groups of palm oil mills following production factors and wastes quantities generated: small, medium, large and very large mills. These groups produced yearly on average respectively 12.4, 31.3, 132.7, and 800.7 t of EFB; 5.6, 13.6, 135.2, and 637 t of fibre and 15.1, 40.9, 233.4, and 572.6 t of POME. They differed in nature, plantations size, and capacity to employ people. About 80% are small producers. The use of all POME generated depend on waste quantity produced.

Key words: Palm oil mills, wastes, system production.

INTRODUCTION

The palm oil tree (*Elaeis guineensis* Jacq) is a native of the humid tropics of West Africa. It is one of the major oil crops in the world, producing more oil than all other plants oil (Adeoluwa and Adeoye, 2008). Palm oil is used mainly for cooking (cooking oil, margarine, shortening, etc.) (Baharuddin et al., 2009). It is an important source for edible oils, as raw material for cosmetics and detergents and more recently for biodiesel production (Wicke et al., 2008; Lim, 2010; Hirsinger, 1995; Stalmans, 1995). In Benin Republic, palm cultivation was developed under the Guezo's Kingdom (1818 - 1858). Western African countries were the main outlet of palm oil in order to feed their soap mills. In 1848, palm oil gradually replaced slave trade. The production system

*Corresponding author. E-mail: vkindomihou@yahoo.fr. Tel: +22995023058. Fax: +22921303084.

was entirely traditional until half of the twentieth century. Thereafter, it was industrialized from 1950 to 1975 with 44000 t of palm oil being exported yearly. But this industrialization failed due to the Asian markets competition and decline of plantation productivity (Fournier et al., 2001). However, the National Living Forces Conference in 1990 resulted to the adoption of a liberal economic system of the development of palm trees and small private nuts productive units for women under supports from government, donors, and NGOs. In this context, plantation self-developers and palm oil self-producers might reverse threatening the profitability and hence, enable some small scales producers to survive in the coming years (Carrere, 2010). After the large-scale supply of improved seeds in the early 1990s, the industry was fairly supported; nonetheless, every attempt to recover was unsuccessful. However, the oil palm production developed with the involvement of associations such as the Regional Union of Palm Oil Producers (RUPOP) and the Communal Union of Palm Oil Producers (CUPOP) in 2004. Moreover, several private mills were also created and developed. These sources supplied mainly Nigeria' soap mills. This sector impacts several households in Benin. Indeed, palm oil and sodabi (that is, local palm wine) highly contribute to the income and social capital accumulation; this also discriminates operators and their households socially and economically. In Southern Benin, as oil palm acreage expanded, so did farmers' income (Adegbola et al., 2009). Nowadays, palm oil belongs to the main agricultural production chains which are planned to be nationally promoted. Palm oil production generates residues or wastes, such as empty fruit bunches (EFB), palm oil mill effluents (POME), palm kernel cake, palm kernel shell and fibre from the mesocarp. Every ton of crude palm oil produced causes the emission of 46 m^3 (that is, 32.9 kg) of methane, corresponding to 384 m^3 (that is, 756 kg) of CO_2 (Schuchardt et al., 2007). The POME waste induced methane is the major environmental pollutant (Schuchardt et al., 2006). The raw POME has biological oxygen demand (BOD) values averaging 25.000 mg/L, making it about 100 times more hazardous than domestic sewage (Maheswaran and Singam, 1977). Therefore, the palm oil industry is the single largest polluter in Malaysia contributing to 83% of total pollution, and this might be similar where ever palm oil is produced (Ojonoma and Nnennaya, 2007). Ensuring effective and sustainable management of palm oil mills wastes is important while enjoying the reverse from production (Ojonoma and Nnennaya, 2007). Also, the appropriate waste management might become the major contributor for reducing overall global greenhouse gas emissions (Sudirman et al., 2011). Hitherto, quantitative data are still lacking on waste generated through the local production systems. Here, a monograph of the palm oil mills waste production system was assessed throughout the processes description and quantitative analyses in Southern Benin.

METHODOLOGY

Study site

This study was carried out in Southern Benin Republic and covered the departments of Atlantic, Mono, Couffo, Oueme, and Plateau (Figure 1). The South of Benin Republic extends from the coast at 6° 25' to 7°30' N latitude. This part of Benin Republic belongs to the Guinea-Congolese zone. The climate of this part is sub-equatorial with two rainy seasons (March to June and September to mid-November) and two dry seasons (July to September and November to March). The annual rainfall varies between 1,100 and 1,400 mm. The average daily temperature ranges from 25 to 29°C and the average daily humidity from 69 up to 97%. The Guinean zone is the area of deep Lateritic soils of low fertility (700 000 ha), and that of more fertile Alluvial soils and heavy clay soils (360 000 ha) located in the river valleys of Mono, Couffo, Oueme, and in the Lama depression (Adjanohoun et al., 1989).

Sampling

This study considered palm oil mills which belong to RUPOP. In each department, the CUPOP was contacted and palm oil producing villages, comprising significant numbers of plantations and mills were selected. In each village, only mills which produced palm oil for commercial income were considered. Mills which buy nuts from CUPOP mills members and produced for commercial income were also surveyed. Three hundred and thirty five palm oil mills were surveyed (Table 1).

Survey

The survey was carried out from November 2011 to March 2012. A semi-structures questionnaire was used and complemented with personal observations. The questionnaire evaluated information concerning the biodata (name, sex, age, ethnic group, main activity), the production factors (palm oil production process, number of employees, production period, property of palm plantation, palm plantation area, external supply of nuts by buying, variety of nuts used, average monthly quantity of oil produced) and palm oil mills waste management (proportion of palm oil mills waste quantity used by the producer, proportion sold and proportion discarded were determined using the matrix notation method). The discussion with the producers was completed with direct observations on the surroundings of the mills.

Palm oil mills wastes (POMW) quantification

According to the type of machine used for palm oil production in a partial or total process, palm oil mills processes were classified into 4 categories (Figure 2):

i) Traditional palm oil process: Producers do not use any machinery. All the steps of production were made by feet and hands;
ii) Semi mechanized or improved palm oil process: Producers have only digester engine in their mills. Whereas all other steps were made by feet and hand, digestion step was made with machine;
iii) Motorized or modern palm oil process: Producers have a digester engine and almost all of them use the DECAM press;
iv) Semi industry palm oil process: Only threshing step was made by feet. Producers possess big cookers, presses, sterilizers, clarifiers and other big facilities for oil production.

