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Full Length Research Paper

Physical, chemical, and microbiological evaluation of a compost conditioned with zeolites

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Zeolites are minerals characterized by high cation exchange capacity (CEC). This study evaluated possible effects of zeolites on improving the composting process in terms of the physical, chemical, and microbiological quality of compost and the productivity of the test plant, *Raphanus sativus* L. Compost enriched with 1.5% ground zeolites exhibited 62% greater degradation of organic particles sized less than 0.125 mm and 26% higher CEC than compost prepared without zeolites, while total coliform and *Escherichia coli* content were 96 and 36% lower, respectively. Moreover, the dried root mass of *R. sativus* was 21% higher with zeolite-enriched compost, subject to complementary mineral fertilization with macronutrients. The addition of zeolites to the composting process improved the quality of obtained organic compost as well as the productivity of plants grown using this compost. Given that zeolites are used mainly in the treatment of effluents in Brazil, and considering their mineralogical properties, more study is necessary to explore the effects of zeolite-enriched composts on crops with longer phenological cycles and grown in low-fertility soils.

Key words: Composting, clinoptilolite, rock dust, radish, *Raphanus sativus* L., cation exchange capacity.

INTRODUCTION

Zeolites are a group of hydrated crystalline aluminosilicate minerals of alkaline or earth alkaline metals, structured in tridimensional, rigid crystalline nets, formed by tetrahedrons of AlO_4 and SiO_4 and variations of TO_4 (T = Si, Al, B, Ge, Fe, P, Co, etc.), bonded by oxygen atoms (Arimi, 2017). Annual production of natural zeolites in 2016 was estimated to be 2.5 - 3 million tons, with cost varying from USD 50 to 800 per ton, in the USA, depending on granulometry and rock purity (US Geological Survey, 2016). However, exploitation in Brazil is still incipient and more research in terms of geological prospection is necessary (Luz, 1995; Resende and Mello Monte, 2008).

The main characteristic of zeolites is their high cation exchange capacity (Luz, 1995). Consequently, they have great potential as physical and chemical soil conditioners, and research investigating the effects of these minerals on soil and in crop productivity is urgently needed

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(Sangeetha and Baskar, 2016; Eroglu et al., 2017). To the best of our knowledge, few studies have thus far evaluated the effect of these minerals in soils. As reported in the limited literature available, zeolites have been found to increase the efficiency of nitrogen fertilization, reducing losses of NH_3 by volatilization from 76 to 24% (Giacomini et al., 2014; Villarreal-Núñez et al., 2015). An increase of up to 67% in available water capacity of an Entisol was also observed (Bernardi et al., 2009), and the addition of 2.5% zeolites was reported to increase water retention in Latosols (Barbosa et al., 2014). Furthermore, as zeolites can retain heavy metals in the treatment of effluents, when applied to cultivated soils, these could function as "filters" in the migration of cations and anions down the soil profile (Eroglu et al., 2017; Taamneh and Sharadqah, 2017). Further research regarding the agricultural value of these minerals could therefore stimulate large-scale exploitation and commercialization of zeolites in Brazil.

In view of the abovementioned qualities of zeolites in soil, the hypothesis of the present study was that incorporating these minerals during a composting process would improve the physical, chemical, and microbiological qualities of organic compost, consequently improving the productivity of plants cultivated using such compost. In this context, this study aimed at the following:

i) Evaluating the effects of the addition of zeolites on the physical, chemical, and microbiological qualities of organic compost.
ii) Evaluating the response to the addition of different dosages of organic compost produced with and without zeolites and with and without complementary fertilization on the productivity of radish (*Raphanus sativus* L.), using sandy soil as substrate.

MATERIALS AND METHODS

Study area

The experiment was carried out in the Faculty of Agronomy and Zootechnics of the Federal University of Mato Grosso (UFMT), Campus of Cuiabá, Mato Grosso State, Brazil (15.61156ºS, 56.06484ºW; altitude: 170 m). The climate in the region is Aw (tropical wet and dry or savanna), based on the Köppen–Geiger climatic classification (Alvares et al., 2014). The mean annual precipitation in Cuiabá is 1342.3 mm, and the annual average temperature is 25.8°C, with a mean relative humidity of 73.1%.

Collection of organic residue

Organic residue was collected from a horse stable at the Veterinary Hospital of UFMT. The residue was composed of feces and alfalfa remains (*Medicago sativa* L.), a leguminous plant, and tifon-85, a gramineous sterile hybrid resulting from the crossing of tifton-68 (*Cynodon nlemfuensis*) with *C. dactylon* from South Africa. These residues were kept in a stationary Brooks-type damp-cart with a volume of 7 m^3. The estimated density of the material on the day of collection was 270 kg/m^3 humid base and 188 kg/m^3 dry base (Kiehl, 2002).

Physical and chemical characterization of zeolites

Mineral zeolite material was acquired commercially from Brazil and presented predominantly coarse granulometry, i.e., ≥ 1.0 mm. The material was ground to increase the surface area until its texture was similar to that of rock dust, before the following tests were conducted: granulometry and routine chemical characterization (Silva, 2009), electrical conductivity at a 1:2 soil : water ratio (Gartley, 2011), and mineralogical analysis by X-ray diffraction (Moore and Reynolds, 1997). A D8 Advance instrument (Bruker, Billerica, MA, USA) was used, having a range of 5 - 90° at increments of 0.02° and an incidence time of 0.4 s per step. The instrument had a Cu2Ka goniometer with wavelength of 1.5406 Å and a dispersive slit width of 1.00 mm. Interpretation of the diffractograms was conducted using DIFFRAC.EVA version 3.1 software, with the database "Powder Diffraction File" produced by the International Center for Diffraction Data (ICDD, 2018). All analyses were carried out at the Laboratory of New Materials Characterization at UFMT.

Mounting and monitoring the composting process

Equine organic residues were placed in two compost bins, 5 m long and 1.20 m wide, with a lateral barrier 0.40 m high. To mount the layers, organic residue was homogenized manually with a rake and hayfork. A layer of leaves, about 0.10 m, obtained from the sweeping of the university gardens was deposited at the bottom of the compost bins to allow drainage of the liquid produced at the beginning of the composting process, thereby ensuring adequate gas exchange (Kiehl, 2002). Equine residue was then added until a 1 m high layer was reached. The initial volume of each pile of compost was approximately 5.25 m^3 and the estimated dry base mass was 987 kg (Kiehl, 2002).

Fifteen kilograms of dust powder rich in zeolites was pulverized, intercalating with the organic residue in one of the compost bins in a proportion of 0.015 kg of zeolites per kilogram of dry base compost. This proportion was defined on the basis of the high cost (about USD 2.50 per kg) of commercial product in 2016, without considering grinding cost for increasing surface area. The material in the compost bins was homogenized, that is, turned out, weekly. The composting process was initiated on July 26, 2016 and finalized on November 28, 2016, with the process thus totaling 126 days.

The temperature of the residue under decomposition was measured daily at 7:30 and 14:00 with a calibrated digital thermometer inserted in the material after removal of about 0.10 m of the superficial layer; a mean value was subsequently calculated. If the surface layer of the compost was dry, 10 L of water was applied to both compost bins using a garden watering can.

Physical analysis of the compost

At the end of the composting process, 10 simple samples of approximately 4 L were drawn from the surface and base of the compost and were mixed to produce one sample. Part of this sample was used for chemical and microbiological analysis, while the remainder was used for granulometry determination using sieves with mesh sizes of 8, 4, 2, 1, 0.5, 0.25, and 0.125 mm and a base to collect particles of < 0.125 m. For each compost, an aliquot of approximately 1 L was placed on the upper sieve and submitted to water jets until the eluate reached the last sieve. The material retained in each sieve was dried at 60ºC for 72 h. This

Table 1. Physical and chemical characterization of the 0 - 0.20-m soil layer.

Sand[1]	Silt	Clay	Ca	Mg	Al	H	CEC	OM	pH	P	K	BS
(%)			($cmol_c\ dm^{-3}$)					($g\ kg^{-1}$)	($CaCl_2$)	($mg\ kg^{-1}$)		(%)
81	8.4	10.6	3.55	1.25	0.0	1.6	6.53	27.1	6.6	18.1	50.6	75.5

[1]Sand, silt and clay content was determined using the pipette method (Donagema et al., 2011); exchangeable Ca, Mg, and Al were extracted with KCl 1 mol L^{-1}; H and Al were extracted with a solution of calcium acetate at pH = 7; CEC is cation exchange capacity; OM (soil organic matter content) was determined by burning in a muffle furnace at 600°C for 6 h; pH in $CaCl_2$ 0.01 mol L^{-1} was determined at a proportion of 1:2.5 (soil:$CaCl_2$); exchangeable P and K were extracted with a 0.05 mol L^{-1} HCl solution and a 0.025 mol L^{-1} H_2SO_4 solution (Mehlich I); BS is percent base saturation (Silva, 2009).

determination was done in eight replicates.

The dry and humid color of each sample of compost was determined on with the basis help of the "Munsell Soil Color Book" as well asand the wet consistency of the material in terms of plasticity and stickiness (Santos et al., 2013ab).

Chemical analysis of the compost

1 L aliquot of each compost was dried in an oven at 60°C for 72 h and then passed in a Willey-type knife mill coupled to a 0.30 mm sieve. Two sub-samples were dried at 105ºC for 12 h to reduce water content. To determine the amounts of inorganic residue (ash) and incinerated organic material, 0.5 g of each ground material, in triplicate, were submitted to calcination at 600ºC for 6 h in a muffle furnace (Silva, 2009).

To determine the nutrient content (P, K, Ca, Mg, S, Zn, Cu, Fe, Mn, and B), another subsample of 0.5 g was oxidized with hot acid solution (HNO_3:$HClO_4$ ratio of 3:1) to eliminate organic carbon and then passed through filter paper, retaining the insoluble residue. The filtered solution, referred to as mineral soluble material, was used to determine nutrient content using inductively coupled plasma optical emission spectrometry, previously calibrated with ISO 9001 certified standard solutions (Silva, 2009).

Determination of N-NH_4^+ was done using the Kjeldahl method, consisting of acid digestion with H_2SO_4 followed by distillation in NaOH, with collection of NH_4^+ in H_3BO_3 solution, which was titrated with HCl with an indicator (Silva, 2009).

Microbiological analysis of compost

We quantified coliform bacteria in 10 replicates for each compost. This bacterial group was selected because of the origin of the organic material, comprised primarily of equine feces, among other materials, that naturally contain high concentrations of these organisms. In the laboratory, 10 g of each compost was added to 90 mL of sterile extracting solution (0.1% sodium pyrophosphate and 0.1% Tween 80), with one vigorous agitation to obtain a 10^{-1} dilution. From this solution, new dilutions were prepared. Determination of coliform bacteria (total coliforms and *Escherichia coli*) was done using the chromogenic–fluorogenic substrate method (system Colilert®/Quanti-Tray – INDEXX® Inc., Westbrook, ME, USA), with results expressed in most probable number (MPN) per 100 g.

Plant experiment

The plant experiment was carried out in vases in a vegetation house with a plastic roof and lateral net for protection against insects. We adopted the complete randomized design with four replicates in a triple factorial arrangement (5 × 2 × 2): (i) compost dosage; (ii) with and without zeolites; and (iii) with and without complementary mineral fertilization, with 80 vases in total. The vases had a diameter of 10 cm and were 35 cm high. The response variable evaluated was the dry mass of the tuberous radish root (*R. sativus,* Hibrido Genius F1 100 GR – Tecnoseeds, Uberlândia, Brazil).

The doses of each compost were 0, 15, 30, 45, and 60%, with vase height of 35 cm considered to be 100%. Structurally loose (sandy) soil was added to each vase, and the height was filled with compost. The content of the vase was withdrawn and placed on a tray, carefully mixed, and returned to the vase. This procedure aimed at simulating compost spreading applications on soil, allowing further incorporation of the compost into the soil. The soil used in this experiment was collected from the 0 - 0.20 m layer of Cambisol Haplic Eutrophic Tb, corresponding to Cambisols based on the Food and Agriculture Organization's World Reference Base for Soil Resources and to Inceptisols based on the US Department of Agriculture's Soil Taxonomy system (Santos et al., 2013a). Soil physical and chemical characteristics are given in Table 1.

Mineral fertilization of the soil followed technical recommendations for *R. sativus*, that is, doses of 10, 100, 40 kg ha^{-1} of $(NH_4)_2SO_4$, MAP, and KCl, respectively. Vases were then irrigated until the water started to drain. One hour after the draining stopped, one extra vase for each treatment was used to determine water content at field capacity (kg kg^{-1}) (Pereira, 2011). In sequence, five radish seeds were placed in each vase at a standard depth of 1 cm, and five days after germination, the plants were thinned out, keeping only the most vigorous plants.

The vases were watered every day at 8:30, with the amount of water administered based on the difference between water content at field capacity and the daily weight of one replicate of each treatment, thus replacing water lost by evapotranspiration. Weekly, extra vases were weighed (in each treatment) to calculate losses and to minimize irrigation errors due to the growth of aerial parts and roots. After the radish harvest on the 32nd day after seeding, plant productivity was calculated as the dry mass of the *R. sativus* tubercle (dried at 60°C for 72 h) (g $plant^{-1}$).

Statistical analysis

The normality of data distribution was tested using the Shapiro–Wilk test ($p > 0.05$) (Hair et al., 2009). Once this was confirmed, compost granulometry, total coliform concentration, and the dry mass of the *R. sativus* tubercles were submitted to variance analysis at error probability of 5%. If a significant F-test result was obtained, the Tukey test was applied to compare mean values ($p < 0.05$) (Hair et al., 2009).

RESULTS AND DISCUSSION

Zeolite characteristics

There was a reduction in zeolite particle size after

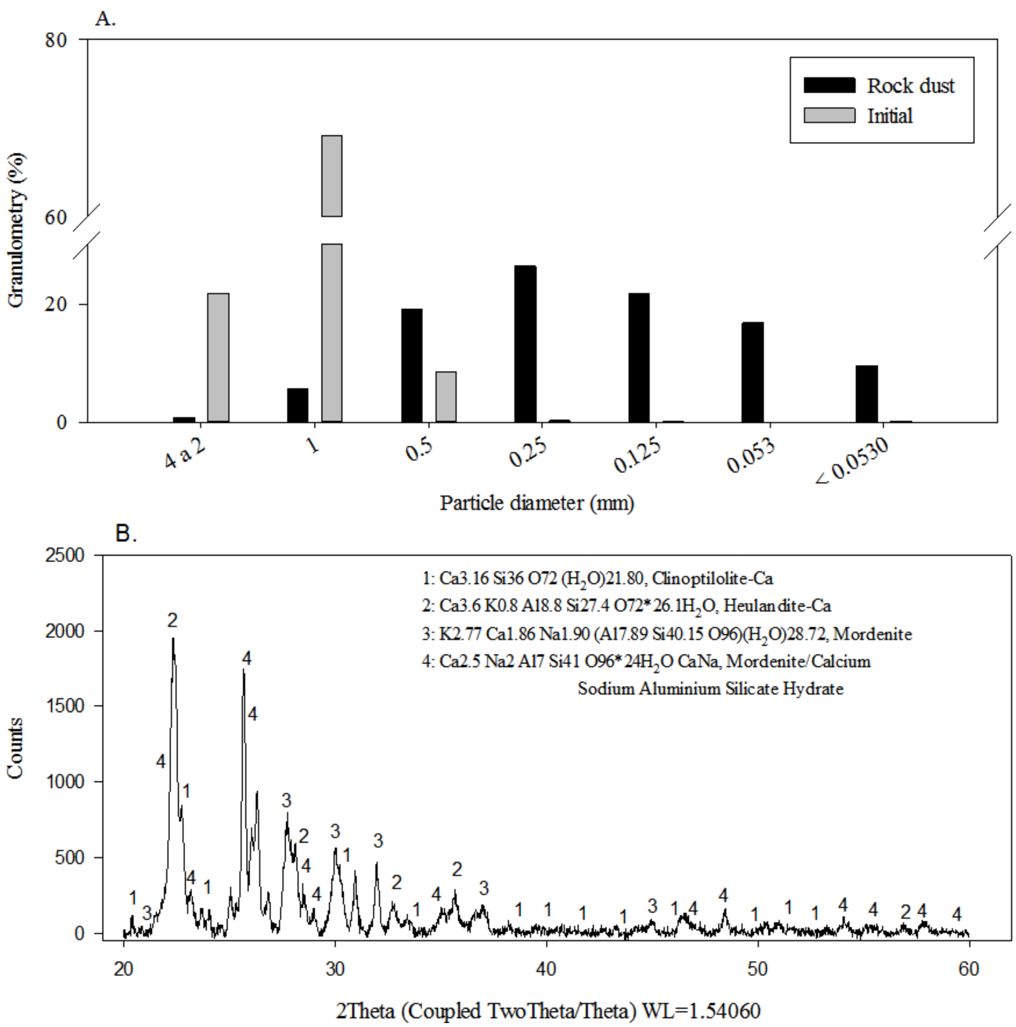

Figure 1. (A) Granulometry of zeolites before and after grinding, and (B) X-ray diffractometry of ground zeolites.

Table 2. Physical and chemical attributes of rock dust rich in zeolites.

pH[(1)]	P	K	Ca^{2+}	Mg^{2+}	Al^{3+}	H^+	CEC	OM
(C_aCl_2)	(mg dm^{-3})		(cmol$_c$ dm^{-3})					(g dm^{-3})
6.1	57.1	0.553	66.0	6.64	0.0	1.7	74.9	11.2

[(1)]pH in $CaCl_2$ 0.01 mol L^{-1} was at a proportion of 1:2.5 (rock dust:$CaCl_2$); exchangeable P and K were extracted with a 0.05 mol L^{-1} HCl solution and a 0.025 mol L^{-1} H_2SO_4 solution (Mehlich I); exchangeable Ca, Mg, and Al were extracted with 1 mol L^{-1} KCl; H and Al were extracted with calcium acetate at pH = 7; CEC is cation exchange capacity; OM (soil organic matter content) was determined by the oxidation method with potassium dichromate and colorimetric determination (Silva, 2009).

commercial zeolites were ground, with a more homogeneous distribution in size classes (Figure 1A). The mineralogical characterization of particles showed that zeolites were rich in Ca (Figure 1B), considering that these zeolite minerals are classified as a function to the predominant alkaline or earth-alkaline metal ions (Arimi, 2017; Eroglu et al., 2017).