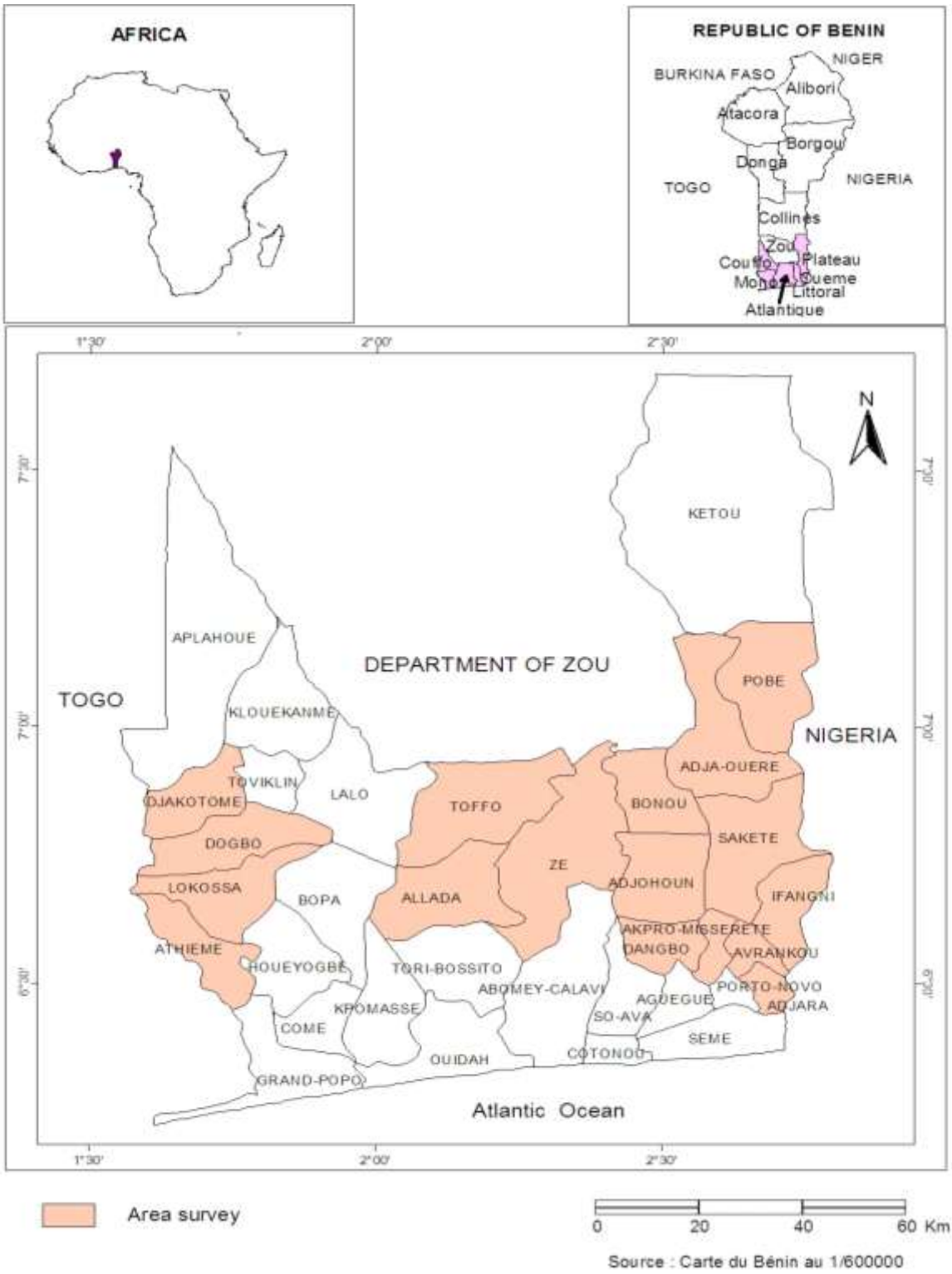

Figure 1. Location of experiment site.

All semi industrialized mills know their extraction rate and waste proportion. For the 3 other groups, 15 mills were randomly chosen in order to assess their palm oil mills waste quantities. Seven traditional mills, 4 modern mills and 4 improved mills were monitored three times during their transformation process. These numbers were chosen because the engine used among modern mills and improved mills were similar. At each producing step, waste weight was assessed. The waste quantities Q (t) of each mill

Table 1. Palm oil mills responders.

Department	Respondents	
	Number	Percentage
Atlantic	80	23.9
Couffo	56	16.7
Mono	63	18.8
Oueme	90	26,9
Plateau	46	13.7
Total	335	100

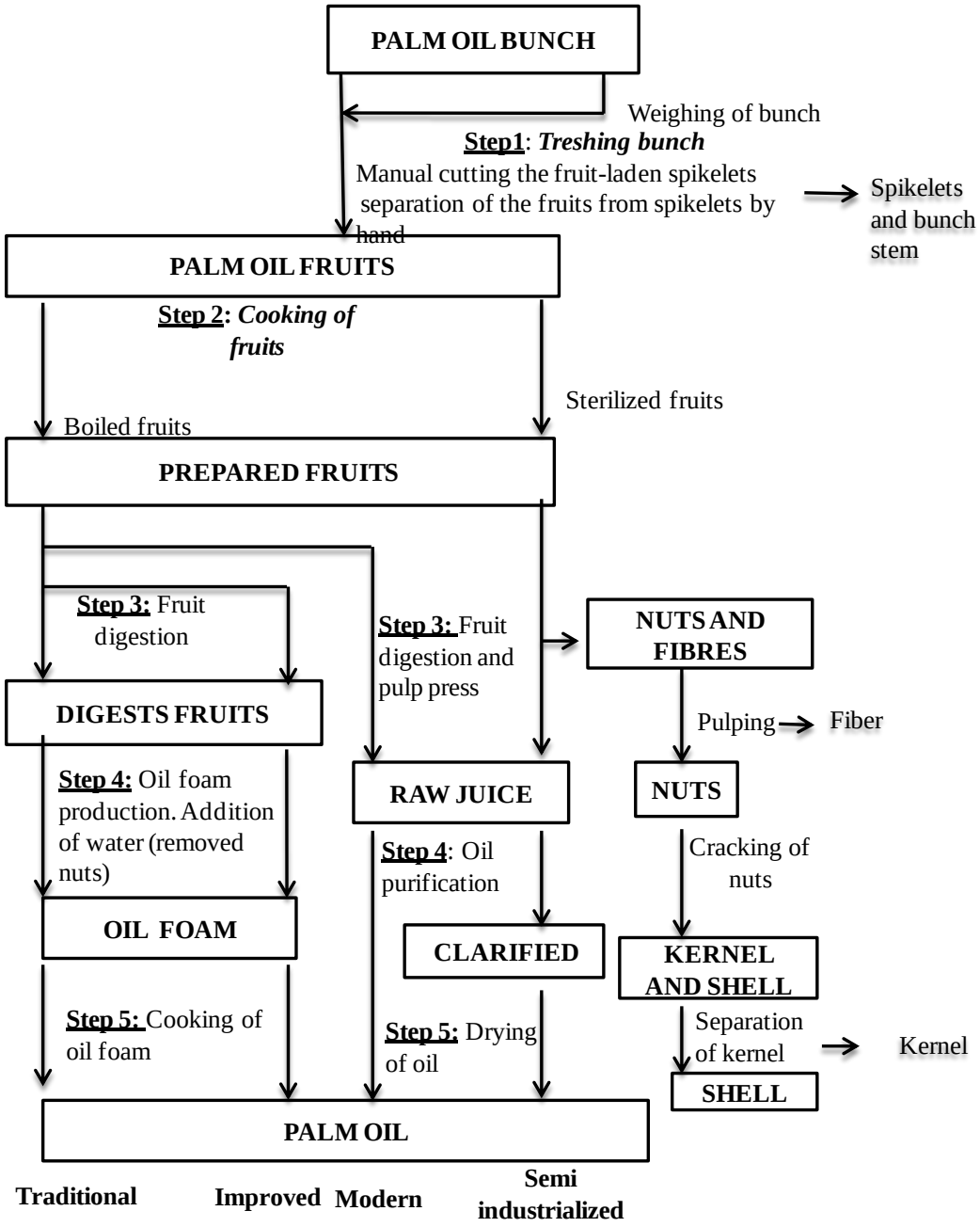

Figure 2. Palm oil processing types.

Table 2. Palm oil mills owners: Characterization and activities sector classification.

Sex		Age		Sectors activities					
				Agriculture		Buisiness		Industry	
Male	Female	23 < X ≤ 50	X > 50	Oil	Oil and tree	Oil	Oil and tree	Oil	Oil and tree
247 (75)	88 (25)	211 (63)	124 (37)	51 (15.2)	234 (69.9)	2 (0.6)	7 (2.1)	12 (3.6)	29 (8.7)

( ) in percentage.

were calculated as:

$$Q = \frac{q_{wi} * q_{prod}}{q_{ri}}$$

where q_{wi} stands for the quantity of waste produced by 1 t of full fruits bunches (FFB) according to the oil production category "i" that mill belongs to, 1 t of FFB according to the production category "i" that mill belongs to, q_{prod} is the annual palm oil quantity (t) produced by the mill or $q_{prod} = q_{ri} \times F_i$ and where F_i = the total quantity (t) of FFB according to the production category "i" that mill belongs to.

Statistical analyses

The cluster analysis was realized with Ward “minimum variance cluster procedure” to classify palm oil mills according to the waste production system. The palm oil mills production factors (employees number, property of palm plantation, palm plantation area, nuts buying, variety of nuts used, the average quantity of oil produced by month) and the waste quantities generated EFB quantity, the fibre quantity and POME quantity) were considered for this classification. A data matrix comprising the waste quantities produced and waste production systems was submitted to a Principal Component Analyses (PCA) to evaluate the linkage between waste quantities and their production system among palm oil mills. Analysis of variance was used to compare different averages of waste quantities, and homogeneous groups were determined using Tukey honest significant difference (HSD). These analyses were performed using SASv9 software. Chi-square analysis with one classification criterion (4 groups) was performed on the observed group frequencies with respect to the expected rate 1:1:1:1.

RESULTS

Socio demographic characterization of palm oil mills’ owners

Table 2 presents palm oil producers according to their sex, age and activities sectors. Two-thirds of mills surveyed belonged to men and 63% of owners were less than 50 years old. Although almost all mills’ owners are farmers, 15% of them are involved in business and industry sectors. Seventy one percent of them own palm plantations and produce palm oil.

Evaluation of palm oil mills waste quantities

In the mills, producers collect all the FFB that they have. These FFB originate from different palm oil plantations of different ages. From 1 t of FFB, each palm oil mill produced an average of 712.1 kg of fruits, 254.7 kg of EFB, 399.8 kg of palm kernel cake, 114.9 kg of fibre and 240.4 L of POME, 152.3 L of crude palm oil. Table 3 presents the average of palm oil mills waste quantity generated by each kind of mills. The semi industrialized process produced significantly more EFB, fibre and less palm kernel cake than the traditional process.

Characterization of palm oil mills wastes production systems

The cluster procedure for palm oil mills waste production systems identified 4 groups of palm oil mills wastes production systems according to the waste quantities generated and production factors. R^2 was 53% for this analysis and 61% for the PCA used to describe these groups with the first two axes. Table 4 shows the coefficients of correlation between the factors palm oil production and the first two PCA axes. This table shows that axis 1 is correlated positively with quantity of palm oil, palm oil mills waste quantity generated (EFB quantity, fibre quantity, POME quantity, palm kernel cake (PKC) quantity), the process oil production category, employees number and palm oil plantation area. Axis 2 explains palm oil plantation possession, the variety of nuts used and the buying of nuts. Figure 3 shows the projection of the different palm oil mills waste production systems in both axes 1 and 2.

Group 1 is composed of 64 mills owned by people in the business or industry sectors. These owners do not possess palm oil plantations (98%) and need to buy the nuts for feeding the palm oil plant (Table 5). Most of them (56.3%) use a local variety. These mills use modern processing (59.4%) or improved processes (28.1%) and employ 7 persons to produce 16.71 t of oil per mill and per year on the average during 6 months of production activity. To produce this palm oil quantity, each mill generated during the production period 31.34, 13.64, 40.87 and 81.17 t, respectively of EFB, Fibre, POME and PKC on average. Group 2 contains 264 mills whose owners belong equally to all activity sectors (37.5, 42.4, and 20.1%, respectively are farmers, palm oil producers, and other sectors). They all possess 5.98 ha palm oil plantations on average and most of them buy nuts (63.6%) in addition.

Table 3. Palm oil wastes and crude palm oil quantities generated from 1 t of full fruit bunch.

Type of palm oil mills	Traditional	Improved	Modern	Semi industrial
Palm oil fruits (kg)	786.9 ± 34.0[a]	777.1 ± 31.8[ab]	783.9 ± 14.9[ab]	656.6 ± 31.7[b]
EFB (kg)	213.1 ± 34.0[a]	222.9 ± 31.8[ab]	216.1 ± 15.0[ab]	343.3 ± 31.7[b]
Palm Kernel Cake (kg)	458.9 ± 57.9[a]	674.8 ± 118.1[a]	558.6 ± 140.8[a]	161.1 ± 18.2[b]
Fibre (kg)	87.4 ± 15.6[a]	91.6 ± 14.0[ab]	94.5 ± 20.3[ab]	155.6 ± 13.0[b]
POME (l)	282.1 ± 33.9[a]	285.4 ± 55.5[a]	281.9 ± 38.1[a]	169.4 ± 26.3[a]
Crude Palm Oil (l)	97.5 ± 15.6[a]	145.8 ± 14.7[a]	114.6 ± 20.5[a]	214.4 ± 12.4[b]

Table 4. Correlation between the characteristic parameters (palm oil mills waste production system and production factors) and the first 2PCA axes (in brackets is the proportion of variation explained by each axis, expressed in percentage).

Parameter	Axis 1 (45.88%)	Axis 2 (15.13%)
POME quantity	0.95566	- 0.08615
Palm oil quantity	0.91932	- 0.05404
Fiber quantity	0.90909	0.00630
Palm kernel cake quantity	0.87000	- 0.12840
EFB quantity	0.88058	- 0.07075
Palm plantation area	0.62837	0.30984
Employees number	0.56996	0.34230
Process category of palm oil production	0.45773	- 0.20286
Palm oil plantation possession	- 0.00785	- 0.80801
Nuts buying	0.00789	0.78425
Nuts variety used	- 0.02764	0.33152

They use more a mixture of selected and local (47.7%) or local (39.4%) nut varieties to be processed either into improved (43.6%) or modern (39.0%) production lines. Each mill in this group employs 12 persons to produce palm oil. In these mills, there are of 4 mini industry productions. These mills are rarely installed and do not run at optimum level. The mills in this group produce less palm oil mills waste quantities than group 1 (Table 5).