The predominance of exchangeable Ca was confirmed by chemical analysis of the rock powder, which showed a high exchangeable amount of this metal, associated with a high cation exchange capacity (CEC) of 74.90 cmol$_c$ dm^{-3} (Table 2). This zeolite mineral was classified as non-saline since the aqueous electrical conductivity of the ground zeolite sample was only 0.077 mmhos cm^{-1} (< 0.40 mmhos cm^{-1}) (Gartley, 2011). High CEC was therefore caused mainly by adsorption and not by the

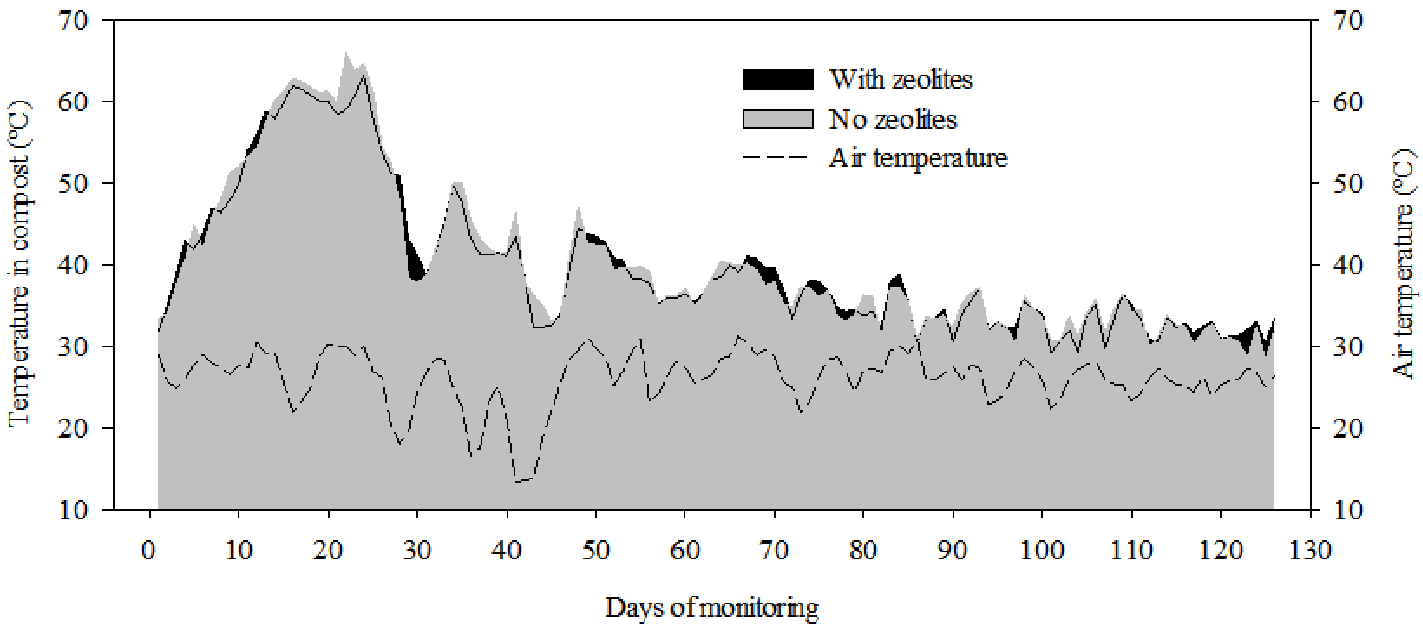

Figure 2. Temperature of air and of compost, with and without zeolites.

presence of soluble salts.

The CEC of zeolite powder was about four times that of tropical clayey soils with high CEC, that is, with CEC > 18 $cmol_c$ dm^{-3} (Sousa and Lobato, 2004). Moreover, the soluble P content of the zeolite powder was high (> 25.0 mg dm^{-3}) and three times higher than the P content of soil used in this experiment (Table 1). Depending on the amount of zeolites mixed with the soil and whether the addition was made to a sandy soil with low CEC, a considerable increase in CEC was therefore to be expected, with benefits for development of plants cultivated in sandy soils (Bernardi et al., 2009; Barbosa et al., 2014) of natural low fertility, also contributing to reduced fertilizer losses by leaching (Taamneh and Sharadqah, 2017).

Physical characteristics of composts

No significant difference was observed in the daily temperatures of compost with and without zeolites. In fact, readings were close and strongly correlated ($r = 0.9852$; $p < 0.0001$). However, no correlation was observed between the temperatures of air and compost when considering all sampled days ($r < 0.12$; $p > 0.9889$) (Figure 2). The mean temperature of zeolite-enriched compost was 39.89°C (standard deviation, s = 8.86), whereas that of compost without zeolites was 40.10°C (s = 9.36). Nevertheless, a moderate and significant correlation between compost and air temperatures was observed ($r = 0.4625$; $p < 0.0001$, with zeolites, and $r = 0.4318$; $p < 0.0001$, without zeolites) after the 60th day, during the humification (final composting) stage, which experiences lower biological activity (Ozimek and Kopeć, 2012).

The humification stage is characterized by a reduction in heat production and release mainly of carbon dioxide and water vapor (Kučic et al., 2013). This is because the availability of easily degradable organic matter declines over time, with the accumulation of recalcitrant material diminishing biological activity and hydrolysis of organic compounds, thereby diminishing heat release (Kiehl, 2002). Conversely, at the beginning of the composting process, compost temperature increases. The thermophile phase (40 - 65°C), which in the present study occurred from the 4th to the 41st day, is followed by the mesophile phase (predominance of temperatures from 30 to 40°C), observed from the 41st day onwards (Figure 2). The most critical phase is the latter, since it is responsible for destroying most pathogenic microorganisms. The temperature observed in the present study was within established quality criteria for aerobic composting, that is, temperatures of > 40°C for at least 14 days (National Council for the Environment, 2006).

Despite the similarity between the temperatures of both composts (Figure 2), treatment with zeolites allowed accumulation of particles with smaller diameters (Figure 3). This indicates that decomposition was favored in this treatment probably because of more adequate equilibrium between water content in the compost and its oxygenation over time (Kiehl, 2002). Since the amount of water used for irrigation was the same in both composts, the probable reason for the presence of coarser particles in the compost without zeolites was poorer oxygenation,

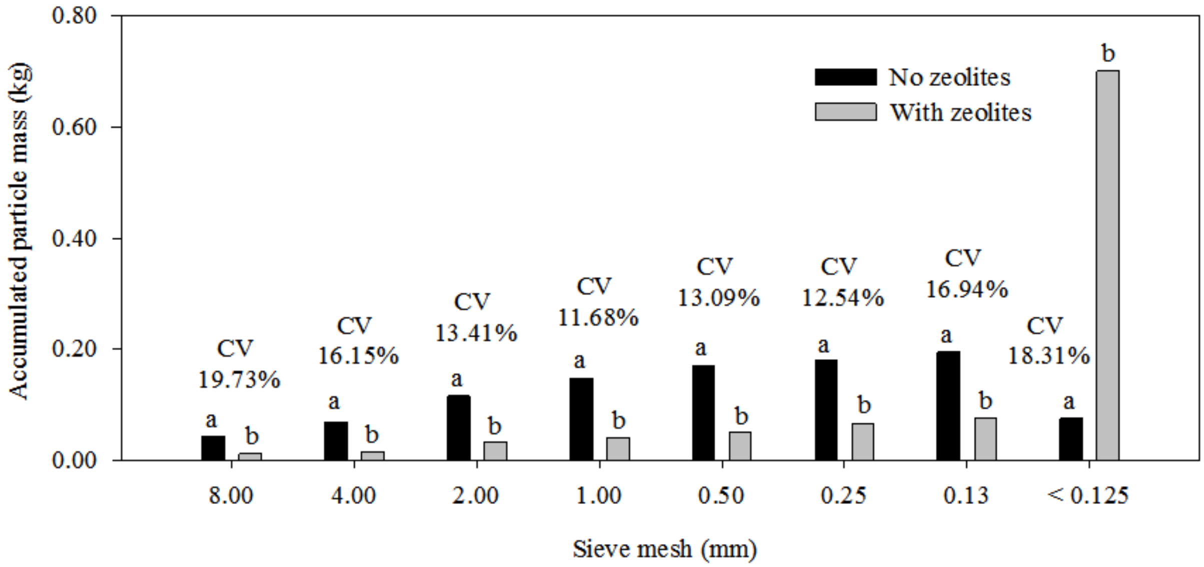

Figure 3. Granulometric curve of compost with and without zeolites, determined for a 1 kg sample. Mean values for the same sieve mesh with the same letter do not differ significantly, according to the Tukey test results ($p < 0.05$); CV: coefficient of variation.

given that the rate of anaerobic degradation of organic matter is slower than that of aerobic degradation (Erses et al., 2008). This hypothesis is justified by the higher concentration of total soluble Fe and Mn in the compost without zeolites, as discussed next.

Furthermore, whereas the humid color of zeolite-enriched compost was “dark brown” (7.5R 3/2), the color of that without zeolites was “black” (10YR 2/1), and whereas zeolite-enriched compost did not show any stickiness or capability to form a 4 cm diameter ball, the compost without zeolites showed both these characteristics. Mature organic material does not stick to the hands and does not flow through the fingers when compressed in the hand (Pons and Zonneveld, 1965), as observed with the zeolite-enriched compost. The opposite was observed for compost without zeolites, with this having low stickiness, behaving as a quasi-matured material. These observations therefore confirm reduced aerobic conditions in the compost without zeolites, that is, with slower decomposition.

Chemical compost characteristics

The greatest difference observed was a 26% increase in CEC and a reduction in soluble micronutrients (particularly Mn and Fe) in the zeolite-enriched compost, as compared to the compost without zeolites (Table 3).

It has been reported that natural zeolites promptly adsorb almost all exchangeable metals (Villaseñor et al., 2011), retaining metals by ion exchange and adsorption processes when these are released from the decomposition of organic matter (Stylianou et al., 2008). It is therefore likely that the 81.53 and 43.64% reductions in Mn and Fe solubility, respectively, in the treatment with zeolites were related to both the adsorption capability of the zeolites and the effect of aeration caused by zeolite addition. The compost without zeolites was always more humid, favoring anaerobic chemical reactions and causing the occurrence of reduction reactions in the compost, leading to higher availability of Fe (Chérif et al., 2009) and Mn (Millaleo et al., 2010).

Microbiological compost characteristics

The addition of zeolites to the composting process reduced the concentration of total coliforms by 96% and the concentration of *E. coli* (Figure 4) by 36%. It is likely that this result was related to zeolite adsorption properties. When used as a filter in sewage treatment, a natural zeolite was found to remove approximately 70% of total coliforms present in residual water. However, this removal was not selective to a specific organism (Cifuentes et al., 2012), meaning that the high adsorption capacity of zeolites leads to retention of all types of coliforms, due to the honeycomb-like microporous structure of these minerals (Smit and Maesen, 2008; Corona et al., 2009).

E. coli does not reproduce outside the intestinal tract of warm-blooded animals (Gruber et al., 2014), that is, its presence is an indicator of recent fecal contamination. The limited reduction in *E. coli* of only 36% may be related to possible contamination of the water used for

Table 3. Chemical characterization of compost produced with and without zeolites.

Observed variables	Results in dry base		
	With zeolites (a)	Without zeolites (b)	Difference
	.... 105°C		(a) - (b)
Total mineral residue (g kg^{-1})	447.8	474.6	-2.68
Insoluble mineral residue (g kg^{-1})	208.3	276.6	-6.83
Soluble mineral residue (g kg^{-1})	239.6	198.1	4.15
Total organic matter (combustion) (g kg^{-1})	552.2	525.4	2.68
Organic carbon (g kg^{-1})	172.9	143.5	2.94
pH $CaCl_2$ 0.01 moL^{-1} (1:5)	6.7	6.4	0.3
Total nitrogen (g kg^{-1})	19.5	18.9	0.06
C_{total}/N_{total} ratio	17/1	16/1	1
C_{org}/N_{total} ratio	9/1	7/1	2
Total P_2O_5 (g kg^{-1})	13.2	12.90	-0.03
Total K_2O (g kg^{-1})	20.9	16.50	-0.44
Total Ca (g kg^{-1})	24.9	18.70	-0.62
Total Mg (g kg^{-1})	6.7	5.70	-0.1
Total S (g kg^{-1})	2.9	2.50	-0.04
Total Zn, mg kg^{-1}	167.28	182.56	-15.28
Total Cu, mg kg^{-1}	37.62	47.23	-9.61
Total Mn, mg kg^{-1}	50.07	271.23	-221.16
Total B, mg kg^{-1}	106.1	121.7	-15.6
Total Fe, mg kg^{-1}	199.21	353.52	-154.31
CEC, $mmol_c$ kg^{-1} [(1)]	670	495	175

[(1)] CE: cation exchange capacity.

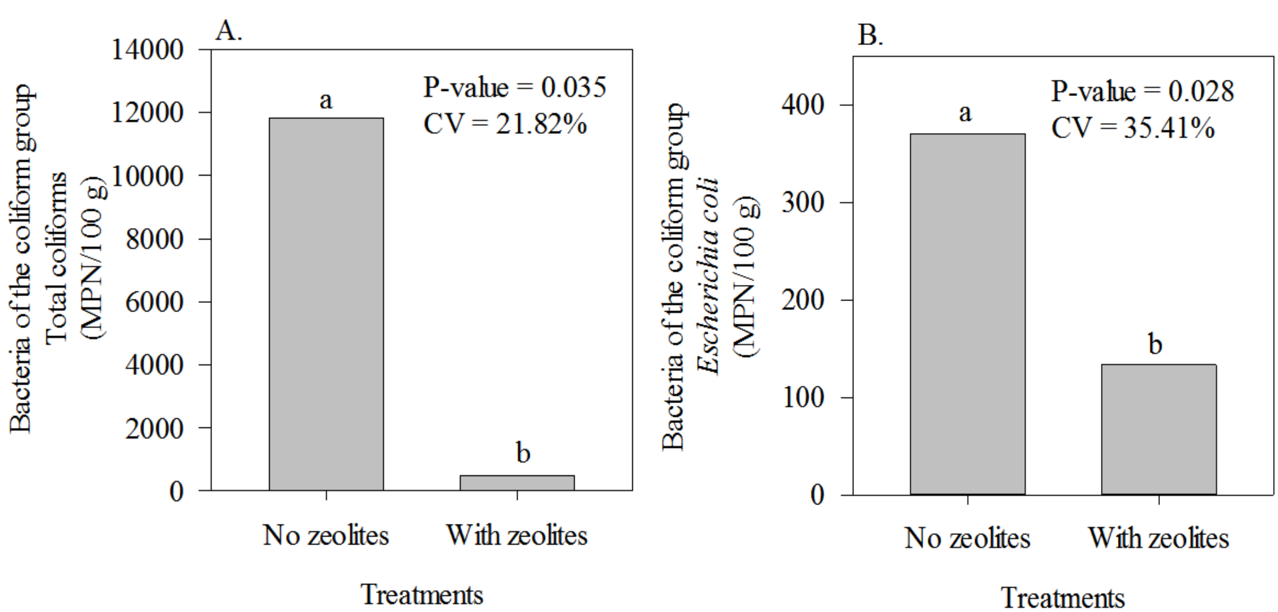

Figure 4. Mean coliform bacteria concentration in compost with and without zeolites: (A) total coliform (B) *Escherichia coli*. CV: coefficient of variation; MPN: most probable number of microorganisms.

irrigation, which was stored in water boxes placed on the soil and was used for garden watering by university employees. Nevertheless, the use of zeolites for pathogen removal seems promising. The efficiency of

Table 4. Mean comparison test for dry mass (g) of radish roots (*Raphanus sativus*); factor 1: compost dosages; factor 3: complementary fertilization with NPK.

Doses (%)	Factor 1: General mean	Complementary fertilization with NPK	Factor 3: General mean
0	0.31063b[1]	With zeolites	0.73500a[1]
10	0.77250a	Without zeolites	0.58075b
20	0.82688a	Dms	0.07467
40	0.70500a	CV (%)	25.18
60	0.67438a	Probability F-test	< 0.0001
Dms[2]	0.16603		
CV (%)[3]	23.09		
Probability F-test	< 0.0001		

[1]Mean values followed by the same letter do not differ statistically, according to the Tukey test ($p < 0.05$) results; [2]Dms: minimum significant difference; [3]CV(%): coefficient of variation. Data showed normal distribution according to the Shapiro–Wilk test ($p > 0.05$).

total coliform removal by coarse particle zeolites (> 2.36 mm) has already been demonstrated (Cifuentes et al., 2012), and an increase in the powder surface area may intensify this removal efficiency, as observed in our study, where the addition of only 0.015 kg of zeolites per kg of compost (dry base) caused a reduction of 96% in total coliforms.

Production of radish root

Statistically, no interaction between the studied factors was observed in the variance analysis. However, the general means of compost dosage and complementary fertilization with NPK differed significantly (Table 4).

In the Tukey test, factor 2, that is, treatment with and without zeolites without NPK, did not cause significant variations in radish root dry mass. However, the use of composts with zeolites, which were reinforced with base fertilization to correct soil NPK deficiencies, significantly increased root dry mass. Furthermore, the addition of only 10% compost, with or without zeolites, was important to increase radish root dry mass (Table 4). The addition of compost to soil is recommended for any greenery because of improvement in physical, chemical, and biological soil properties resulting from organic matter (Walsh and McDonnell, 2012). Similarly, an adequate amount of organic matter in soil is essential, since this enhances CEC, reducing nutrient leaching and ensuring a more constant supply of nutrients to plants (Maia et al., 2013).

Zeolites, particularly when rich in ground clinoptilolite, have been used for more than a century in Japan to improve the quality of agricultural soil because of their ability to enhance the adsorption of nutrients and water retention in soil (Luz, 1995). However, in Brazil, zeolites are mostly used in effluent treatments and, to the best of our knowledge, this is the first study evaluating the effects of zeolites on the composting process (Luz, 1995). More research is needed to evaluate the effect of different dosages of zeolites on composting and in soil for crops with longer phenological cycles. Moreover, it is important to assess the possible role of zeolites as pesticide-retaining filters, possibly limiting contamination of groundwater. It is therefore likely that zeolites will play a more important role in agricultural practice and food safety in the near future (Eroglu et al., 2017).

Conclusions

The addition of zeolite minerals with clinoptilolite, heulandite, and mordenite to a composting process using equine organic residues improved the physical, chemical, and microbiological quality of the obtained organic compost. Regardless of the addition of zeolites to the compost, the dry mass of radish root depended upon the proportion of organic compost mixed into the soil. However, a dose of 10% gave a dry mass similar to the 60% dose. Considering the long timeframe for producing compost using traditional processes, the use of the lower dose is recommended for radish cultivation. The dry mass of the radish tuberous root was also dependent upon complementary base fertilization (NPK); when this was applied to the zeolite-enriched compost, there was an increase in root dry mass, regardless of compost dosage. Therefore, when zeolites are used in soil, it is necessary to correct its macronutrient content, including N, P, and K.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Socio-economic and environmental impacts of invasive plant species in selected districts of Bale Zone, Southeast Ethiopia

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Understanding the socioeconomic and environmental impacts of invasive plant species from the affected communities' perspectives is essential to design and plan sustainable control and prevention strategies. Hence, understanding the socio-economic and environmental impacts in the infested and susceptible areas such as Bale zone is very crucial. Therefore, the objective of this study was to assess socio-economic impacts of in lowlands of Bale zone, Southeastern Ethiopia. House hold survey, focus group discussion and key informant interview to understand socio-economic and environmental impacts invasive plant species were used. Statistical package for social sciences (SPSS) (v. 20) was used for data collection. The results showed that a total of 12 invasive plant species were recorded, and of which *Parthenium hysterophorus*, *Xanthium strumarium*, *Argemone ochroleuca*, *Ceasalpinia spp*, *Acacia bussie*, *Acacia mellifera, Acacia seyal* and *Acacia tortolis* were highly distributed in the study areas. Respondents reported that heavy infestation of invasive plant species were found on the roadsides followed by arable land. The invasive plant species has also certain economic and ecological benefits. The local communities blame the invasive plant species for their negative impacts on biodiversity, degrading ecosystems, livestock and livestock products, crops, animal and human health. The study result showed that the local community utilizes chemical, mechanical and biological methods to reduce and control the impacts even though the percent of households that were trying to control is very low. Community perception showed the invasive plants species infesting grazing lands, crop lands, road sides, frosts and settlement areas. However, much has not been done to alert the local people on the danger of environmental impacts on biodiversity, agriculture and health. The menace of the species is increasing at an alarming rate, thus control methods have to be designed to stop further spreading into Bale Mountain National Parks.

Key words: Environmental impacts, invasive alien species, socio-economic impacts, Southeast Ethiopia.

INTRODUCTION

Globalization has brought social and economic benefits to many people, but it has also presented new challenges of which invasive alien species (IAS) are among the most significant. At no time in history has the rate of biological invasion (Mack et al., 2000) or the diversity and volume of these invaders been so high and the consequences so great (Reaser et al., 2007).

Ethiopia has a long history in the introduction of alien plant species, especially those which were found to be productive elsewhere and offered potential economic benefits to the country. In many other countries in the tropics, hundreds of alien plant species have entered Ethiopia intentionally and unintentionally (Abdulahi et al., 2017). In the country, there are many invasive plant species that are posing negative impacts on native biodiversity, agricultural lands, range lands, national parks, water ways, lakes, rivers, power dams, road sides and urban green spaces with great economy and social consequences being reported (Reaser et al., 2007; Abdulahi et al., 2017).

Invasive plant species reduces the effectiveness of development investments by choking irrigation canals, fouling industrial pipelines and threatening hydroelectric schemes thus, contributing to social instability and economic hardship, placing constraints on sustainable development, economic growth, poverty alleviation and food security (Habtamu, 2015; Abdulahi et al., 2017).

Previous works conducted in Southeastern Ethiopia indicated presence of the invasive plants for a long period of time (Takele, 2006; Teshome, 2006). Invasions of invasive plants hinder crop production through claiming agricultural lands and serving as a hiding place for crop pests and wild animals. The livestock feed shortage is also further complicated by the introduction and expansion of unwanted bushes and invasive weeds (Abate et al., 2010). The existing biodiversity and peoples livelihood is a threat because of invasive plant species (Mohammed et al., 2016). Therefore, there is a need to take a concerted look at the likely effects of invasive alien weed species on socio-economics of the community, and devise appropriate measures to mitigate the effects of these invasive plant species (Habtamu, 2015).

Community based knowledge plays a significant role in management and preventions of invasive plant species (Mulugeta, 2006; Herrie, 2014; John et al., 2014). People influence plant distribution and need management of invasions to reduce impacts and enhance benefit thus an understanding of social perspectives is important (Mulugeta, 2006). In recent years, growing bodies of literatures (Oba, 1998; Angassa et al., 2012; Tilahun et al., 2016) have tried to inform policy makers and development practitioners to recognize community's knowledge for sustainable management of their environment. Previous studies (Feye, 2007; Angassa et al., 2012; Tilahun et al., 2016) have also shown that communities' knowledge has a role to play in the advancement of scientific research and attainment of sustainable development goals.

Although knowledge of the communities has provided basis to design and plan sustainable control and prevention strategies, such knowledge has been overlooked in different areas of southeast Ethiopia. Studying the socioeconomic and environmental impacts of invasive plant species from the affected communities' perspectives is essential to design and plan sustainable control and prevention strategies. It would enable one to identify the communities' perception regarding the plant, determine the negative and positive impacts as perceived by the community and understanding the solutions in the context of the local social, cultural and environmental conditions.

Therefore, the objective of this study was to assess socio-economic and environmental impacts of invasive plant species in lowlands of Bale zone, Southeastern Ethiopia.

MATERIAL AND METHODS

Description of the study areas

The study was conducted in the selected districts of Bale zone, Oromia National Resional State, Southeast Ethiopia (Figure 1). The selected districts were Raitu, Dalomana, Ginir and Maddawalabu of Bale zone, Southeast Ethiopia. Rayitu district covers an area of about 6139 km^2 of land. Its climate varies from hot to warm sub-moist plains (Sm1-1) sub-agro ecological zone. The rainfall pattern is bimodal (March - June and September - October) with mean annual rainfall about 450 mm. The production system in the district is pastoral. Dallomana district covers about 9,543 km^2. The rainfall pattern is bimodal (March - June and September - October) with mean annual rainfall about 600 mm and mean annual temperature ranges between 26-42^oC. Madawalabu district covers about 9,543 km^2. The rainfall pattern is bimodal (March - June and September - October) with mean annual rainfall about 600 mm and mean annual temperature ranges between 26-42^oC. Ginir district is located 07^{0}08'357'' north and 040^{0}43'178''East. Also bounder is in the Eastern part of Bale zone. The rainfall pattern is bimodal (March – April and September - October) with mean annual average rainfall is 700mm whereas the minimum and maximum rainfall is 200 and 1200mm respectively.

Study design

Four selected districts of Bale zone, namely Raitu, Dalomana, Ginir and Maddawalabu, of Bale zone, Southeast Ethiopia, where infestation is high using purposive random sampling technique. For this study, cross-sectional type of study was employed.

Sampling technique and sample size determination

The study was conducted in selected kebeles of Raitu, Dalomana, Ginir and Maddawalabu districts of Bale zone, Southeast Ethiopia. In order to achieve the objective of the study, five (5) of 19 Kebeles of Rayitu, five (5) of 20 Kebeles of Madda wallabu, five (5) of 20 Kebeles of Ginir and 7 (seven) of 28 Kebeles of Dallomana district

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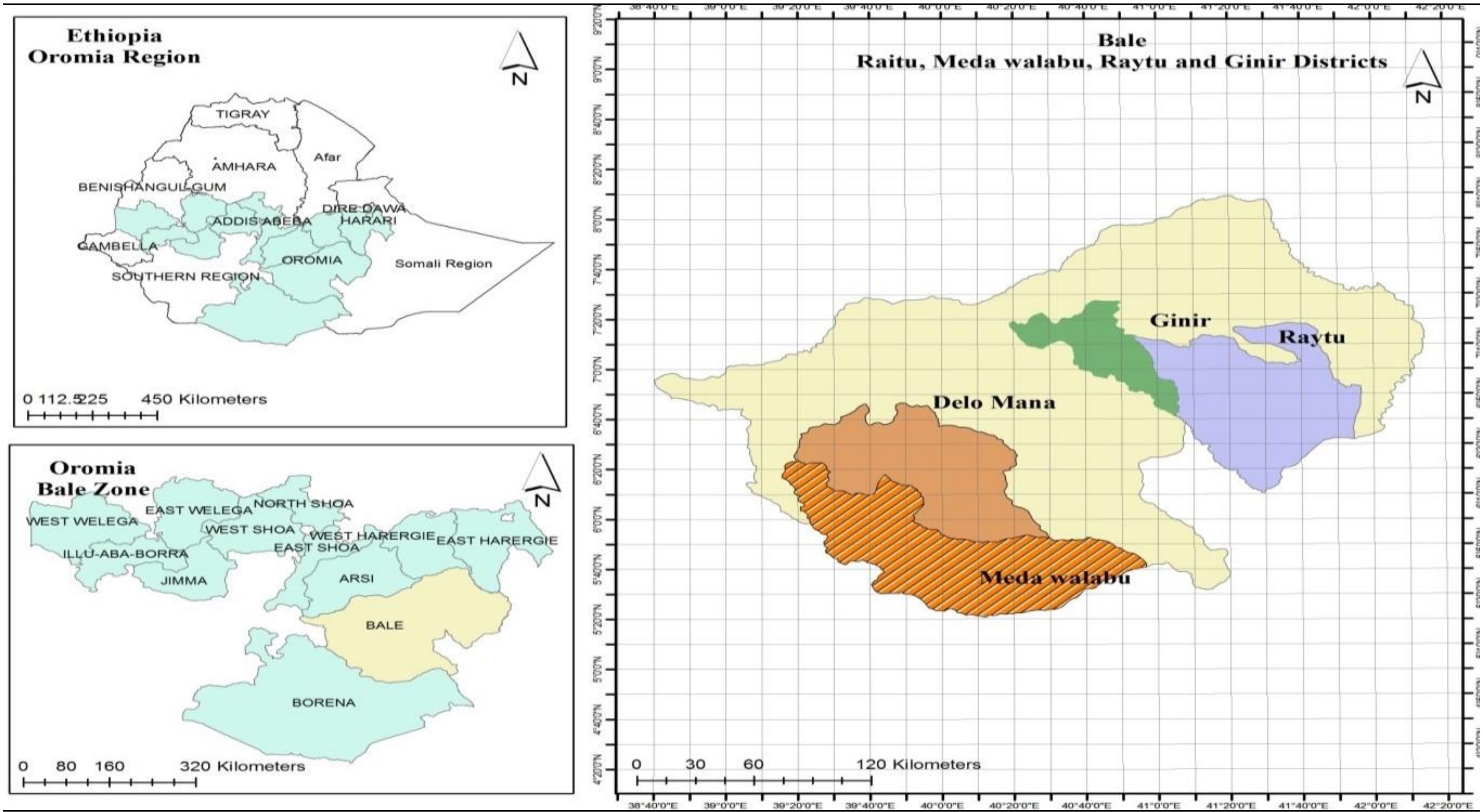

Figure 1. Map of study areas in selected districts of Bale zone, southeast Ethiopia.

were selected purposefully based on their importance to livestock as a major grazing areas due to problems of invasive plant species and representativeness of rangelands. The sample size of households was determined by using the formula recommended by Arsham (2007) for survey studies. Finally, a total of 286 households were selected by using proportional to population size technique from each study of kebeles. The number of households selected per Kebele was fixed based on the proportion of households in each kebele.

Data collection methods

The primary data were collected through household survey, key informant interview, and field observations. Household survey is one method of gathering primary data from horse mouth. By this particular method, 286 households were considered and interviewed as research subject from December 2016 to May 20, 2017.

A semi- structured questionnaire that includes both close and open-ended were designed and employed to generate quantitative data from respondents. The questionnaire was prepared in English language and translated to local language (Afaan Oromo). Three local enumerators who can understand both English and Afan Oromo languages were hired and trained on how to administer questionnaire.

Pretesting of questionnaire was conducted to see about inclusiveness, its validity, relevance and comprehensiveness. Based on the pre-testing feedback, final questionnaire was prepared and administered accordingly (Mussa et al., 2017).

During key informant interviews, key informants having long standing knowledge regarding invasive plant species were purposefully selected with the help of development agent and Kebele administrators. These informants are mainly elders, clan leaders of local communities, administrators and development workers (Tsegaye et al., 2010). Key informant interviews were conducted with these knowledgeable community representatives. For the key informants' interview, a total of 4 individuals were included in the study from each selected kebele.

The other method employed to collect firsthand information from the respondents were focus group discussion. It is one of the most important research methods to get varieties of information from different segments of the community for qualitative data which was conducted to get general information about the impacts of invasive plant species on pastoralist livelihood. In this particular research, focus group discussion with key informants containing 12 individuals were conducted in each Kebeles selected from each districts. A check-list prepared for and key informants interview, and an open kind of discussion were held. The information obtained from focus group discussions were analyzed and checked with those obtained by other methods for triangulation.

Field observation method was used during the whole period of field work activities by informally discussing with the people; observed by different activities carried out by the community to control the impacts of invasive plant species. During observation, field note was taken and issues were raised during focus group discussions and key informants interview to get insight about the issue under investigation. The secondary data sources that were used in this research were both hard copies and online materials such as published articles, unpublished documents, proceedings and project reports available at kebele, district, and zonal, regional, national and international levels.

Data analysis

The socio-economic data were coded, entered and analyzed using Statistical Package for Social Sciences (SPSS v.20). For data that does not require analysis, simple descriptive statistics were employed. Descriptive statistics such as mean, percentage and standard deviation were used to present the results.

RESULTS AND DISCUSSIONS

Demographic characteristics

Two hundred and eighty six households were successfully interviewed about their knowledge of the invasive plant species in south east Ethiopia. Of the total 286 respondents involved in this study, 252 (88%) were male households and 34 (12%) were female households. Among the households interviewed, 51 households were from Rayitu, 72 from Delomana, 72 from Ginir and 85 from Maddawalabu. The result of household survey showed tan average family size of 9.43±1.38 (± SD) (Table 2). This result is comparable with that reported for Rayitu district (9.45) by Mussa et al. (2017) but less than that reported for the Borana pastoralists (13) (Alemayehu, 1998).

In contrast to that of Borana pastoralists, the average family size in the study areas was higher than the ones reported for Afar (7.87) (Mohammed and Abule, 2015), of Borana (7.32) (Elias et al., 2015) and for South Omo zone (6.83) (Worku and Lisanework, 2016). The high family size might be associated with the cultural practice of polygamy for most of the pastoralists of the study area (Tables 1 and 2).

The age range was 31 to 89 years (mean 48±9.44) (Table 1). The level of education attained by the respondents was low, about 72.7% of the respondents had never been to formal school, only a few (26%) had attained primary education and even fewer (1.3%) had attained a secondary education (Table 4). Out of the interviewed households, 8% were uneducated and 64% of households had not attained formal education which indicated a low level of education which might be the case in many pastoral areas of Ethiopia (Admasu, 2006; Abule et al., 2007). This might suggest that such phenomena encountered in the study areas might impede technology transfer, intervention to be made and the need for introduction of pastoralists based education. During group discussions, pastoralists in the study areas indicated that due to shortage of trained staff, low motivation of teachers, mobility and cultural taboo towards sending girls to school the level of education is very low; this situation is in line with the report of Beruk (2003). Pastoralists do not allow their children especially girls to attend school mainly due to their cultural conviction; that if they send their children to school, they will be overwhelmed by their own livestock.

Table 4 presents the main occupation of the local community in Raitu, Ginir, Delomana and Maddawalabu districts of Bale zone, Southeast Ethiopia. The result of this study indicated that, about 80% of the households were agro-pastoralist (mixed farming system), 12% were pastoralist, while about 5.6% were pure agriculturalist and the rest 2.1% were involved in petty trade (Table 4). This is well documented in the earlier reports of Mussa et al. (2017) for these study areas. The majority of the people are engaged in both animal production and crop cultivations. The result of this survey revealed that livestock were used mainly for both traction and income generation from the sale of live animals.

Human population pressure, decrease in livestock feed, commercialization and settlement were the main reasons for increasing dependency on crop cultivation and decreasing dependence on animal rearing. According to the reaction of the respondents, the government was

Table 1. Sex, educational backgrounds and main occupation of sample respondents.

Variable		Number	Percentage (%)
Sex	Male	252	88
	Female	34	12
Main occupation	Pastoralism	34	12
	Agro-pastoralism	229	80
	Pure agriculturalist	17	5.9
	Petty trade	6	2.1
Education	Illiterate	23	8
	Elementary	74	26
	High school	4	1
	Diploma and above	0	0.0
	Some mosque education	185	65

Table 2. House hold size and age of sample respondents.