Group 3 contains 3 mills which encompass a big palm oil plantation area (209.1 ha on average) and buy nuts again to increase their production. They employ 83 persons on average and almost all of them use the two nut varieties as well as a more selected variety through a semi industrial process attaining 159.2 t palm oil per mill per year. This kind of production generates more waste than the first two groups. One of these mills uses an improved process with only 20 employees with more selected varieties whereas those which have 200 employees use only local varieties through a mini industrialized process.

Group 4 comprises 4 mills differing from the other groups by its number of employees (54). Each mill of this group uses only nuts provided by their selected palm plantation (85.3 ha), producing more oil (252.9 t) and waste than other groups (Table 5). Three of them are semi industrialized and use a mix of local and selected nuts. One of them uses a modern process with only selected nuts in order to produce palm oil.

In the study area, most of palm oil mills surveyed belong to Group 2 (Figure 4). More than 84, 85.18 and 73.05% of traditional, improved and modern palm oil mills belong to Group 2 and the rest in each category belong to Group 1. However, rarely improved mills belong to Group 4. Forty four percent of the semi industrialized mills are component of Group 3 while the rest belong to Group 4.

Relation between the group and use of all waste quantities

All waste materials generated were used for many purposes by mills' owners. In fact, EFB are used as fertilizer, cooking fuel or to produce snail (Table 6). The χ^2 analysis between waste production groups and use of all waste quantities show that the use of all EFB and FIBER quantities produced by mill does not depend on group. But for POME, most mills which belong to Group 2 use all the quantities of this waste produced. For palm kernel, almost all mills use all the quantities by producing palm kernel oil or selling to palm kernel oil producers.

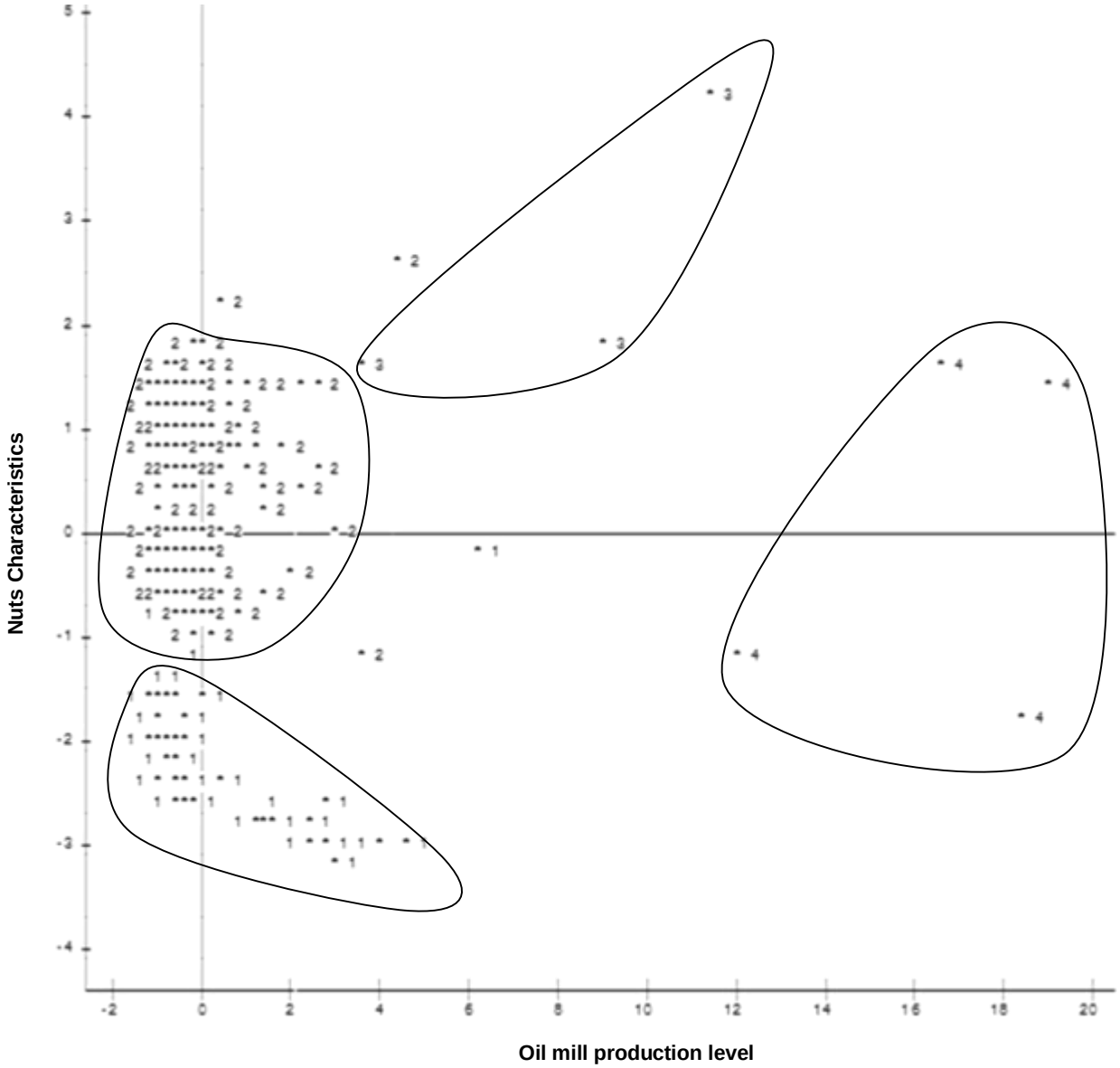

Figure 3. Projection of the different palm oil mills wastes production system in the system Axes 1 and 2.

Table 5. Wastes usages and the heterogeneity of group frequencies testing results.