Variable		Mean ±SD
House hold size		
Sex	Male	10.22±1.39
	Female	8.24±1.29
	Total	9.43±2.38
Age of the respondents		48±9.44

inducing people from other Woredas to come and settle on the vast rangeland to expand commercial crop farming, especially sesame.

Introduction and dispersal mechanisms of invasive plant species

Invasions of invasive plant species are perceived to be widespread and increasing in many different environments in the study areas. All respondents (100%) mentioned that invasive plant species was present on their grazing lands, road sides, settlements and croplands. The majority of the local community indicated that invasive plant species had been in the area for long period of time. The result of this study is in line with the reports from highlands and lowlands of Ethiopia (Lemma et al., 2015; Tola and Tessema, 2015; Belayneh et al., 2016).

According Table 3, most of the respondents (26.5%) reported that, the plant is dispersed by wind, 20% by flood, 29% by animals, while 13.3% by vehicle and 11.2% by others (donkey). In line with the study, local community in Borana and Guji zone agreed that animals are major dispersal agents of invasive plant species (Lemma et al., 2015). Moreover, invasion of invasive plant species is aggravated by the aid of different dispersal agents, such as donkey, harvesting vehicles and road construction (Abiyot and Getachew, 2010). Parthenium is widely used as a shade during the transportation of Opuntia (Shooka) from Sawena (Parthenium infested) district to Ginir. Invasive plant species is also distributed with sands used for road construction and vehicle during harvesting. Some of the respondents also said that the increased importance of browsers such as camels and goats in the pastoral production system also resulted in the expansion of unwanted Acacia species. They also said that because the camels are highly browsers on Acacia species, their seeds are not digested but released through feaces and then regenerate again in masses.

Hence, Acacia species were believed to be widely distributed to districts of the study area with the introduction of camels in to the area which was totally absent in previous time. This result supports the report of Lemma et al. (2015).

Impacts of invasive plant species

According to the informants, invasive plant species has several harmful effects on the inhabitants of the study area (Table 4). Around 45.1% of the respondents replied that, the plant is threatening the local plants (biodiversity) whereas 23.4% of the informants perceived that the plant destroys the ecosystem. The rest of the respondents (17.8%), mentioned the plant has problematic thorns that cause problems to both humans and animals, about 5.7% respondents replied that the plants decreasing the productivity of crops, and other 8% agreed that the species negatively affects the animal products. Invasive plant species is one of the major threat to Ethiopian rangeland ecosystems and it diminish the biodiversity (Mussa et al., 2016). Furthermore, the study reported by

Figure 2. Invasive plant species invaded different environments in selected districts of Bale zone, southeast Ethiopia.

Table 3. Dispersal mechanisms of invasive plant species in the locality.

Dispersal mechanism	No. of respondents	Percentage (%)
Animals	83	29
Vehicle	38	13.3
Wind	76	26.5
Flood	57	20
Others	32	11.2

Table 4. Harmful effects of invasive plant species in the locality.

Harmful aspects	No. of respondents	Percentage (%)
Threatening local plants (Biodiversity)	129	45.1
Degrade ecosystem	67	23.4
Decrease crop productivity	51	17.8
Problematic thorn (towards human and animal)	16	5.7
Decreasing the quality of animal products	23	8

Lemma et al. (2015) and Belayneh et al. (2016) has also reported similar harmful impacts of invasive plant species on pastoralists and agro-pastoralists. The thorns of the plant are inflicting wounds on legs, hands and eyes causing blindness, lameness and even amputation of legs and hands due to infection of wounds. In the study areas, the local community expressed their views about the effect of parthenium on quality of animal products. They reported that the milk had bitter taste. This result confirms the reports of Lemma et al. (2015) and Archer (1997a). However, the pastoralists and agro-pastoralists said that the cattle feed on parthenium only during shortage of pasture (Figure 2).

Benefits of invasive plant species

As mentioned by most of the respondents in the Table 5,

Table 5. Economic and ecological use values of invasive plant species.

Economic benefits	Respondents		Ecological benefits	Respondents	
	N	Percentage (%)		N	Percentage (%)
Source of fodder and nectar	95	33	Combat desertification	73	26
Hedge (live and dead)	51	18	Decrease soil erosion	121	49
Fire wood	40	14	Reduce wind speed	41	14
Medicinal value	29	10	Shelter for wild life	13	5
Food	18	6	Shade tree	38	13
Construction	31	11	-	-	-
Charcoal	22	8	-	-	-

Table 6. Perceptions of local community on invasive plant species.

Perceptions on invasive plant species	No. of respondents	Percentage (%)
Disadvantage	246	86.2
Advantage	40	13.8
Should be completely removed (agreed)	265	92.7
Should proper management adopted (disagreed)	21	7.3
Attempted to remove or control (yes)	72	25
Attempted to remove or control (No)	214	75

invasive plant species has certain economic and ecological benefits. The results of this study showed that,invasive plant species is used as a source of fodder and nectar (33%), hedges (18%), fire wood (14%), medicine (10%), as food (6%), construction (11%) and used for charcoal (11%). The result of this study also showed that, invasive plant species has ecological functions such as combat of desertification (26%), soil erosion control (49%), reduce of wind speed (14%), shelter for wildlife (5%) and shade tree (13%) (Table 5). Invasive plant species such as acacia species are widely used by pastorals as a fodder for browser, nectar for bees, charcoal, medicine against different disease, fuel wood and construction material (Shackleton et al., 2017).

Perception of local community on invasive plant species

Table 6 presents the perceptions of local community on invasive plant species. The result of this study showed that most of respondents (86.2%) blame invasive plant species for the socio-economic problem facing them, and most of the respondents (93%) need complete removal of invasive plant species. The rest of the respondents (13.8%) mentioned the plant has important socio-economic importance as discussed earlier, and around 7% of respondents want proper management of invasive plant species. The local community specifically the pastorals in dry land of the study areas used woody plant species for charcoal, medicine, fuel wood, and fodder for browsing animals (Table 5). Despite the fact that residents recognized the negative impacts caused by invasive plant species, only 25% of respondents mentioned that they had attempted to remove or control the spread of invasive plant species from their cropping lands. This shows the lack of awareness of the local communities regarding the invasive plant species. Table 7 presents control methods of invasive plant species in the in Raitu, Delomana, Madda Walabu and Ginir districts of Bale zone. Most of the respondents (56%) agreed that, chemical control is a controlling mechanism for invasive plant species. On the other hand, 43% of the informants perceived that, mechanical methods such as burning is the best mechanism to eradicate invasive plant species. Whereas 2% of the informants reported that, biological methods should also be used to minimize invasive plant species (Table 7). Consistent with this study, pastoralists and agro-pastoralists and farmers in Borana and Afar uses the aforementioned controlling mechanisms (Shetie, 2008; Lemma et al., 2015). Similar studies has also reported burning (Archer, 1995) and having population of large browsers in invaded areas (Archer, 1995) which can be used to reduce bush encroachment. The local communities use chemicals (herbicide) and mechanical methods such as weeding and cutting in combination with burning to control the weed from their croplands. The use of chemical to control weed from rangelands is uncommon, and they use the mechanical ones to control the invasive plant species

Table 7. Controlling mechanisms of invasive plant species in the locality.

Controlling mechanism	No. of respondents	Percentage (%)
Chemical method	160	56
Mechanical	120	43
Biological	6	2

from their lands.

CONCLUSIONS

The spread of invasive plant species is now recognized as one of the greatest threat to the ecological and wellbeing of the planet. Pastoralists and agro-pastoralists and farmers in the study areas are affected by invasive plant species differently in different habitats. From composite data, a total of twelve invasive plant species were recorded.

Heavy infestation of invasive alien plant species was recorded on the roadsides followed by arable land. On the other hand, natural forest is the least infested habitat in the study area. Generally, disturbed habitats are more infested by invasive alien plant species than natural habitats. Acacia species have taken over vast grazing, crop land areas, blocks movement routes and access to available pasture and water points. They are seen to be very difficult to control and almost impossible to remove as they are easily spread and re-invade a cleared land unless a strict measure was taken.

Invasive plant species are dispersed by wind, flood, animals, vehicle and other types of transporting mechanism. Invasive plant species has also certain economic and ecological benefits. It is used as a source of food and fodder, nectar, hedges, fire wood, medicine, construction and charcoal. Combating desertification, soil erosion control, reduce of wind speed, shelter for wildlife and shade tree for human and animal are the ecological benefits of the species. The local communities blame the invasive plant species for their negative impacts on biodiversity, degrading ecosystems, livestock and livestock products, crops, animal and human health. The local community utilizes chemical, mechanical and biological methods to reduce and control the impacts of invasive plant species even though the percent of households that were trying to control is very low.

Hence, it was concluded that much has not been done to enlighten the local people on the danger of invasive plant species causing impacts on biodiversity, agriculture and health. Based on the results obtained, the following recommendations were drawn: Findings of this study can be used as part of the baseline information in managing the threat of invasive plant species; impacts of invasive plant species on human beings should be investigated in the study area; very little is known about the impacts of invasive plant species on the biodiversity in the study area and this should be given urgent attention to create awareness and research should be conducted in the area of invasive plant species in order to develop appropriate management systems.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Agricultural Knowledge and Technology Transfer Systems in the Southern Ethiopia

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Agriculture is the mainstay of the Ethiopian economy. However, the growth of the sector is constrained by different factors. Low level of technology development, inefficient technology dissemination, low utilization of improved production inputs and shortage of research proven production practices are among the most important factors hindering the growth of the sector. The objective of this study was, therefore, to assess the status of the current agricultural knowledge and technology transfer systems. For this purpose, a combined effort of literature study, expert elicitation and questionnaire based survey were carried out. The results of the study showed that a multitude of factors are constraining the system in the area. Limited economic capacity and awareness of farmers, lack of motivation of stakeholders, lack of motivation and knowledge level of development agents to support the transfer system are within the limiting factors. Weak linkage amongst the concerned actors, negligence of farmers' indigenous knowledge, and resistance of farmers to newly introduced technologies are also among the main factors hindering the efficiency of the system in their respective orders. Based on the findings of this study, it can be concluded that practicing participatory research approach, capacity building training, and mobilization of farmers are in urgent need for improving the efficiency of the transfer systems. Equipping with knowledge and skill and establishing a motivation scheme for development agents also a central solution to improve the systems. On top of this, timely dissemination of agricultural technologies, and information considering farmers indigenous knowledge needs to get due attention so as to improve agricultural knowledge-information and technology transfer system as well as smallholders' livelihood.

Key words: Agricultural information, agricultural technology, Southern Ethiopia, technology and knowledge transfer.

INTRODUCTION

Agriculture is the mainstay of the Ethiopian economy. The sector contributes 50% of the country's Gross Domestic Product (GDP). It directly supports about 85% of the population and generates 88% of foreign exchange earnings (Ayalew et al., 2015). The differences in production environment among regions enable the country to produce a variety of crops and rear different species of livestock. However, the production system is dominated by smallholder farming under rain fed condition. The farming systems are also traditional with subsistence crop and livestock mixed farming system, with an average per capita land area of 0.2 ha in 2008 (Francesconi and Heerink, 2010; Spielman et al., 2010). Although, the country is characterized by diverse agro ecological zone and endowed with ample natural resources which support successful crop and animal production, the agricultural sector has low productivity (Sewnet et al., 2016). By most measures, the growth and innovation of the sector is weak. For instance, between 1996 and 2005, agricultural GDP per capita grew only by 0.48 per year.

Various factors contributed to the low productivity of the agricultural sector and food insecurity in the country. Of all the barriers, the low level of agricultural technology development, dissemination, utilization of modern production inputs and the low adoption rate of proven research technological production packages by smallholder farmers are among the important factors (Sewnet et al., 2016). For instance, only 37% of the farmers use inorganic fertilizers, with a very low application rate of 16 kg per hectare. Moreover, the uses of improved varieties are very limited in the country (Spielman et al., 2010).

In the last decades, agricultural information has increased rapidly; however, the effective transfer of agricultural knowledge and technological package system is a bigger challenge. The main factors affecting the effective transfer of agricultural systems to the end-users are knowledge level of the information users, access to information of end users and readiness of farmers for adoption (Carrascal et al., 1995). Therefore, comprehensive transfer of research knowledge and production technology is demanded to impact the livelihood of the end users; farmers (Sewnet et al. 2016; Carrascal et al., 1995). Agricultural innovation system approach is already recognized as a best means to use as a comprehensive framework for analyzing the status of the agricultural system in developing countries (Klerx et al., 2009). Up-to-date and structured data coupled with open information transfer system in parallel with interactions among the stakeholders are necessary for improved agricultural information transfer system (Bouma, 2010) and agricultural production up-lift (Sewnet et al., 2016; Pezeshki and Dehkordi, 2006). According to Van Crowder and Anderson (1997), knowledge generation is considered as the mandate of researchers and extension agents, but to have effective information system, active participation of farmers, and other agricultural innovation actors need to be considered in the system.

Involvement of all innovation actors in the information/knowledge exchange, and the use of farmer's indigenous knowledge and farming systems are crucial (Aflakpui, 2007; Ashraf et al., 2007) to hasten information transfer, technology adoption rate of farmers, and make genuine decision. Therefore, strengthening the linkage between all the innovation actors is important to hasten the agricultural knowledge and technology transfer system and also to increase the effectiveness of the developed and disseminated agricultural technologies (Pezeshki and Dehkordi, 2006). Furthermore, establishing efficient knowledge and information transfer system in agriculture would help to attain efficient operation of agricultural systems (Carrascal et al., 1995).

Technology transfer is the main component of technology development; this is because for the developed technology to be applied effectively, it needs to reach the end-users of the technology with its full package and also the feedback need to reach the developer of the technology so as to involve the idea of all actors on the decision making. Considering technology dissemination as a main part of technology development and research coordination was started in 1960 in Ethiopia (EARO, 1998), and since then a number of efforts were made to improve technology transfer system and linkage between different partners like research, extension, farmers and other stakeholders (ICRA, 2010). Although extension has long history in Ethiopia, the coverage is very low and the linkage of the actors of the system is very poor (Sewnet et al., 2016; Davis et al., 2010), which is the main reason for low adoption of improved agricultural technology/production systems and inputs (Sewnet et al., 2016). Moreover, the extension agents are not accessible for farmers, and the interaction between different agricultural innovation actors is very limited in the country (Gildemacher et al., 2009). This poor linkage of the stakeholders coupled with disregarding farmers' indigenous knowledge in extension program and during the policy development process make the linkage ill-functioning (Kassa and Alemu, 2017; Sewnet et al., 2016). This calls for improvement of the linkage between the different agricultural innovation actors and information and knowledge transfer system, so as to alleviate poverty, improve the livelihood of the smallholder farmers in particular and the overall economic status of the country. Therefore, the main purpose of the current study was to describe the existing agricultural knowledge and the aforementioned issues a combined effort of literature

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limiting the efficiency of the system, and thereby forward suggestions for improvement of the system. To deal with study, expert elicitation and questionnaire based survey were implemented.

METHODOLOGY

Description of the study area

The study was conducted around Hawassa area, Southern Ethiopia. Hawassa, where the expert elicitation was made is located at about 273 km south of the capital city, Addis Ababa, Ethiopia with a geographical position of 7°4' North latitude and 38° 31' East longitude. The altitude of the area is about 1700 m.a.s.l. The average rainfall of the area is about 900-1100 mm annually, whereas annual maximum and minimum temperature are 27 and 12°C respectively. The site is characterized by sandy loam soil with 7.9 pH value, which is of volcanic origin and described as flovisol. Farmer based survey was conducted in Wondo Genet District, Sidama Zone, Southern Ethiopia. The Wendo Genet district is located in the Sidama Zone of the SNNPR with a latitude and longitude of 7° 1′ 0″ N, 38° 35′ 0″ E and an elevation of 1723 m above sea level. Two kebeles-small administrative units namely Banja Fabrica and Wetera Genda were selected from Wendo Genet district. These kebeles are located about 42 and 65 km respectively from Hawassa, the capital of SNNPR state.

Data collection techniques

The data were collected using three approaches. Literature study, expert elicitation, and questionnaire-based survey were carried out to solicit the required data. Each method is detailed next.

Literature study

To elicit data on agricultural information-knowledge and technology transfer system in the world and at country level, published and grey research papers were reviewed indepth. The literature review was useful to obtain general understanding of the research activities and the agricultural information system ahead of the questionnaire-based survey. Moreover, reviewing the secondary information helped to understand the main problems or issues and gaps that need to be emphasized and addressed during this particular study.

Expert elicitation

Following the literature review, interview was conducted with different professionals from the university, Agricultural Research Institute, Regional and Zonal Agricultural offices and nongovernmental organizations. All inclusive information like the way the information reach the farmer, inter-organizational interaction have their involvement level in agricultural information/knowledge and technology transfer system, level of contact they have with farmers, the way the farmers' demands are considered and development agents activities and the interaction farmers and development agents have, etc., were discussed during the interviews. Moreover, the main problematic issues hindering efficient transfer of agricultural information/knowledge and technology as well as the impact the technologies brought on the technology transfer system, identify the main factors livelihood of farmers were stressed.

Quantitative survey

Following expert elicitation, a questionnaire-based survey was executed. The questionnaire was filled by agricultural experts from Sidama Zone Agricultural Office of Southern Ethiopia, researchers from Southern Agricultural Research Institute, Research and Development Directorate Office of one public university in the zone, researcher from one public university in the zone and non-governmental organizations. The quantitative survey was conducted to quantify the important parameters related to the objective of the study. Quantification of these important parameters was used to confirm the qualitative information obtained during qualitative data collection stage, assess issues untouched during qualitative data collection stage and present the findings with empirical evidences. The questionnaire was divided into different sections. Under each section of the questionnaire, the respondents were asked to mention the main constraints concerning that particular section of the questionnaire before starting with the next topic to avoid mix-up or overlapping of ideas, so as to contribute for the general prioritization of the constraints. The questionnaire also included sections for listing the main factors inhibiting efficient knowledge and technology transfer and to access opinions of respondent's on the improvement of the system as a whole.