Parameter	Different uses	Number of users				χ^2	P
		G_1	G_2	G_3	G_4		
EFB	Mulch, cooking fuel, soap, snail	36	154	2	1	1.9	0.5833
FIBER	Cooking fuel, mulch, fire starting cake, fertilization	49	205	2	1	6.289	0.0984
POME	Pig alimentation, fire starting cake, fertilization	26	184	1	1	22.496	<0.0001

DISCUSSION

Palm oil tree is cultivated by many farmers and retailed to secure a decent retirement. It is also an agricultural source of employment for many rural women. In some mills that employ more than 200 persons, per season each year, women are in majority. This is confirmed by Olagunju (2008) in Nigeria who reported that, palm oil

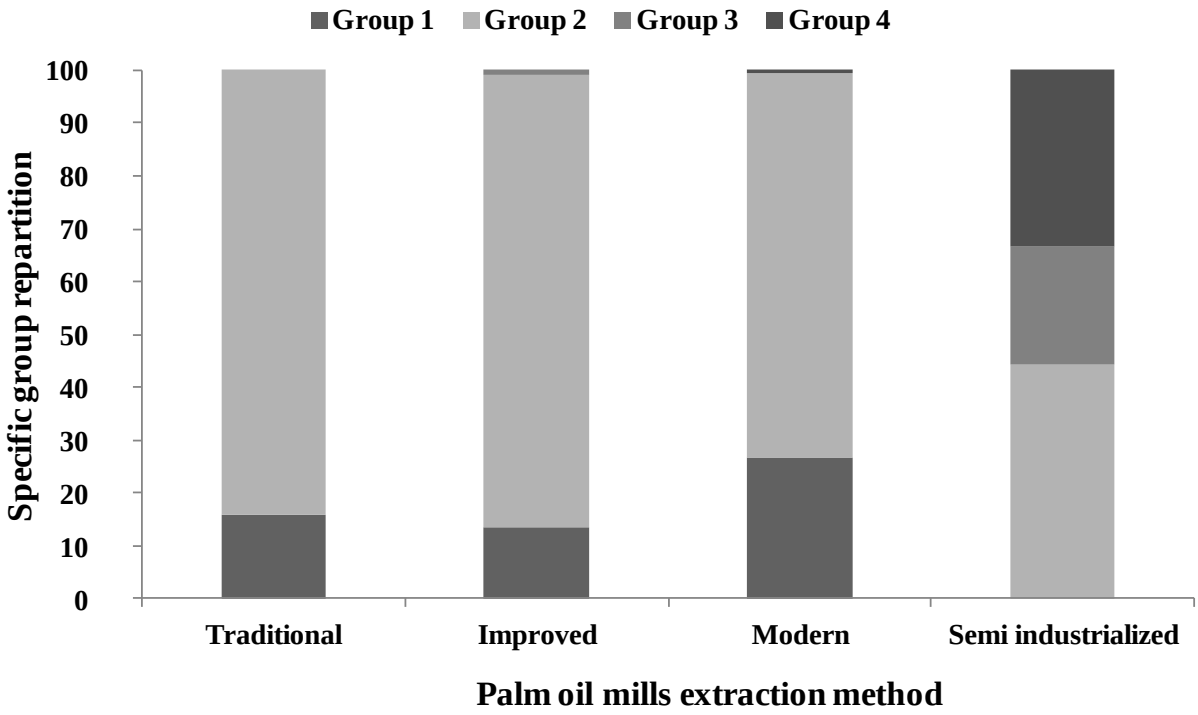

Figure 4. Palm oil mills investigated classification in each department.

mills employ 4 million Nigerians in about 20 palm oil growing states and indirectly to other numerous people involved in processing and marketing. Results show that, 1 t of FFB generates on average 712.1 kg of fruits, 254.7 kg of EFB, 399.8 kg of palm kernel cake and 114.9 kg of fibre. These values are similar to those observed in Malaysia and in Indonesia. In Malaysia, Maheswaran and Singam (1977) found that 1 t of FFB composed of 230 to 250 kg of EFB, 130 to 150 kg of fibre, 60 to 65 kg of shell, 55 to 60 kg of kernel, and 160 to 200 kg of crude oil. Hayashi (2007) found that in Indonesia, palm oil mills produce 22.5, 14.3, 6.7, 54.8, 5.4 and 21.8%, respectively for EFB, fibre, shell, POME, kernel and crude palm oil with 1 t of FFB. Palm kernel cake weight is higher in Benin than Malaysia. In fact, producers in Benin Republic use the local (Dura variety) and selected (Tenera x Dura variety) nuts. Palm kernel cakes obtained by mini industrialized mills are less and not different from those obtained in Malaysia, because these mills use selected nuts, Tenera. According to Twumasi et al. (2014), Tenera palm kernel is smaller than the Dura kernel, although the Tenera bunch is much larger than Dura. Jimoh and Olukunle (2013) reported that Dura' possesses more shell than the kernel while 'Tenera' possesses more kernel than the shell. The EFB, fibre and kernel shells generated by 1 t of FFB are approximately the same as those found by Sudirman et al. (2011). Among these palm oil mills solid wastes, palm kernel cake and EFB are more produced. These results differed from those reported by Rupani et al. (2010) and Najafpour et al. (2005), who found that POME and EFB are more produced in Malaysia mills. For transforming 1 t of FFB, small scale mills produce more POME than medium scale mills (mini industrialized). Medium scales mills produce more POME per season each year, because they produce more oil than small scale mills. The production of 240.4 L POME by transforming 1 t of FFB is less than 0.5 to 0.75 t estimated by Yacob et al. (2005). The Principal Component Analysis (PCA) reveals 4 waste production groups: small (Group 2), medium (Group 1), large (Group 3) and big (Group 4) mills waste producers, respectively. In the country, most of the mills are small waste producers. Medium, large and big mills produced the wastes quantities of 2, 11 and 66 small mills. The more important is the oil produced by mills, the more important are also the wastes produced by them. The palm oil productions depend not only on palm plantation, but the use of selected nuts and the money invested in the production. According to Twumasi et al. (2014), Tenera is a much better variety for industrial and economic purposes. Large mill (Group 3) employed more persons and had the big plantation area but produce less than very large mill (Group 4). This shows that large mills producers have some problems that do not permit them to reach their maximal capacity of production. EFB is more used as cooking fuel or incinerated for soap or as fertilizers by some mills. Fibre is used as cooking fuel and starting cake and also as fertilizers. Using all EFB and fibre quantities generated was not dependent on their quantities produced. In fact, small mills waste producers

Table 6. Production factors and waste quantities according to palm oil mills wastes production groups.

Parameter		Group 1	Group 2	Group 3	Group 4	P
Palm mills number		64	264	3	4	-
Employees number		7±7	12 ±12	83 ±102	54 ± 35	<0.0001
Total production months		6 ±1.6	6.5±1.8	7.3 ± 4	7.8 ± 2.2	-
Possession of plantation	Selected plantation	0	117	2	3	-
	Local plantation	0	46	0	0	-
	Selected and local possession	0	101	1	1	-
Source of nuts	Plantation	0	168	1	3	-
	Buying	64	0	0	0	-
	Plantation and buying	0	96	2	1	-
Characteristics of nuts	Local variety	36	104	1	1	-
	Selected variety	5	34	0	0	-
	Local and selected varieties	23	126	2	3	-
Average Plantation area (ha)	Selected plantation	0	4.1 ± 6.7	207.4 ± 39.4	83 ± 91.2	-
	Local plantation	0	3.5 ± 7.9	5	5	-
	Total areas	0	6 ± 9.2	209.1 ± 39.1	84.3 ± 89.7	<0.0001
Average Full fruits bunches (T)		144.9 ± 242.7	57 ± 94.5	942 ± 463	3723.6 ± 944.2	<0.0001
Average Palm oil quantity (T)		16.7 ± 28	7.2 ± 12.3	159.2 ± 98.5	252.9 ± 89.7	<0.0001
Average Wastes quantities produced per year (T)	EFB	31.3 ± 52.8	12.4 ± 22	132.7 ± 59.1	800.7 ± 418.1	<0.0001
	Fiber	13.6 ± 23.1	5.6 ± 10.3	135.2 ± 95.2	637 ± 312.6	<0.0001
	POME	40.9 ± 28	15.1 ± 23.7	233.4 ± 172.1	572.6 ± 90.3	<0.0001
	PKC	81.2 ± 136.4	30.3 ± 46.8	218.3 ± 80.8	937.5 ± 399	<0.0001

do not use all their wastes produced as the waste management is highly questionable in this area. How to manage waste is important as it is a source of pollution (Oyelola et al., 2009; Yacob et al., 2005). Contrary to other wastes, POME becomes a problem when so much was produced. Our study reveals that only small mills wastes producers in this location arrive to use all the waste produced by their systems. Otherwise, so much attention is needed where large and very large mills wastes are produced, this means that all semi industrialize mills and some modern and traditional mills that belong to medium wastes producers need to be improved in order to avoid environmental pollution, because this type of waste pollutes the environment 100 times more than domestic sewage (Schuchardt et al., 2007; Singh et al., 2010; Maheswaran and Singam, 1977).

Conclusion

Palm oil production globally contributes to the environment pollution. This production system monograph shows small, medium, large and big mill waste producers. Majority of mills in Benin Republic are small oil plants, with some big producers. The large quantities of EFB and fibre are not used while POME which is produced in a little quantity is easily valorised. But the pollutant nature of the latter requires proper management of critical priority. Further studies are needed for the relevant approach to manage the wastes generated and their effects.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Efficacy of some local *Bacillus thuringiensis* isolates against soil borne fungal pathogens

Al Banna L.[1], Khyami-Horani H.[2]*, Sadder M.[3] and Abu Zahra S.[1]

[1]Department of Plant Protection, Faculty of Agriculture, University of Jordan, Amman- 11941, Jordan. [2]Department of Biology, Faculty of Science, University of Jordan, Amman-11941, Jordan. [3]Department of Horticulture and Crop Sciences, Faculty of Agriculture, University of Jordan, Amman-11941 Jordan.

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Seven Jordanian strains belonging to the bacterium *Bacillus thuringiensis* (*Bt*) were evaluated for their antifungal effects on soil borne plant pathogenic fungi under laboratory conditions. The antifungal effects of total soluble proteins of *Bt* stains on the growth of two isolates of the fungus, *Fusarium oxysporum* (isolated from roots of wilted peach trees and tomato plants), *Fusarium proliferatum* (isolated from roots of wilted palm trees) and *Rhizoctonia solani* (isolated from infected tomato seedling) were investigated. Results showed that *B. thuringiensis thuringiensis* (J23), was the most effective strain on the two fungal species; *F. proliferatum* and the peach fungal isolate of *F. oxysporum*. *B. thuringiensis entomocidus, Bt* (J115) showed the highest activity on the tomato fungal isolate of *F. oxysporum*. While *B. thuringiensis pakistani* (J107*)* was the most effective on *R. solani*. The *Bt* (J139) was the least effective strain. Soluble proteins of all *Bt* strains showed variable potential inhibitory effects on the tested fungi. Soluble proteins of the most effective *Bt* strains can be developed for potential antimicrobial applications; however, these findings necessitate a step to test the efficacy of these soluble proteins as soil drench to suppress soil borne fungi under field conditions.

Key words: *Bacillus thuringiensis*, inhibition, *Fusarium oxysporum, Fusarium proliferatum, Rhizoctonia solani.*

INTRODUCTION

Species belonging to the genera *Rhizoctonia* and *Fusarium* are the most common and persistent soil borne fungi, attacking economic plants and causing serious damages (Agrios, 2005). The fungus, *Rhizoctonia solani* attacks several crops causing pre and post emergence damping off, in addition to the root rot on fruit trees (Agrios, 2005). Furthermore, several pathovars of the wilt, *Fusarium oxysporum* and *Fusarium proliferatum* attack vegetables, fruit trees, field crops and ornamentals (Abdalla et al., 2000; Agrios, 2005; Armengol et al., 2005). The infected plants show yellowing and wilting of leaves, and eventually cause the death of the entire plant. Several methods are generally used to suppress the fungi and reduce their harmful effects; these methods

*Corresponding author. E-mail: horani-h@ju.edu.jo. Tel: 00 962 (6) 5355000 (22010).

Table 1. Species of *Bacillus thuringiensis* isolates used in the study.

Species	Strain	Source	Location
Bacillus thuringiensis autoagglutinated	J71	Tomato	Al Shuneh
Bacillus thuringiensis entomocidus	J115	Lentil seeds	Jordan University
Bacillus thuringiensis jordanica	J 112	Soil	Jordan Valley
Bacillus thuringiensis kurstaki	J6	Water	Al Khirbah Al- Samra
Bacillus thuringiensis pakistani	J107	Tomato seeds	Amman
Bacillus thuringiensis pakistani	J139	Water	Jordan Valley
Bacillus thuringiensis thuringiensis	J23	Chicken manure	Gawr Kabed, Jordan Vvalley

include seed treatment, soil sterilization, and/or use of resistant cultivars (Abu-Blan et al., 1990; De Cal et al., 2005; Zhang et al., 2013; Chang et al., 2014). Resistance of cultivars was reported only against certain races of the wilt fungus, this resistance can be broken by several other means including certain nematodes in the soil (Sidhu and Webster, 1977; Naji and Abu-Gharbieh, 2004). However, once these fungi are established in the soil, it would be rather impossible to eradicate them. Although soil solarization alone is generally used to suppress these fungi, several reports have shown that integrating the use of bioagents like *Bacillus thuringiensis* (*Bt*) as a component of integrated pest management (IPM) was the most effective method (James, 2008; Naranjo, 2011; Tabashnik, 2008). It was reported that corn cultivars that have been engineered with the *Bt* strain (Bt corn) lowered the severity of ear rot caused by the fungus *F. oxysporum* (Folcher et al., 2010; Nedělník et al., 2012). Investigators stated that the *Bacillus* spp. may assume their antagonistic effects by producing cell-bound antifungal compounds (Edwards, 1993; Walker et al., 1998) or indirectly by inducing plant resistance mechanisms. Edwards and Seddon (2001) identified the antifungal compound exhibited by the bacterium, *B. brevis* against the fungus *B. cinerea in vitro* as gramicidin S. The Jordanian *Bt* strains showed insecticidal and nematicidal effects **(**Khyami-Horani et al., 1999; Al-Banna and Khyami-Horani, 2004; Abu-Dhaim et al., 2006). Herein we aimed at investigating the effect of Jordanian *Bt* on the growth of some *F. oxysporum, F. proliferatum* and *R. solani* isolates.