Data management

Relevant information obtained from all data collection stages (secondary data collection, interview, professional and farmers based survey) were systematically organized and summarized. For the quantitative data obtained from the quantitative survey, the questionnaire was tabulated, and Excel spread sheet and descriptive SPSS were implemented.

RESULTS AND DISCUSSION

Socio-economics and education on knowledge-information and technology transfer efficiency

The study revealed that farmers' education level has its own impact on information flow efficiency and rate of technology adoption. Of the surveyed farmers, 55.6% are illiterate, 27.8 got first cycle-primary education, while only 16.7% had secondary education (Table 1). Farmers with lower education levels are less likely to seek for information about improved technologies and application of the technology. As the majority of farmers (55.6%) are illiterate, their interest to learn about and adopt technologies is likely to be low. This is because information seeking, information giving capacity and use of improved technologies were determined by awareness level of farmers. Low education level also limits designing of extension activities, when compared with the so-called model farmers (Adugna, 2013). Farmers' reading and understanding ability of written materials used to transfer

Table 1. Education status of the sampled respondents.

Classification	Frequency (%)
Illiterate	55.6
First cycle primary school	27.8
Secondary school	16.7
Total	**100**

information. The result supports the previous finding of Soleiman and Saed (2013) who indicated the significant relationship of farmers' education characteristics with the rate of technology adoption. The income level of farmers was also found to influence their purchasing power for production inputs, especially of improved technologies which are relatively expensive. Significant proportion of the respondent farmers indicated that the cost of inputs is among the factors that hinder them from practicing the newly emerging technologies.

Farmers' level of involvement in technology development

When the sample respondent farmers were asked whether they were involved in the development of agricultural technology they use in their farming practice or not, they indicated that none of them were involved in any technology development processes including the one they are using in their production system. According to them, they have never visited a research centre to discuss the problem they are facing on the ground, to share the indigenous knowledge they have and to acquire improved technologies and trainings. This finding is in agreement with the study of Clark (2002) who indicated the lack of opportunities for farmers to invest on technologies to improve agricultural productivity. Sewunet et al. (2016) also revealed the existence of top down approach in the extension and research management system of Ethiopia, where the research and technology transfer system are designed and implemented without consideration of farmers' local knowledge, experiences and opinions. Especially, the poor and marginalized farmers are neglected in the According to the respondent farmers, they did not receive the technologies directly from the research institutions, but from other farmers and sometimes from the offices of agriculture. Since it is necessary that technologies be properly packaged to meet the needs of the targeted clients and achieve the desired productivity (Aflakpui, 2007), non-participation of the research institutions in the technology transfer process could hinder the continuous improvement of technologies through feedback. On the other hand, 44% of the sample farmers indicated absence of training on production management of the technologies they are already practicing. To ensure the optimal application of technologies the capacity of the farmers need to be strengthened through hands-on training.

The survey carried out on agricultural professionals indicated the significance of field day as means of information and technology transfer. This is supported by the report of Aflakpui (2007), who pointed out that organizing field day visit is one of the most effective dissemination methods of agricultural technologies. In relation to this, 83% of the surveyed farmers pointed out that they have a trend of visiting neighbor farmers' field practicing agricultural technologies. Farmers also share the information they have with family members (11%) and relatives (6%) (Table 2). This is in-agreement with the finding of Spielman et al. (2002) which states the potential actors who play a role in sourcing information including public sectors (research, extension, universities, enterprises etc.), private sectors (traders, entrepreneurs, companies), farmers cooperatives, NGOs, farmers, families, rural communities.

Farmers also pointed out different factors constraining them from getting information about the agricultural technologies they are practicing. Of these factors, absence of information about the agro-technology from the original sources, that is, professionals, research institutes takes the great share by contributing about 83% of the reason, whereas, lack of interest of farmers to share the information contributed to 17% of the reasons why information is scarce. This is in agreement with the finding of Day et al. (1994) who explains the extent to which lack of efficient communication hinders dissemination of research results to the desired user and effective application of the technology as proved by research.

Farmers also indicated their own knowledge limitation as a constraining factor to share the information they have. This can be improved through increasing the farmers' awareness and knowledge level. Moreover, the emphasis needs to be given by professionals to reach timely and proper information about the technology they are developing to improve the information system in the area as well as its effective application; because of the developed technologies. Since the absence of efficient communication about the technologies could result in failure and deficiencies in the dissemination of research results thereby the technologies remain without addressing the desired objectives and impacting the end user (Day et al., 1994; Aflakpui, 2007).

Farmers' sources of information about agricultural knowledge and technology

The survey made on the professional based survey

Table 2. Sample respondent indigenous methods of agricultural technologies dissemination.

Items/description	Frequency (%)
Visiting neighbor farmers' fields practicing improved agricultural technologies	83
share the information with family members	11
Share the information with relatives	6.0
Total	**100**

indicated the use of different means to transfer information about agricultural technologies or best practices, such as written materials (brochure, leaflets, pamphlets, manuals, journals, and proceedings), workshops, training, field day, and demonstration. This is in line with the study of Aflakpui (2007), which presented increasing publication, publishing production guides and farmer's handbooks, publishing in local language so that the farmers could understood it, ensuring the accessibility of publications to technology transfer agents, organizing field days and demonstrations as a means to increase technology adoption rate thereby productivity.

However, the response from sampled farmers showed the dominant use of neighbor farmers and agricultural development agents as a source of information. Information exchange between neighbor farmers contributes 60% of the information sources, which is supported by previous studies of Van Crowder and Anderson (1997) indicating the essentiality of farmers' involvement in technology development and transfer in ensuring acceptability and effectiveness of technologies. Forty percent of the information exchange or transfer is contributed by agricultural development agents. This shows the significant contribution of farmer to farmer or informal information exchange system. This is the appropriate system from the view point of ensuring successful information transfer and application of technology, since it avoids the hierarchical knowledge transfer system, which assign researchers in the top, next extension and farmer knowledge at the bottom of the hierarchy (Douthwaite et al., 2010; Van Crowder and Anderson, 1997). Ensuring the efficiency of farmer-to-farmer information system demands reaching them with tangible information which can be done through increasing farmers' awareness on the importance of having information about the technology they are practicing and also exchanging information with others.

Agricultural knowledge-technology transfer and stakeholders' involvement

In Ethiopia, Research Institutions and Universities are the most responsible actors for technology development, whereas Agricultural Offices and Non-governmental organizations are involved in technology dissemination. Research institutes and universities perform the pre-scaling up and first phase of dissemination activities through establishing demonstration trial in addition to developing technologies. After developing the technology, it carries out the pre-scaling-up activities. Thereafter, if the technologies become successful on farmers' fields, the technologies will be conveyed to the Office of Agriculture, who then does the scaling up at a region level. The technologies are further disseminated to the agricultural office of the lower administration unit, and then to farmers through extension agents. This clarifies the absence of involvement of all the concerned actors from the initiation of the technology development process. This process is in agreement with Aflakpui (2007) and Van Crowder and Anderson (1997), who described a system where information and knowledge flows from research organizations to farmers through extension agents without full involvement of the stakeholders from the beginning; which is called linear/traditional knowledge and information flow system. This finding is also supported by Clark (2002), who describes linear information flow as top down transfer of technology; in which the agricultural practices are diffused in one direction without any complex information exchange between different actors and without giving room for farmers' knowledge. It is a conventional practice allowing only one way flow of information, which is condemned in the modern approach (Spielman et al., 2010).

This off-course shows the absence of participatory technology development system. A review research in Ethiopia on the similar topic by Sewunet et al. (2016) discussed the separate administration of research institutions and extension sector resulting with a limited work relationship between these actors. To some extent the different actors are involved during dissemination of the technologies as compared to technology development stage. This might contribute towards the ill-functioning of the agricultural knowledge and information system in the area. This is because the other party involves in the dissemination process without having deep knowledge about that particular technology because of absence of involvement during the development of the technology.

This might affect the effectiveness of the developed technology as well as the agricultural information/knowledge and technology transfer system (Douthwaite et al., 2000). This is because the actors who are not involved during the technology planning and development process might have low understanding about the technology. The process also clearly shows the negligence of farmers demand and indigenous knowledge which can be solved through practicing participatory research approach. Moreover, pre-research problem assessment needs to get due attention so as to respond to farmers practical problem.

As per the new research structure of Ethiopia, agricultural research activities are executed in a case team bases, in which professionals from all the concerned departments are involved. The case team includes at least agronomist, soil scientist, agricultural extensionists and socio-economic professionals all having their role in technology development and dissemination process. This was designed to develop the technology with its full production package. This of course helps to have uniform understanding by all the concerned actors about the technologies developed. This system found to increase the participation level of different professionals from the beginning of the technology development, and thereby hasten the dissemination of the developed agricultural technologies.

For agricultural information/knowledge and technology transfer from research to practice and vice versa to be effective, the involvement of all the concerned stakeholders is crucial. According to the expert elicitation result, different actors were involved directly or indirectly in the information and technology dissemination process as compared to the technology development activity. Once the technology scaling up is done by research institutions, the technology reaches farmers through the office of agriculture and in some places non-governmental organizations also take part in the dissemination process. According to the response of the sampled professionals, office of agriculture, research institutes, NGOs, Universities, administrative peoples at different level, seed enterprises, media, cooperatives and marketing promotion were the stakeholders involved in agricultural information/knowledge and technology transfer system in their respective order. The office of agriculture is the main body who is mainly reaching the information to farmers followed by research institute.

However, the actors apart, research institutions and universities join the process after the technology development and scaling up processes are over. This is in different line with the modern system which gives room for farmers' involvement through actively participating, forwarding their perception about the existing problems, indigenous knowledge and farming practices of farmers (World Bank, 2006). Moreover, involvement of farmers' indigenous knowledge and their actual demand is negligible during technology development process. The same phenomenon is reported by Sewnet et al. (2016) and Davis et al. (2010). Rather, farmers are mostly involved by giving feedback about the technologies which they already practiced; whether the technology is effective or not. This one way approach, might decrease the acceptability of the technologies by farmers as well as the know-how of the other stakeholders about the developed technology. This can be improved by involving all the concerned stakeholders including farmers from the beginning of technology development since farmer's involvement plays indispensable role to overcome the failure of the developed technologies (World Bank, 2006).

Institutional linkage on agricultural knowledge and technology transfer

In the current research area, the concerned agricultural institutions were not strongly interconnected from the beginning of technology development process, which inclines to one way communication of information-knowledge and technologies. According to the results of this study the stakeholders cooperate in some part of dissemination processes rather than having strong interaction throughout the technology development and dissemination activities. Research institutions and Universities totally take the technology development part. After developing the technology they communicate to the Office of Agriculture about what technology they already developed and the need to give responsibility of technology scaling up at a large scale or regional level to the Office of Agriculture.

This shows the lack of strong linkage among agricultural stakeholders from the beginning of technology development though dissemination of the developed technologies. However, for agricultural information-knowledge and technology transfer system to be effective, it needs to have purposive and strong institutional linkage (Spielman et al. 2010). Similar situation was also described by Spielman et al. (2010), on their study of rural innovation system and networks in Ethiopia, elaborating the linear information-knowledge and technology flow process in which the information-knowledge and technologies only transfers from the scientists to extension agents to farmers. Kassa and Alemu (2017), in their study on "Agricultural Research and Extension Linkages: Challenges and Intervention Options in Ethiopia" noted a similar situation. Spielman et al. (2010) also indicated the importance of having diverse actors and interactions between these different actors to address efficient information-knowledge and technology transfer and increase agricultural innovation.

Feedback system on disseminated agricultural knowledge and technologies

After the technology is practiced by farmers, the feedback whether the technology is effective or not flows back to the sources of the technology through different channels. To get feedback whether the technology is effective and the practice as well as the farmers' selection process went well, a team from Zonal Office of Agriculture goes to the area where the technology is disseminated (direct system). This team observes the practice on ground and discusses with district agricultural office, development agents, focal person and farmers and check if it is done properly, from selection of farmers to application of the technology through the impact of the technology in the livelihood of farmers. Finally, the team discusses the feedback to the District Agricultural Office based on the observation result. In other ways, feedback reaches the Zonal Office of Agriculture through report (indirect way).

Farmers also give feedback about the technologies they are practicing; especially, if the technology is not successful they communicate to the office of agriculture. If the technology is effective in the area the neighboring farmers' demand for the technology increases, thereby the information and technology disseminates to a larger scale in the area. In this case, the technology may be disseminated from farmer to farmer or from the source of the technology to farmers, source in this case, is not the owner of the technology but the stakeholders who take the responsibility of disseminating the technology. Farmer to farmer dissemination might be important from an economically, efficient knowledge transfer and technology effectiveness point of view. This is in agreement with the finding of Glenna et al. (2010), stating the dependency of efficiency, acceptability and adoption of agricultural technology on this same issue. This means, if the farmer gets the technology from the neighboring farmer, it reduces the cost of transportation and helps them to get it on time. Furthermore, it increases the information exchanging behavior of farmers and it might increase the efficiency of the information system since they are on the same knowledge level.

Performance of the extension service in the study area

The result of the current study revealed that the extension system in the study area is weakly functioning. A multitude of factors was mentioned for their contribution to the ill-functioning of the extension system (Table 3). Moreover, as explained by the sampled professional respondents, the existing extension system is mostly one way, which might contribute for not having a well serving extension system in the area. Similar finding was revealed by Kassa and Alemu (2017), stating the implementation of a one-way communication model in Ethiopian agricultural extension system.

According to the current Ethiopian extension system, the development agent workers are the main actors that have frequent contact with farmers, and are expected to give theoretical and technical assistance for farmers. In addition to the aforementioned activities they are also expected to facilitate communication between farmers and other stakeholders working on agriculture, facilitate technology dissemination, capacitate farmers with practice of new technologies and information acquired from different sources, and actively participate in technology transfer process. Aflakpui (2007), in his study that deals with the present outlook and transformation in the delivery of agricultural extension services, the implications for research-extension-farmer linkage, listed role for extension agents which are in agreement with the finding of the present study.

However, according to the respondents, the extension agents are not serving as to the demand of the stakeholders including the farmers. As explained by sampled respondents, lack of motivation is the main reason why the development agents are not serving the system properly. This is in agreement with Sewnet et al. (2016) who stated the weak motivation level of the development agents. This same paper pointed out the weak incentive package designed for development agents as a reason why they lack motivation. Davis et al. (2010), in their study on public agricultural extension system of Ethiopia, also reported that development agents leave their position in search of better incentives instead of striving for agricultural development through working with farmers. A supporting finding was reported by Gebremedhin et al. (2006), stating the high turnover of the development agents due to the aforementioned reasons.

On top of the lack of motivation, knowledge and skill gaps of the development agents are frequently cited as a problem which then affects the efficiency of the extension or information and technology transfer system. According to the respondents from non-governmental organizations the development agent workers are not willing enough to respond to the diverse interest of the stakeholders. This might be explained by the low rate of incentives they receive; which thereby contribute to loss of willingness to serve up to their capacity (Gebrehiwot et al., 2012). Generally stating, the role the development agents are playing is incomparable with what they expected to contribute.

In the meantime, the development agents reasoned out the absence of facilities/infrastructure for the lack of interest to reside and work in the rural areas, where

Table 3. Factors influencing the performance of the extension system in the area

Items/description	Frequency in Percent
Absence of improved input supply	44
Lack of interest of DAs to assist farmers	28
Absence of training on improved production packages	17
Bias during farmers selection for training and input distribution	11
Total	100

DAs: development/extension agents.

Table 4. Sampled farmers practices of applying recommended technologies.

Response	Frequency Per cent
Apply the full recommended package	17
Apply only part of the recommended package	83
Total	100

actually the farm is situated in the cases of Ethiopia. This is in line with the report of Davis et al. (2010), discussing lack of the basic infrastructure and resources including fund, operation equipment and input at the farmer training center (FTC) and Woreda/Destrict level.

Technology adoption behavior of farmers

According to the sample respondents, although previously, resistance was observed to shift from the local practice to the research proved and newly introduced technologies, these days' farmers are showing interest towards new technologies. They actively participate in visits of demonstration trials and farmers' field days, and these shows their interest to learn about and adopt emerging technologies. Currently, farmers complain about shortage of improved agricultural technologies, which is again an indicator of their interest towards adoption of improved farming practices, although farmers are interested in adopting new technologies. The majority of them do not apply the full package of the recommended technologies (Table 4). Effective application of the full package of technologies is inhibited by different factors of which economic capacity and knowledge level of farmers takes the major share. Even though, different factors are contributing towards the substandard application of recommended technologies the low income level of the farmer takes the major share in inhibiting the correct application. The income sensitiveness of the farmer might be explained by low market price of the produce which inhibits the farmers' capacity as well as interest to invest on production inputs. The poor system of information flow and farmers' resistance were also found to limit adoption of technologies to some extent. Resistance of farmers might also be associated with high cost of production technologies. In some cases, farmers also want to keep on following their own local practice at least till they see the advantage of the new practices over the local one. The other factor affecting effective application of the new technologies is the weakness of the development agents in providing technical assistance and advice to farmers. Quality of technologies also found to affect farmers adoption rates of newly emerging technologies and agricultural information-knowledge seeking behavior of the farmers. On top of this, absence of farmers' involvement during the development of the technologies is blamed by the respondent for declining technology adoption behavior of farmers. Therefore, farmers' involvement during the development of technologies might help in its acceptance. The finding of Adesoji and Tunde (2012) supports the current result, which witnessed the contribution of farmers' involvement in technology development process for increased technology adoption rate.

Conclusion

The study conducted in Hawassa area, southern Ethiopia to describe the current status of agricultural knowledge and technological packages transfer system, indicated the weakness of the existing system. In the study area, the system is still dominated by one way or linear agricultural knowledge-information and technology communication pathway; which does not allow the involvement of all stake-holders across the stages of the system, especially of the practitioners of the technologies. This shows the urgent need to improve the efficiency of the existing agricultural knowledge-information and technology transfer system. The factors hindering efficiency of the system extends from the initiation of the technology development process through knowledge and technological packages dissemination stage. Thus, to improve the system, the research problem selection and technology development process needs to consider the concerned stakeholders especially of the farmer and development agents. The motivation and responsibility taking behavior of the concerned stakeholders should be improved in the way that it strengthens the linkage among actors so as to have a common understanding on each process of the system, thereby improving the development and dissemination efficiency of the technologies and its impact on the livelihood of the rural poor. Besides, motivation and knowledge and/or skill level of the development agents, awareness and income of the farmers should be improved.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Early prediction of internal bruising in potatoes by biospeckle laser technique

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The internal bruising in potatoes is a kind of damage caused by the mechanical impact, which is the major cause of post-harvest losses. This kind of injury is hard to detect because it occurs due to the internal cellular rupture of the tuber. This research aims at evaluating the applicability of the biospeckle laser technique in order to detect and predict the internal bruising in potatoes caused by mechanical impacts during the early stages where there is no visual perception of the injury. The correlation of Pearson between a quantitative index generated from the biospeckle laser images (BSL values) and the enzymatic darkening in the region of the injury have been calculated. The BSL values was used to compare a group of potatoes subjected to mechanical impacts caused by a pendulum and a group of potatoes that did not suffer any sort of mechanical impacts. Comparison was performed by average and the standard error of the BSL values was obtained: Before impact, immediately after, 2, 4, 6, 24, 48 and 72 h after the mechanical impact. Results showed that the BSL values correlated significantly with the progressive darkening of the potato tissue (r = - 0.79) and this allowed the detection of the formation of internal bruising from 4 hours after the occurrence of a mechanical impact.

Key words: Mechanical impacts, injury detection, optical technique.

INTRODUCTION

As a result of the reduction in the loss of food worldwide, it is essential to improve food security and reduce pressure for natural resources (FAO, 2013). The study also states that in low-income countries, food losses are related to the management of the harvesting and post-harvesting stages of food in general (Sharpley et al., 2015).

Concerning the losses of fruits, vegetables and tubers, one of the major problems is related to the injuries originated by mechanical impacts caused by falls, collisions and handling of fruits, vegetables and tubers. These injuries lead to physiological, metabolic, flavor and

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quality modifications (Li and Thomas, 2014).

In potatoes, the losses by mechanical injuries occur during every stage of production, from harvest to consumption. However, as the injuries are cumulative, the whole process should be monitored, and there should be actions to prevent damages (Shepherd et al., 2015). Ferreira and Netto (2007) reported that in potatoes the superficial damages and internal bruising can get to 40% of the whole production, reducing the sale price and even causing the rejection of the product.

The internal bruising is a common kind of injury in vegetables generated by mechanical impacts during the post-harvest (Opara and Pathare, 2014). Fruits, vegetables and tubers submitted to impacts, even without any apparent damage, develop internal cellular ruptures on tissues. The damaged membranes allow enzymatic oxidation of phenols and the production of melanin, causing internal and external browning of vegetables, called the bruising phenomenon (Hussein et al., 2018).

The detection of internal bruising in vegetables and fruits allows the control of their losses, therefore generating an increase in the efficiency of agricultural production (Li et al., 2013; Bugaud et al., 2014). The potential of the use of non-destructive approaches can be considered a key factor in the development of techniques to get reliable information on monitoring and predicting the internal bruising in tubercles (Rady and Guyer, 2015; López-Maestresalas et al., 2016), and the effects of the mechanical impacts on potatoes (Danila, 2015).

Optical methods present a capacity to provide information on physical, mechanical and chemical attributes of agricultural products due to the fact that the light has a great interaction with the cellular structure of biological materials (Hu et al., 2015). The laser light, more specifically, presents a great power of penetration in cellular tissues and the response of its dispersion after the incidence in foods can be associated to several quality attributes to these products (Romano et al., 2011).

Biospeckle laser technique is based on an optical interference phenomenon that occurs when one beam of coherent light spreads on a surface of the biological sample. Over time, the successive patterns of biosepckle become susceptible to be tracked, and the changes of biospeckle patterns are associated with biological activity (Braga, 2017; Stoykova et al., 2017). The correlation between the biological activity of a biological materials and variation of the biospeckle pattern over time can be observed in several relevant researches in medicine that studied the monitoring of melanoma (González-Peña et al., 2014) in the evaluation of the activity of bacteria and parasites (Ansari et al., 2016; Grassi et al., 2016) and other variety of applications in agriculture (Zdunek et al., 2014; Retheesh et al., 2016; Sutton and Punja, 2017; Arefi et al., 2018). Szymanska-Chargot et al. (2012) and Gao et al. (2016) presented the biospeckle laser as a feasible way to detect bruising process in apples and potatoes after mechanical impacts.

This research presents a non-destructive optical protocol, based on the biospeckle laser technique, to detect and predict internal bruising in potatoes caused by mechanical impacts during the early stages where there is no visual perception of the injury.

MATERIALS AND METHODS

Samples

The potatoes (Monalisa cultivar) used in this experiment were obtained from a commercial bag of 25 kg. The potatoes were acquired after harvesting and before washing, transportation and commercialization to avoid the occurrence of mechanical damages in these steps. The biospeckle laser tests used 15 potatoes previously submitted to a mechanical impact and 5 potatoes as a control, without the occurrence of mechanical impacts.

The determination of pulp firmness, mass and diameter were performed to characterize the preconditions of the amount of 25 kg of potatoes. Firmness was determined using a digital penetrometer with 5-mm diameter tip. The values of firmness for each fruit were calculated using the mean of three readings for each potato. The mass of the potatoes was determined through a precision weight scale and the diameter was determined through a digital caliper. The mechanical impacts were simulated using a pendulum with an arm of 600 mm and positioned at an angle of 90 degrees according to the plane of the sample. On the arm was placed a steel sphere of 180 g with a radius of 20 mm. Similar protocols to realization of the impact in potatoes can be observed in the research developed by Danila (2015). All impacts were located in the central region of the potatoes and were sufficient to cause internal bruising. The damage was black spot of blue-grey pigmentation, with a hollow concavity in the center whit diffuse edges (Noble, 1985). Figure 1 shows an internal bruising after 72 h, resulting in the impact of the pendulum.

A digital colorimeter was used to quantify the darkening of the impact region during the evolution of the damage before each biospeckle laser test. The darkening scale colorimetric had a range of 0-100, where 0 corresponded to the maximum absence of color (total black) and 100 corresponded to the maximum white color (total white).

Biospeckle laser test

After the impacts, the data acquisition of images was carried out during the early 6 h in intervals of 2 h, and after the early 6 h, there were acquisitions after 24, 48 and 72 h from the impact.

After 72 h from the impact, the potatoes were cut in order to check the presence of any dark area. In addition, the control potatoes were also illuminated over the same period and at the end of the experiment were cut to confirm the absence of bruising.

In the experimental configuration, the camera was a CCD (JAI VGA 480 x 640 pixels), with a pixel size of 7.4 µm, using a macro zoom lens in order to focus the Region of Interest (ROI) within the tuber, defined by a square area of 400 mm^2 . The magnification and the distance of the camera (300 mm) was adjusted to guarantee a speckle grain higher (many times) than the pixel size, and with a well-defined area, that is with high contrast. For the illumination, laser HeNe/632 nm with 36 mW was used, with an angle of incidence between the beam and the sample of approximately 45°. The backscattering configuration (camera caught the scattering of the laser light reflected after the incidence on the sample) was completed with polarized filters to the reduction of the illumination intensity, expander lenses,(20x, 20 mm) and mirrors, to target the beam (Figure 2).

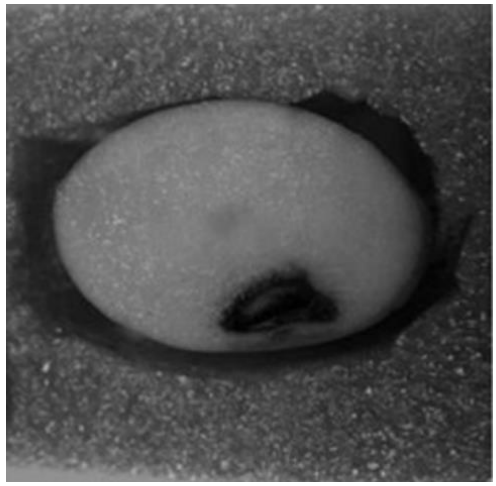

Figure 1. Internal bruising of the type internal crushing in potatoes after 72 h caused by a mechanical impact.

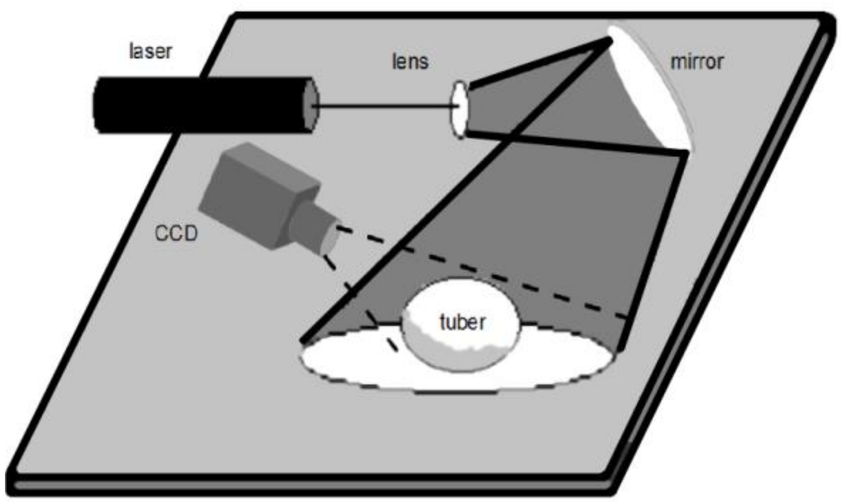

Figure 2. Experimental configuration with a computer, CCD, laser set, lenses and the samples.

Data analysis

A collection of 128 images in 8 bits of speckle patterns was assembled with a time rate of 0.08 s, and the images processed in areas within and out of the damage, and as well with the damaged area merged in the normal tissues (in this latter case the ROI was increased to 2500 mm²). A temporal speckle pattern was built, from the collection of images, generating the Time History Speckle Pattern - THSP (Arizaga et al., 1999). The time history information within the THSP was extracted using a co-occurrence matrix according to Equation 1:

$$COM = [N_{ij}] \quad (1)$$

Where, the values of Nij are the occurrences of the consecutive gray levels throughout the lines of the THSP.

The algorithm of absolute value of the differences (Cardoso and Braga, 2014) was applied to quantify the intensity of the variations present in the COM, based on Equation 2:

$$AVD = \sum_{ij} \frac{N_{ij}}{\sum_{ij} N_{ij}} |i - j| \quad (2)$$

Where, the variables i and j represent the cells in the COM matrix.

Original values of absolute differences (BSL values) were normalized to minimize the effect of variance in the behavior of the values obtained from each sample. The normalization was performed by the relation between the real value of the BSL values at a given instant and the BSL values before the impact occurs.

The values of the darkening scale colorimetric and BSL values were correlated through the Pearson coefficient to 0.05 significance. For statistical analysis, a completely randomized

Table 1. Characterization of 25 kg of potatoes where the samples were selected for the biospeckle laser test.

Attributes for characterization	Range
Firmness (Kgf)	4.00 - 4.75
Mass (g)	70 - 135
Diameter (mm)	60 - 110

design obtained in 8 instants over time (before impact, immediately after, 2, 4, 6, 24, 48 and 72 h after the mechanical impact) with 3 treatments corresponding to the condition of the samples (samples control, impact region and outside of impact region), 15 repetitions of impacted tubers and 5 repetitions of witnesses (control) were used. Measurements in regions outside the impact were obtained on surfaces adjacent to the impact region. Standard error (ϵ) for each treatment was calculated from the Equation 3:

$$\epsilon = \sqrt{\frac{MSE}{r}} \quad (3)$$

Where, MSE represents the mean square of the error obtained in the analysis of variance of instants in each treatment and *r* represents the number of repetition of the treatment.

RESULTS AND DISCUSSON

Characterization of samples

The characterization of the amount of 25 kg of potatoes allowed the estimation of the conditions of the samples before being submitted to the mechanical impact. The potatoes used in the biospeckle laser test were selected within this amount, and therefore, the firmness, mass and diameter were within the ranges shown in Table 1.

When analyzing the ability of biospeckle laser to evaluate the development of damage caused by a mechanical impact in potatoes, there was a significant correlation (Figure 3) between the darkening colorimetric of the injury and the BSL value in the region of impact, where the increase of the darkening colorimetric in the region and the impact is associated to the decline of the BSL value.

The mechanical impact on potato causes a rupture in the cell membrane allowing the entry of oxygen and resulting in darkening in the damaged region (Opara and Pathare, 2014). Quantitative levels of BSL values results from metabolic, enzymatic and respiratory changes due to cell degradation (Zdunek et al., 2014). The gradual reduction of cellular metabolism during oxidation in the impact region was responsible for the decline of BSL values. This is due to low variation of the scattering of light reflected when analyzing the biospeckle patterns over time.

Potatoes subjected to impact reduced BSL values over time, while potato control response remained in a range of constant values (Figure 4). The maximum BSL value in the region of impact, immediately after the impact (0h), is explained by the intense oxygen inflow due to the breakage of cell membranes (Hara-Skrzypiec and Jakuczun, 2013). Between 2 and 72 h, the hypothesis is that the BSL values were associated with cell oxidation in the region of impact over time, which resulted in the decline of cellular metabolism and in the darkening region. The BSL values outside the impact showed a similar response to the impact region, increasing the activity immediately after impact and reducing activity over time. The distinction between the group of control potatoes and the group of potatoes subjected to impact occurred after 6 hours; demonstrating that biospeckle laser has the potential to detect potato damage in the early stages after impact.

The comparison of standard error (ϵ) in the three groups of potatoes presented in Figure 4 indicates the regions where there are developing injury (ϵ = 0.04). BSL values tend to present a common response to the biological reactions resulting from cell oxidation. The peripheral regions or absence of injury (ϵ = 0.08 and ϵ = 0.06, respectively) presented major variation in repeatability in BSL values because they do not characterize a specific biological reaction, which makes the scattering of the laser light more random.

As also shown in Figure 4, biospeckle laser showed the ability to distinguish control potatoes and potatoes with injury, even by analyzing the region outside the impact. Metabolic processes involved in the development of injury and enzymatic darkening can cause a systemic effect in the whole potato as observed in plants by introducing external elements, systemically increasing resistance against diseases and pests (Mehari et al., 2015) or on the effect of toxins in animal cells (Iacobellis, 2015). Thus, when analyzing a potato with internal bruising, it is expected that its final metabolism is lower due to energy loss caused by the emergence of internal bruising. This information is relevant since the visual analysis of potato surface cannot be conclusive on the exact location of internal bruising.

Since it would be impossible to know during inspections if there is an impacted area in the tuber, an analysis was performed comparing healthy and damaged potatoes, but this time with no distinction of areas; however with a larger region of interest being used to compute the BSL values. Comparisons between control and damaged potatoes, with data inside and outside the damaged area are presented at Figure 5, being possible to observe the distinction in 4 h.