MATERIALS AND METHODS

Fungal isolates

Three local isolates of *Fusarium* sp. and one isolate of *R. solani* were used in bioassays. The fungus *F. oxysporum* was isolated from the crown area of infected peach and tomato plants grown in Mafraq area (Jordan Eastern Desert) and in Jerash area (Northern Part of Jordan), respectively. An isolate of *F. proliferatum* was recovered from roots of palm trees grown in Qwarah area (Jordan Southern Desert). Whereas, *R. solani* was isolated from infected tomato seedling grown in the glass house at the University of Jordan campus, Amman. Pure cultures of the fungal isolates were identified to the species level based on their morphology (Booth, 1971; Domsch et al., 1980). The identification of fungal isolates was confirmed by the sequences of the ITS region of the rDNA (Nida Salem, Unpublished). For routine culturing, the isolates were grown on potato-dextrose agar (PDA; biolab, Hungary) (39 g/l; agar: 15.0g, potato extract: 4.0 g and dextrose: 20.0 g) and incubated at 24°C.

Bacterial strains

A total of seven strains of *Bt*, previously isolated from different Jordanian habitats (Khyami-Horani, 2002), were used in this study (Table 1). Glycerol stocks of *Bt* were stored at -80°C. Bacterial strains were streaked on nutrient agar plates overnight at 37°C. Single colonies were used to inoculate 150 ml of modified T3 medium (0.3% Tryptone, 0.2% Tryptose, 0.15% Yeast extract, 0.05 M NaH_2PO_4, 0.005% $MnCl_2.4H_2O$) (Travers et al., 1987). Cultures were incubated for 3 days at 37°C over an orbital shaker. The cells were pelleted for 10 min at 3.212 g and 4°C. Proteins were solubilized in 2.5 ml of pH 10 phosphate buffer (50 mM Na_2CO_3, 10 mM Dithiothreitol, 1 mM EDTA) (Fiuza et al., 1996). The solubilized protoxins were clarified by centrifugation for 5 min at 18.514g and 4°C. The pH of the supernatant was adjusted to 8 with 1 mol l^{-1} HCl and stored at -20°C. The solubilization was confirmed by SDS-PAGE gel electrophoresis.

In vitro assay

For each bacterial strain, four wells (8.5 mm) were made in each PDA plate (pH 8) using a cork borer, a total of 100 µl of the 2.5 ml soluble protein fractions obtained from three day culture (150 ml) were added to three wells. The fourth well was filled with 100 µl sterile distilled water to serve as a negative control. The plates were left overnight to allow the proteins to soak, then eight millimeter diameter of actively growing fungal culture discs from PDA plates of each tested fungi were cut using a sterile cork borer and placed in the centre on surface of the tested PDA media plates. Each *Bt* strain fraction was replicated three times. The plates were incubated at 24°C for one week. The plates were observed daily for fungal growth until the growth reached the edge of the control; the inhibition zone was then measured (mm) and recorded. The fungal growth on both control wells and *Bt* soluble protein wells was also monitored. The growth of the three isolates of *Fusarium* reached the edge of the control well after 4 days of incubation. Whereas growth of *R. solani* reached the edges of the water well after 3 days of fungal incubation.

Statistical analysis

Each treatment was replicated three times in a completely randomized design. The data was tabulated and analyzed using

Table 2. Effect of total soluble proteins of seven Jordanian *Bt* strains on local isolates of *Fusarium oxysporum*, *F. proliferatum* and *Rhizoctonia solani.*

Bacillus thuringiensis (*Bt*) strains	Strain	Inhibition of fungal growth (mm)*							
		F. proliferatum/ palm		*F. oxysporum* / Peach		*F. oxysporum*/ Tomato		*R. solani*	
		After							
		4 days	7 days	4 days	7 days	4 days	7 days	3 days	7 days
Bt autoagglutinated	J71	4.7^{ab}**	1.7^{b}	4.3^{ab}	2.3	3.3^{b}	1.7^{ab}	4.3^{b}	0.0^{b}
Bt entomocidus	J115	4.0^{b}	$0.^{3cd}$	4.5^{ab}	1.0	5.9^{a}	0.3^{c}	5.0^{ab}	3.7^{a}
Bt jordanica	J 112	4.0^{b}	1.7^{b}	3.7^{ab}	1.7	3.0^{b}	1.0^{bc}	5.7^{ab}	0.0^{b}
Bt kurstaki	J6	4.7^{ab}	2.7^{a}	3.7^{ab}	1.7	3.0^{b}	0.0^{c}	4.7^{b}	0.0^{b}
Bt pakistani	J 107	4.7^{ab}	2.7^{a}	3.7^{ab}	1.7	2.0^{b}	0.0^{c}	7.0^{a}	0.0^{b}
Bt pakistani	J 139	2.7^{c}	1.0^{bc}	3.0^{b}	1.0	2.0^{b}	0.0^{c}	4.0^{b}	0.0^{b}
Bt thuringiensis	J 23	5.3^{a}	3.3^{a}	5.0^{a}	2.7	5.0^{a}	2.7^{a}	5.0^{ab}	0.0^{b}
Control only water		0.0d	0.0d	0.0^{c}	0.0	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{b}
Lsd *p*=0.05		1.118	0.914	1.894	NSD	1.424	1.075	2.097	1.413

*Means of three replicates; ** Means followed by the same lowercase letter do not differ significantly ($p \pm 0.05$) according to LSD test.

analysis of variance (ANOVA) and the means were separated using least significant difference (LSD) at $P \geq 0.05$) (Little and Hills, 1974).

RESULTS

The inhibitory effect of *Bt* strains varied within the tested isolates of *F. oxysporum and F. proliferatum*. All *Bt* strain significantly inhibited the *Fusarium* isolates after 4 days of incubation. The bacterial strain *B. thuringiensis thuringiensis* (J23) was the most effective strain against both *F. proliferatum* (palm isolate) and *F. oxysporum* (peach isolates) (Table 2). Nevertheless, all *Bt* strains were effective against the palm isolates even after 7 days of incubation (Table 2).

B. thuringiensis entomocidus (J115) was most effective against *F. oxysporum* (tomato isolate) (Table 2). The strain *B. thuringiensis entomocidus* (J115) in addition to *B. thuringiensis thuringiensis* (J23) showed more significantly inhibitory effect than the other strains after 4 days of incubation with the tomato isolate of *F. oxysporum*. However, the effectiveness of the fractions of *B. thuringiensis* autoagglutinated (J71), *Bt entomocidus* (J115), *B. thuringiensis jordanica* (J112) and *B. thuringiensis thuringiensis* (J23) extended to one week (Table 2).

The fungus *R. solani* reached the control well after 3 days of incubation. The growth was inhibited by all *Bt* strains with the maximal significant inhibition by *B. thuringiensis pakistani* (J107) after 3 days of incubation. Only the solubilized protoxins of the strain *B. thuringiensis entomocidus* (J115) extended the inhibition of the fungal growth to one week and was significantly different from other isolates and the control (Table 2).

Although it was noticed that the inhibition zone was reduced after 7 days of incubation in all tested fungi, a crescent-shaped zone of inhibition of fungal growth occurred around the discs as compared to fungal growth surrounding control wells (Figures 1 and 2). Microscopical examination of the fungal growths of all tested fungi, near the margins of the inhibition zones, showed that the hyphae were distorted and included many vacuoles as compared to normal hyphae in the water control wells.

DISCUSSION

In this study, the total soluble proteins of seven Jordanian *Bt* strains were investigated for their biocontrol potential against some soil borne plant pathogenic fungi belonging to species of *Fusarium* and *Rhizoctonia*. Results showed that these *Bt* strains varied in their suppression of the growth of the studied fungi. Similarly, Raddadi et al. (2009) showed that several *Bt* strains inhibited the growth of *F. oxysporum* and *Aspergillus flavus*; strain *Bt* HD932 showed the widest antifungal activity spectrum**.**

The total soluble protein of strain *B. thuringiensis jordanica* (J112) resulted in the suppression of the growth of the studied fungal isolates. In our laboratory, *B. thuringiensis jordanica* (J112) expressed chitinase activity (Unpublished) which may be one component of the total soluble proteins. It has been reported that *Bacillus* species parasitism operates by degradation of cell walls of pathogenic fungi and using their extracellular lytic enzymes, including chitinase, an insoluble linear polymer of b-1,4-N-acetylglucosamine (GlcNAc), the major component of most fungal cell walls. *B. circulans* (Watanabe et al., 1990), *B. licheniformis* (Takayanagi et al., 1991; Trachuk et al., 1996), *B. cereus* (Pleban et al., 1997), *B. pabuli* (Frändberg and Schnürer, 1994) and *B.*

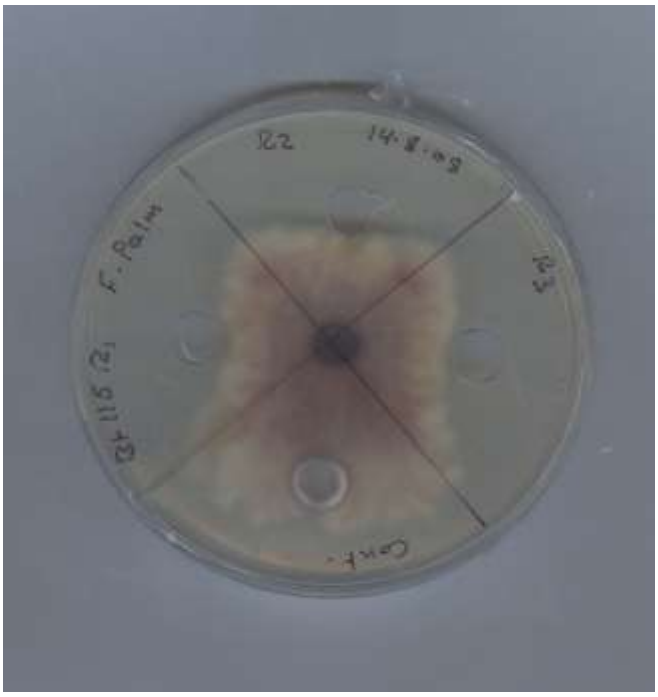

Figure 1. Potato dextrose agar plates showing growth inhibition of the fungus *F*usarium *proliferatum* (palm isolate) after incubation with 100 µl of the soluble protein fractions of the bacterial strain *Bacillus thuringiensis entomocidus* (*Bte* (J115)) that were added to each of the three wells (R1, R2, and R3). The fourth well was filled with 100 µl sterile distilled water to serve as a negative control (cont.). The crescent shaped zone of inhibition of fungal growth is observed around the fungal discs representing three replicates (R1, R2, and R3). While the fungus grew around the control well (cont.)

Figure 2. Potato dextrose agar plates showing growth inhibition of the fungus *Rhizoctonia solani* after incubation with 100 µl of the soluble protein fractions of the bacterial strain *Bacillus thuringiensis entomocidus* (*Bte* (J115) that were added to each of the three wells (R1, R2, and R3). The fourth well was filled with 100 µl sterile distilled water to serve as a negative control (cont.). The crescent shaped zone of inhibition of fungal growth is observed around the fungal discs representing three replicates (R1, R2, and R3). While the fungus grew around the control well (cont.)

thuringiensis (Chigaleichik, 1976) were reported to produce chitinase. Furthermore, Reyes et al. (2004) showed that chitinases from *Bt* suppressed the effect of *Fusarium* on the germination of soybean seeds.

The results showed that total soluble proteins of certain *Bt* strains inhibited the growth of the studied fungi; however, the growth was resumed after 7 days of incubation. Similarly, Kamenek et al. (2012) reported that *B. thuringiensis* delta endotoxins inhibited the growth of several *Fusarium* species, *R. solani* isolates, and *Phytophthora infestans*; and the growth was resumed after 6 days of incubation. These findings suggested that the proteins might be volatile and after sometime they may be reduced or their effect was fungistatic rather than fungicidal. Walker et al. (1998) reported that the suppression of the grey mold fungus, *Botrytis cinerea, in vitro* was exhibited by the *Bt* isolates, and the formation of inhibition zone was due to the metabolites released from the bacteria into the culture medium. Furthermore, Silo-Suh et al. (1994) reported that *B. cereus* also produced fungistatic antibiotics.

In this study, abnormalities of hyphae were observed in all tested fungi at the marginal edge of the disc after incubation with the *Bt* soluble proteins. These abnormalities might be due to lysis of cell walls and other biochemical changes caused by the soluble proteins of the *Bt* strains. Similarly, Sharma and Sharma (2008) reported that the antifungal metabolites of the bacterium *Bacillus subtilis* strain UK-9 caused morphological alterations of the hyphae and spores of the plant pathogenic fungus, *Alternaria* sp.

The inhibition of fungal growth might be due to an increase of respiration rate. Kamenek et al. (2012) reported that *B. thuringiensis* delta endotoxins inhibited the growth of several fungi; and stated that the antifungal inhibitory effect of *B. thuringiensis* delta endotoxin was due to an increase in respiration rate, they also speculated that the antifungal compounds might be linked to uncoupling of oxidative phosphyrylation and respiration in fungal cell.

The current results pointed to potential for biological control of soil borne plant, thus the *Bt* products or *Bt* crops could replace the hazardous or banned fungicides, or even reduce the concentrations of chemical pesticides if used together as part of integrated pest control. However, further studies are indeed required to identify the proteins or other compounds of these isolates to determine which protein or compound is responsible for the inhibition of growth of the specific fungal isolate. In

addition, the effect of the *Bt* endospores together with soluble proteins on germination of *Fusarium* spores should be investigated since Landa et al. (1997) reported that the cell-free culture filtrates of four Bacillus isolates inhibited the conidial germination of the fungus *F. oxysporum* f. sp. *ciceris*.

Thus, further field work should be employed by applying the *Bt* spores or the total soluble proteins as a drench on infested soil and study the effect on the fungus and on the resistance of the plant.

Conflict of Interests

The authors have not declared any conflict of interests.

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