The result is an indication that the prediction of the development of internal bruising in potatoes by biospeckle laser can be obtained in moments close to the occurrence of the mechanical impact, and no need to distinguish regions that suffered impacts from regions that have experienced only systemic effects. Despite the ability of the biospeckle laser to be computed in small areas (Braga et al., 2007), the increase of the analyzed area improved the ability of the biospeckle laser to follow

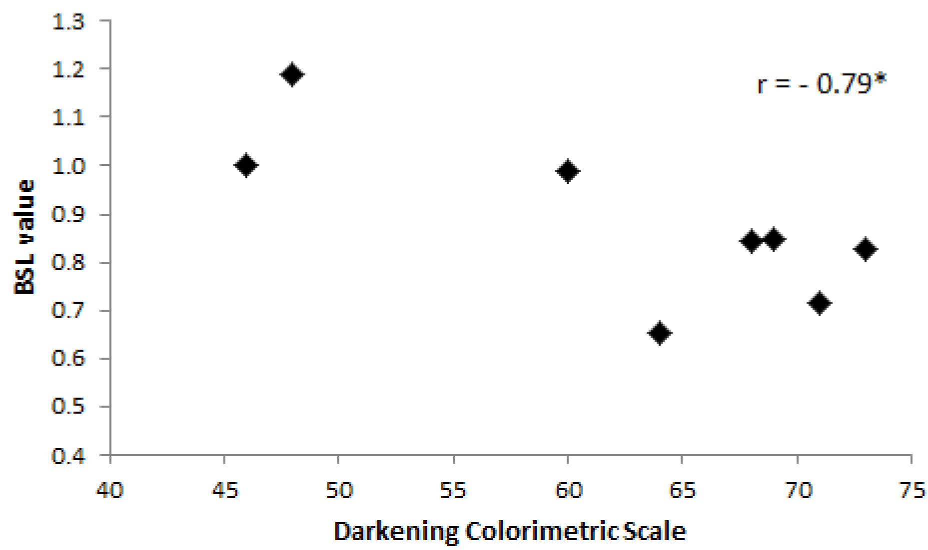

Figure 3. Correlation (r) between darkening colorimetric and BSL value in region of the impact. Significant at 0.05 level (*)

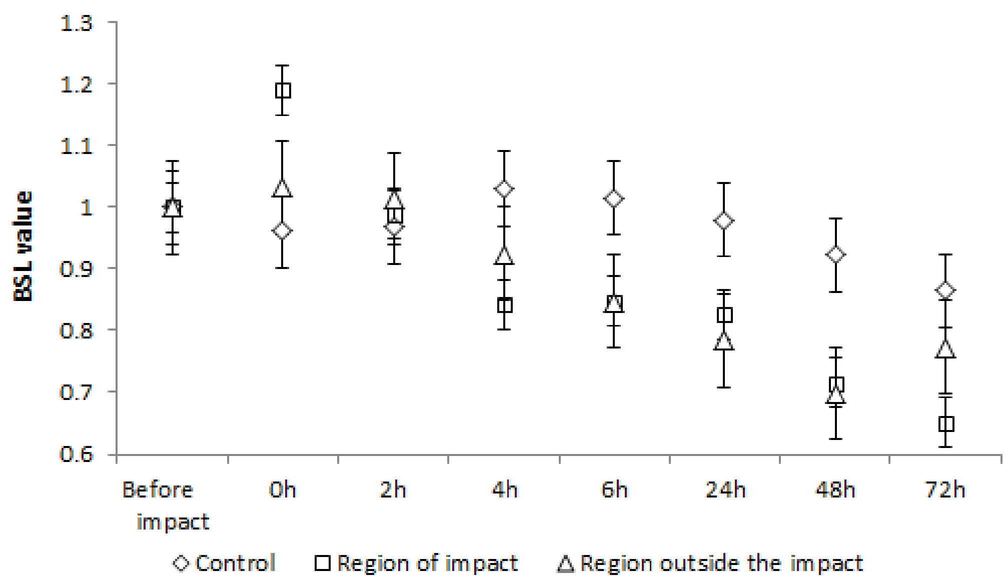

Figure 4. Average response and standard error variation of BSL value in the control samples, region of impact and region outside the impact in potatoes with the occurrence of internal bruising.

the biological phenomena, particularly reducing the dispersion of the results (ϵ = 0.03).

Similarly, López-Maestresalas et al. (2016) were successful in detecting bruising in potatoes, 5 h after impact using of VIS- NIR and SWIR hyperspectral imaging; demonstrating that parameters obtained by optical techniques can be efficient indicators for the detection and prediction of internal bruising. Surface injury on potato tubers on surface skinning was also identified by biospeckle laser within 24 h after the occurrence of an impact (Gao et al., 2016).

Once verified, the early differentiation between healthy potatoes and potatoes that were submitted to some impact during the stages of post-harvest, storage or transportation. A potential tool to detect and predict from the early stages (hours) a bruising process that would cause darkening was presented. From this information, more effective quality control measures can be taken and the percentage of product losses can be reduced, since more rigorous methods of selection and control will lead

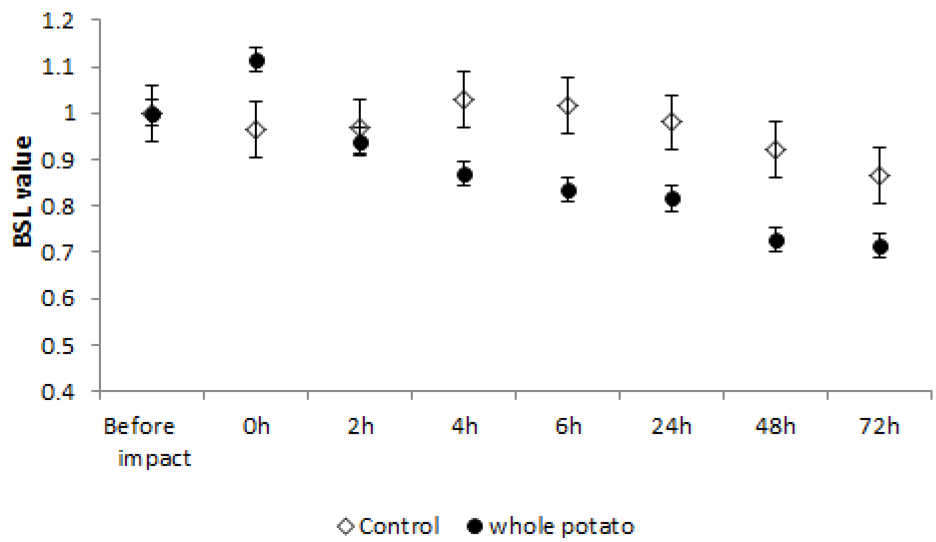

Figure 5. Standard error variation of BSL value without distinction of regions in potatoes with the occurrence of internal bruising and control potatoes (without the occurrence of internal bruising).

to greater care in the handling of products from harvesting to market shelves. In addition, the adoption of a contactless technique with reliable results reinforces the relevance of the research.

Conclusion

BSL values correlated significantly with the progressive darkening of the potato tissue, where the increase of the darkening colorimetric in the region and the impact is associated with the decline of the BSL value. The development of internal bruising was detected from 4 h after the occurrence of a mechanical impact, demonstrating that biospeckle laser technique is a non-destructive alternative to prediction of the occurrence of internal bruising in potatoes, even at times of no visual identification of the injuries.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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Full Length Research Paper

Influence of reducing sugar contents on sprout growth of blackberry cuttings

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The objective of this study was to analyze the use of different doses of indolebutyric acid (IBA) and its influence on reducing sugar contents and the relationship of root and leaf development of mulberry cuttings, using commercial substrates and sand. The experimental design was a completely randomized design, with 3 replicates per plot and 5 cuttings per replicate, in a 3 × 3 factorial scheme, consisting of three IBA doses and three substrates: commercial substrate, sand and 50% substrate with 50% of sand. The cuttings planted on substrate presented a higher percentage of rooting in the treatments with 0 and 1000 mg L^{-1}, differing from the other substrates, which represented increases of 16.68 and 44.45% in relation to the substrate of sand and sand + substrate, respectively. In the control treatment, estimates of the reducing sugar content of the leaves showed interaction between the substrates and doses of IBA, presenting an increasing quadratic effect for the treatment. It is concluded that the use of commercial substrate provides greater development of primary leaves and root length; the concentration of 1000 mg L^{-1} of IBA was higher among the analyzed doses. The reducing sugar content of the cuttings influenced the growth of the shoots.

Key words: Reducing sugars, fit regulator, multiplication, *Rubus*, substrates.

INTRODUCTION

The mulberry tree is a plant belonging to the family, Moraceae, which produces plants of the genus Rubus and Rosaceae, in which there are other important genera (Malus, Prunus, Pyrus among others) for Brazilian fruit growing (Antunes, 2002).

The mulberry tree (*Rubus* sp.) is one of the most

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promising fruit species in the market, having a very promising market due to its palatability, good cultivation and commercialization perspectives (Silva et al., 2016). Due to the low cost of plantation, orchard maintenance and especially, the reduction of pesticide use, the crop is presented as an option in family agriculture (Antunes et al., 2014). This fruit also shows good adaptation in a subtropical climate, where some cultivars have higher yields as compared to cold regions and even has superior fruit quality (Guedes et al., 2013). However, because it is a fruit tree, it is very susceptible to damage mainly after its harvest.

In order to have a structured chain for the cultivation of mulberry, it is necessary firstly to have plantations installed in order to supply the demand of fruits. But for the installation of new plantations, the production of seedlings with good quality is necessary. For the production of quality seedlings, the seminiferous route and vegetative propagation are the most used techniques for the propagation of this plant.

Seminiferous propagation has long been used for production, depending on the species that can arrive for 30 years. Seeking uniformity and speed in the production of seedlings, vegetative propagation is an alternative that may be viable due to the use of cuttings, roots or shoots. The use of cuttings is a widely used alternative to obtain shoots of woody plants with long periods of development through sowing (Antunes and Rasseira, 2004).

However, in order to increase the chances of success of the use of vegetative propagation, the use of rooting promoters is important in increasing rooting rates. Among these promoters are cell growth, root and leaf emission stimulators. It is known that the most used form is the exogenous application of phytohormones, such as indolebutyric acid (IBA), which increases the auxin content in the tissue (Pasqual et al., 2001).

Although, the performance of propagated cuttings is related to genetic, physiological and environmental factors (Fachinello et al., 2005), it is essential to use substrates that promote root formation and shoot (Kämpf et al., 2006; Yamamoto et al., 2013; Hussain et al., 2014). In general, black mulberry tolerates a wide range of soils, but it develops best in sandy soils with good organic matter content (Grandall 1995; Gazda and Kochmanska-bednarz, 2010).

The objective of this study was to analyze the use of different doses of IBA in reducing sugar contents and in the promotion of root and leaf development of blackberry cuttings, using commercial substrates and sand.

MATERIALS AND METHODS

The experiment was conducted between October and November 2016, for 40 days, at the School Farm of Dom Bosco Catholic University, Campo Grande, MS. Herbaceous branches of mulberry plants were collected from the orchard of the farm. The propagation material was kept in a protected environment (agricultural greenhouse) under automatic intermittent irrigation system by micro sprinkler, in order to maintain the relative humidity close to 90%, avoiding the dehydration of the cuttings.

After the collection, the branches were prepared in cuttings with 10 to 15 cm length and diameter of 3 to 5 mm. The cuttings were treated with 0, 1000 and 2000 mg L^{-1} IBA for 15 s (concentrated solutions of the immersion method). After the application of IBA, the cuttings were placed in PVC tubes using 3 types of substrates (commercial substrate, sand and 50% substrate + 50% sand). For the control concentration, only distilled water was used.

The experimental design was a completely randomized one, with 3 replications per plot and 5 cuttings per replication, in a 3 × 3 factorial scheme, consisting of the three IBA doses and the three substrates. In order to obtain the results in each IBA dose, the average of the three concentrations and substrate types was considered, considering the basic principles of experimentation: repetition, randomization and local control (Gomes, 1990).

The variables analyzed were the percentages of rooted cuttings, the number of rooted cuttings, the average length of the rooted cuttings, the weight of the fresh biomass of the rooted cuttings, the weight of the fresh root biomass per cutting, dry matter of the biomass of the primary leaves of the rooted cuttings and weight of the dry biomass of the roots per cutting. Dry matter (DM), calculated according to the methodologies described by Silva and Queiroz (2002) were used to determine the dry biomass of leaves and roots.

The methodology described by Somogy (1945) and Nelson (1944) was used to determine the reducing sugar contents. The apparatus used was the spectrophotometer, and the reading was performed at 535 ηm. Data were subjected to analysis of variance (ANOVA, P <0.05), linear regression, ACP and Chi-square, according to Levine (2000), using the statistical program SAS version 9.1 (2004).

RESULTS AND DISCUSSION

Analysis of the number of cuttings with sprouting of mulberry was significant (P <0.05) between the substrates (Table 1). There was no interaction between the different concentrations of IBA and substrates, nor effect between IBA doses.

The cuttings planted in substrate presented a higher percentage of rooting between treatments of 0 and 1000 mg L^{-1}, differing from the other substrates, which presented an increase of 16.68 and 44.45% in relation to the substrate of sand and sand + substrate respectively, with the control treatment. For the treatment with 2000 mg L^{-1}, lower amount of rooted cuttings was observed in sand substrate with 53.3% of rooting. Possibly, the stakes that did not root and did not increase in the other variables, ended up reducing their reserves, thus leading to cell death. Porosity of the substrate influences the amount of retained water and aeration, having an effect on the percentage of rooting and the number of roots formed (Fachinello et al., 2005). A good substrate should have low density, good water absorption and retention capacity, good aeration and drainage to avoid excessive moisture accumulation (Kämpf, 2005). Yamamoto et al. (2013) reported that IBA has no influence on the survival of mulberry saplings. An effect (P <0.05) was observed between the substrates for root length (Table 2). For the number of leaves, there was no difference between the substrates.

Table 1. Number of cuttings of mulberry sprout treated with different concentrations of IBA and different substrates.

Treatment (mg L^{-1})	Commercial substrate	Sand	Sand+ Substrate	P
0	10^a	9^a	6^b	0.01
1000	13^a	8^b	9^b	0.01
2000	10^a	8^b	10^a	0.01

P- Effect of treatment; mean of the same line with different lowercase letters are significant by the Kruskal-Wallis test (P <0.05) effect of hormonal treatment.

Table 2. Number of leaves and root length of mulberry cuttings on different substrates.

Variables	Substrate	Sand	Sand + substrate	EP	P
Number of leaves	3.71	3.6	4.04	0.212	0.372
Root length	8.08^a	6.10^b	5.91^b	0.172	0.001

Standard error (EP); P- treatment effect; averages of the same line with different lowercase letters are significant by the Waller-Duncan test (P <0.05) substrate effect.

Table 3. Weight of the biomass and reducing sugar of mulberry botanical components subjected to different substrates.

Variables	Substrate	Sand	Sand + Substrate	EP	P
Wet mass of leaves (mg)	337	295	354	0.021	0.281
Wet mass of roots (mg)	253	252	186	0.026	0.096
Wet mass of cuttings (mg)	1.084	1.078	1.119	0.078	0.834
Dry mass of leaves (mg)	87^a	66^b	84^a	0.005	0.041
Dry mass of roots (mg)	63^a	78^a	47^b	0.006	0.047
Dry mass of cuttings (mg)	419	556	405	0.029	0.028
Reducing sugar of leaves (g 100 g^{-1})	14.51^b	14.48^b	19.44^a	0.389	0.001
Reducing sugar of cuttings (g 100 g^{-1})	16.04^a	6.63^b	16.94^a	0.835	0.001

Standard error (EP); P- treatment effect; Same line averages with different lowercase letters are significant by the Waller-Duncan test (P <0.05) substrate effect.

The cuttings that rooted with commercial substrate presented greater amount of primary leaves, in relation to the other substrates (Mendonça et al., 2010). The leaves obtained carbohydrate through photosynthesis and also a small amount of natural auxins present in the leaves, helping in the rooting and sprouting of cuttings (Hartmann et al., 1990). The dry biomass values of the leaves and roots obtained in this study had an effect (P <0.05) between the substrates (Table 3). However, no significant difference was observed for the values of natural biomass of the leaves and roots.

The commercial substrate and substrate with 50% sand + 50% substrate did not differ from each other, showing a yield of 87 and 84 mg kg^{-1} dry leaf biomass, respectively, and 22.8% more efficient than the substrate sand. This result is related to the physical characteristics of sand, which is considered as an inert material with a low water retention capacity (Pasqual et al., 2001; Mendonça et al., 2010).

According to the analysis, there was a quadratic effect (P <0.05) of treatment for fresh leaf biomass weight. The absence of IBA in the culture medium resulted in lower biomass of the leaves, although higher production was observed with the use of 1000 mg L^{-1} (Figure 1). Figure 1 shows that there was an increasing linear effect (P <0.05) for the roots with increase of IBA concentrations applied on the cuttings. This fact is possibly associated with auxin stimulation. Silva et al. (2012) reported that the best IBA concentrations for rooting promotion is about 1634 mg L^{-1} and Hussain et al. (2014) reported that 2,000 mg L^{-1} of IBA is considered the best option for the increase of roots in cut of blackberry. Dias et al. (2011) stated that IBA contractions of 250 to 500 mg L^{-1} provide a higher volume of root shoots.

However, when dry biomass yield is evaluated, quadratic effect (P <0.05) is observed for leaf and root weight per cutting (Figure 1). Therefore, in the absence of auxin, there is a reduction in the dry biomass values of

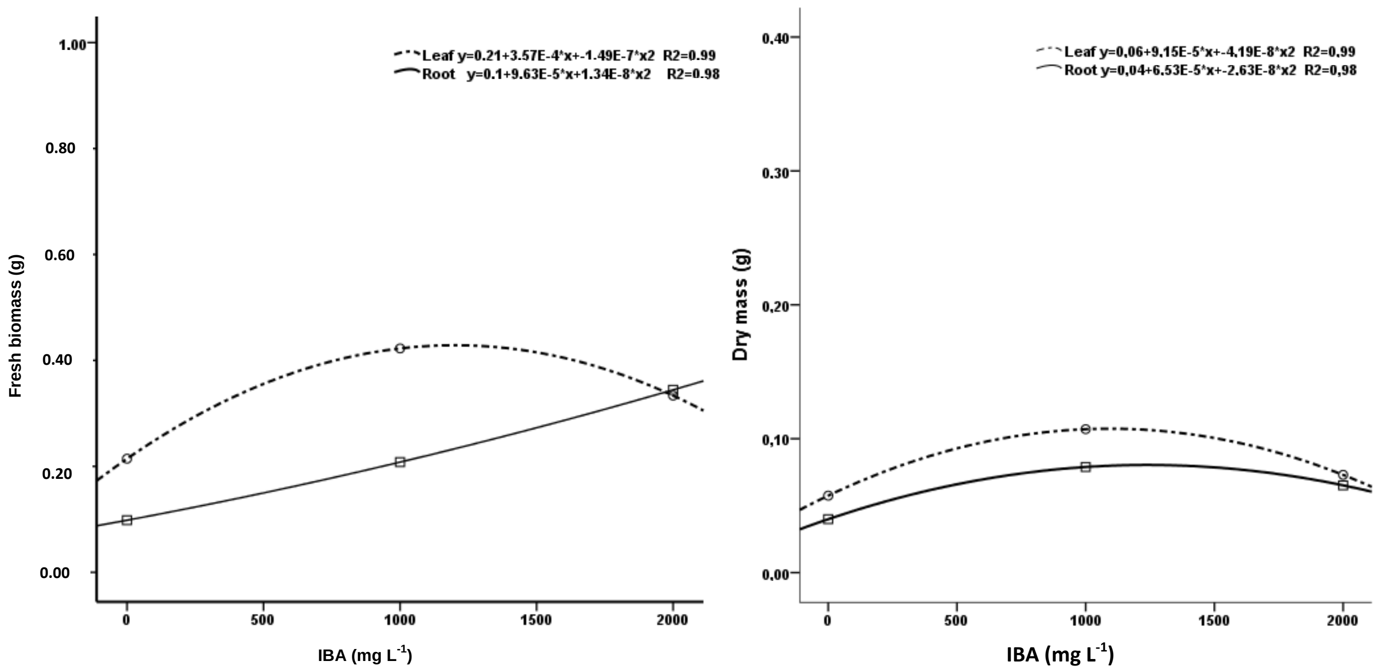

Figure 1. Weight of fresh biomass (left) and dry mass (right) of leaves and roots of mulberry cuttings subjected to different doses of IBA.

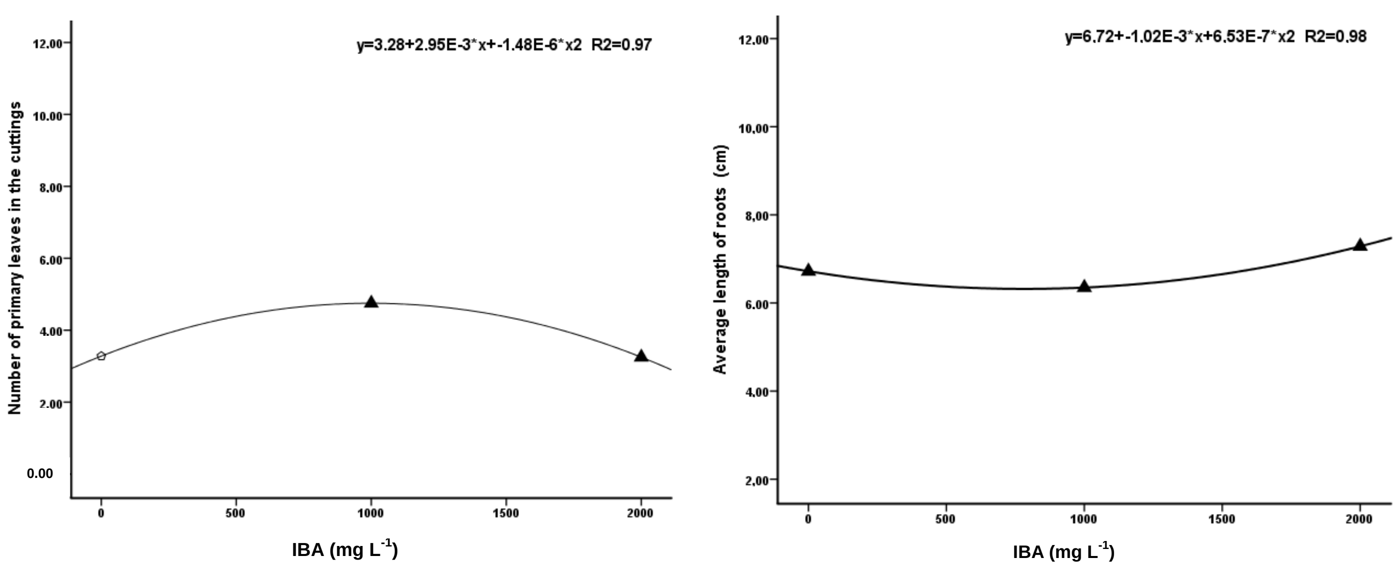

Figure 2. Number of primary leaves in the cuttings (left) and average length of roots per mulberry cutting subjected to different doses of IBA.

leaves and roots, and the same behavior can be observed after the use of 1400 mg L^{-1} of IBA, the plant did not respond.

As the IBA concentration increased, there was an increase in rooting, consequently, roots had longer length (Figure 2). However, the exogenous supply of AIB, above 1200 mg L^{-1}, may not favor the number of primary leaves (Figure 2). The estimates of the reducing sugar contents (AR) of the leaves showed interaction between the substrates and doses of IBA (Table 3), presenting an increasing quadratic effect for the treatment with 50% sand and 50% commercial substrate, which showed 18.76 g 100 g^{-1} reducing sugar content with the AIB dose of 672 mg L^{-1} (Figure 3). The same behavior was observed with 100% commercial substratum, however, with lower contents. When only sand was used as the

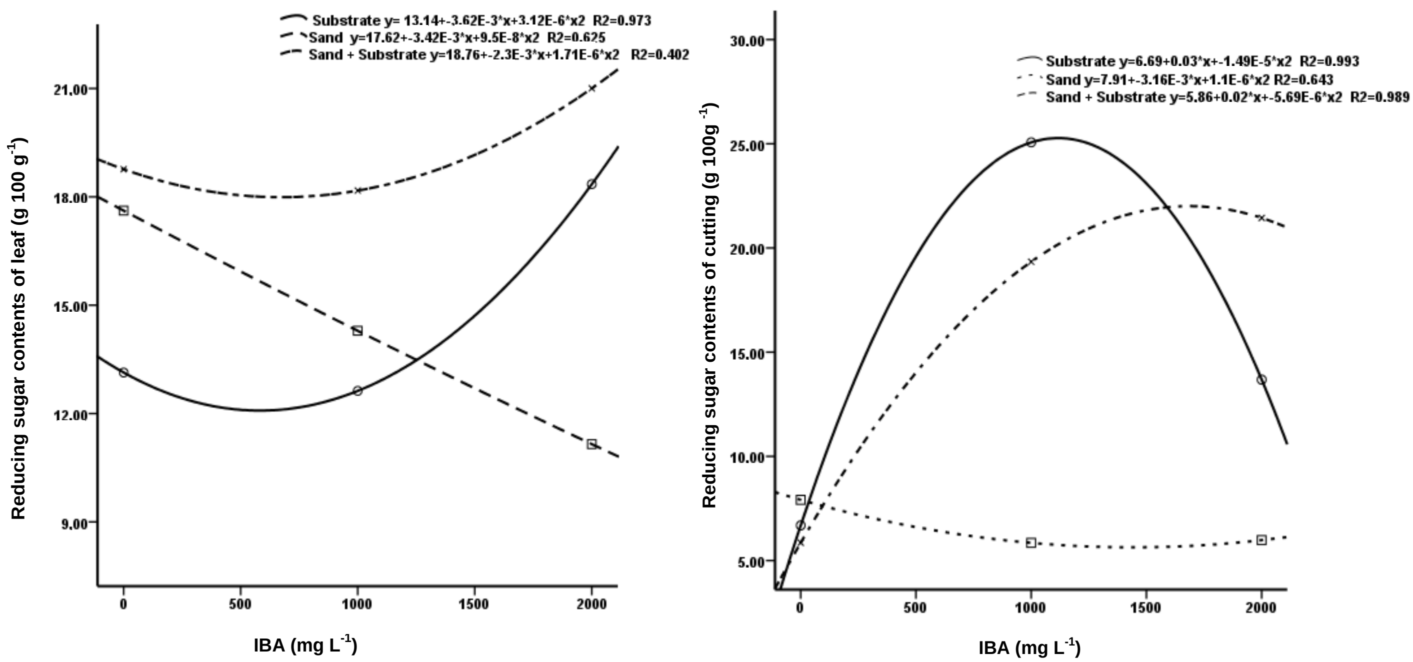

Figure 3. Reducing sugar contents of leaf and cuttings in relation to the substrates used for mulberry cuttings subjected to different doses of IBA.

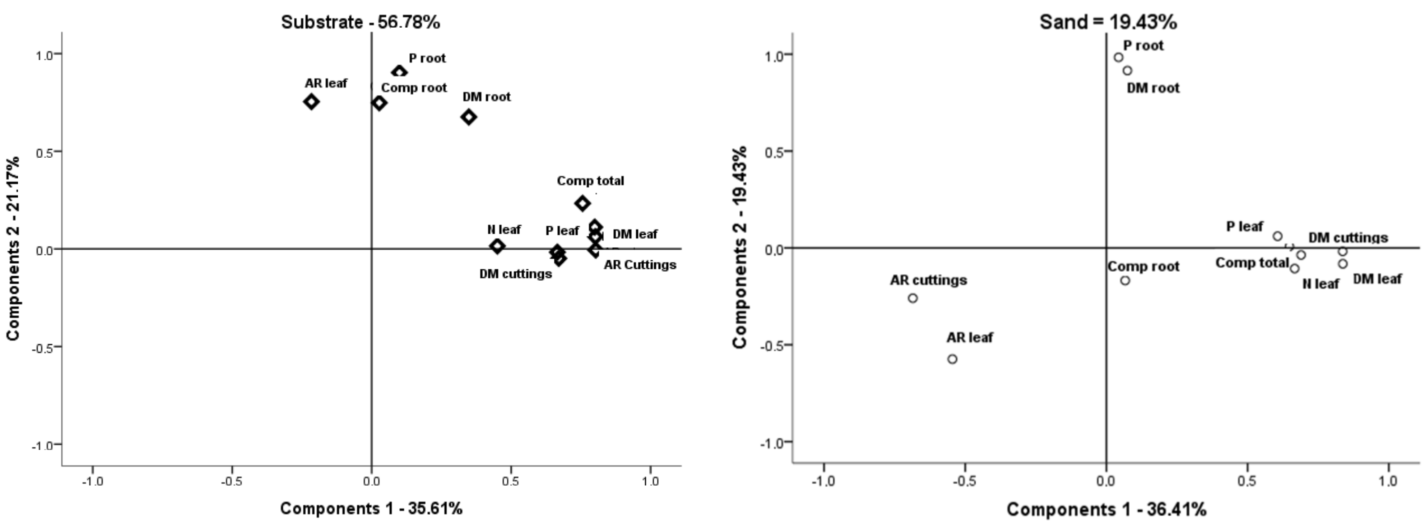

Figure 4. Variation graph for the main components between the levels of reducing sugar in the leaf (AR leaf), stem (AR cuttings), and fresh leaf mass (P leaf), fresh mass of roots (P root), dry leaf mass (DM leaf), root dry mass (MS root), root length (Comp root), number of leaves (N leaf) and total length of the plant (Comp total) in relation to the commercial substrates and sand used for mulberry cuttings subjected to different doses of IBA.

substrate, the doses of IBA did not provide an increase in the levels of RA.

In relation to the AR content of the stakes, interaction between IBA and substrate doses was observed (Figure 3); it can be observed that the commercial substrate use had the highest efficiency of the IBA hormone with a dose of 1,006 mg L^{-1} with respect to the contents (21.79 g $100g^{-1}$) present in the cuttings. However, with the use of sand as a substrate, there was a greater demand for hormone for the maintenance of RA in the system. This fact is related to water retention and dark environment at the base of the stem obtained by the use of the substrate influencing the rooting (Figure 4) as well as the type of roots formed (Hoffmann et al., 1996).

Correlations of the factors related to development of the botanical components of the cuttings and their characteristics studied (Figure 4), are indicated in cluster 1 with commercial substrate, and the reducing sugar levels of the cuttings influenced the morphological development of the aerial part of the plant.

On the other hand, in group 2, the root growth of cuttings with substrate use is positively correlated with the amount of carbohydrates metabolized in the leaves. In the sand substrate treatment, development of the aerial part is related to the greater biomass of the stake. It is necessary to increase the amount of endogenous reserves of the cuttings, because the leaves that will be formed during the rooting process may act as a drain, decreasing the reducing sugar content of the cuttings (Dias et al., 2011).

Mendonça et al. (2010) emphasized that the substrate is fundamental for the rhizogenesis of cuttings; in addition to being a support for the cuttings, it retains water supplied via a longer period of time.

Conclusion

The results of this work showed that the use of commercial substrate provided greater development of primary leaves and root length; the concentration of 1000 mg L^{-1} of IBA was effective among the analyzed doses. The reducing sugar content of the cuttings influenced the growth of the shoots.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Fungistatic activity of essential oil of *Lippia sidoides* Cham. against *Curvularia lunata*

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Synthetic pesticides used in agriculture to control pathogens are being widely questioned for their harmful effects on human health and the environment. Therefore, the objective of this study was to evaluate the fungistatic activity of *Lippia sidoides* Cham. essential oil on alternative control of *Curvularia lunata* (Wakker), the causal agent of spot in maize plants. The fungitoxic contact effect was successful, resulting in a strong inhibition of *C. lunata* at the concentration of 50 mg mL^{-1}. Complete adverse effect on the germination of the conidia of *C. lunata* was obtained at concentrations of 5 and 7.5 mg mL^{-1}. In the preventive assay in maize plants, that is, effect of essential oil on *C. lunata*, a reduction in the progression of the disease was observed at the oil concentration of 7.5 mg mL^{-1}. Regarding the curative effect in maize plants, no satisfactory result was obtained at concentrations from 0.625 to 7.5 mg mL^{-1}. Cytotoxic test in animal cells was also performed by the viability test using MTT assay, which showed that none of the analyzed concentrations was toxic to the cells. These results demonstrate the potential effect of the essential oil of *L. sidoides* on the prevention and inhibition of mycelia and conidia germination, controlling the phytopathogenic fungus *C. lunata*.

Key words: Phytopathogenic fungus, effect preventive, effect curative, cytotoxicity.

INTRODUCTION

The production of maize (*Zea mays* L.) can be affected by a number of factors such as climate, cultural practices, and especially, phytopathogenic microorganisms. All the plant species are potential hosts of fungi that can be primarily associated to different parts of the plant (Agrios, 2005). A majority of them cause negative impact by causing diseases in plants and, therefore, affecting the yield of the harvested products (Bennett, 1998). This necessitates research for the development of new alternatives for the control of pathogenic organisms,

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where productivity and quality can be high (Okumura et al., 2011).

Several filamentous fungi are cultivated and exploited commercially for the production of enzymes and metabolites. Since the past few years, bioactive compounds have become popular and are the target of numerous surveys (Archer et al., 2008; Sun et al., 2010). In addition, several plants, whose primary mechanism of action may be the inhibition of mycelial growth, are being studied for their fungitoxicity in the control of pathogens (Milanesi et al., 2009). According to the literature, Brazil is the third largest consumer of pesticides in the world, besides being the third in mortality rate due to cancer diseases (Krawczyk et al., 2017).

Synthetic substances used in agriculture to control pathogens such as benzimidazoles, aromatic hydrocarbons, and inhibitors of the biosynthesis of sterols are being widely questioned for their harmful effects on human health and the environment. These adverse effects such as pollution of air, water, and food and the growth of resistance of microorganisms such as *Pyricularia grisea* (Sacc.), *Aspergillus* species, and *Colletotrichum musae* (Berk. & M.A. Curtis) fungi have led to the development of resistance to the most widely used fungicides (Aguiar et al., 2014).

Recent studies indicate an increase in diseases caused by the fungus *Curvularia lunata* (Wakker) in maize cultivars in China, and consequently, a major loss in maize production (Gao et al., 2012). The maize leaf disease destroys the photosynthetic tissues and directly affects the formation of grains. Among the maize diseases, the spot of *Curvularia* occurs less often in places of warm climate, but it is prevalent and causes losses in tropical regions (Reis et al., 2004). The destruction of 25% of foliar area of maize in its terminal portion, near the flowering period, can reduce 32% of the production (Fancelli, 1998). There is a shortage of studies on this disease in Brazil, and there are hardly any reports of *Curvularia* species. in Brazil. However, the literature reports a marked increase in the incidence and severity of this disease. Currently, several new biobased products with antimicrobial action have been studied, especially essential oils from medicinal plants (Muthaiyan et al., 2012; Vaz-De-Melo et al., 2010).

Considering the growing importance of *Curvularia* spp. in maize, the few studies conducted for the control of this disease in tropical areas, the growing search for alternative methods of control, and the potential antifungal agents from vegetable oils from medicinal plants, the objective of this study was to evaluate the fungistatic activity and cytotoxity effect of *Lippia sidoides* essential oil on *Curvularia* spp.

MATERIALS AND METHODS

This study was carried out in the Laboratory of Phytopathology of the Federal University of Tocantins, Gurupi Campus, Tocantins, Brazil.

Morphological and molecular characterization of *C. lunata*

The fungus *C. lunata* was initially isolated from maize plants from the experimental field of UFT -Gurupi with symptoms of the disease and grown in potato dextrose agar (PDA) culture medium and deposited in a collection library of CENARGEN (Embrapa Genetic Resources and Biotechnology) under the number CEN 1095. The morphological characterization of *C. lunata* was verified through observations of the macroscopic and microscopic characteristics according to previous studies (Ferreira et al., 2010; Kern and Blevins, 1999). For the molecular characterization done according to Hou et al. (2013), DNA extraction was performed using a cetyl trimetyl ammonium bromide (CTAB) method described by Zolan and Pukilla (1986). The primers used were P1: (5'-ATG GAC GAG AAC AAC AGG ATAA CGA-3') and P2: (5'-CTA CCA GCA TTT GAA TTT ACT CCAG-3'). The amplification was conducted in a Techne TC-5000 Thermal Cycler (Techne). PCR was carried out using Taq DNA polymerase (Invitrogen) and the PCR program was performed as follows: 4 min at 95°C, 30 cycles at 95°C for 1 min, annealing at 55°C for 2 min and 72°C for 2 min, and a final step at 72°C for 5 min, to obtain the PCR products.

Plant material and steam distillation

L. sidoides originating in Ceará was collected in Gurupi (11°44'48" latitude S, 49°02'55" longitude W), Tocantins, Brazil. Taxonomic identification was confirmed by experts at the herbarium (Federal University of São João Del Rei, Brazil), where samples were deposited with reference number 8303. *L. sidoides* essential oil was extracted from the leaves by steam distillation method in a Clevenger-modified apparatus, as described by Guimarães et al. (2008) and stored at 4°C until further analysis, before antifungal experiments were conducted.

Gas chromatography-mass spectrometry (GC-MS) analysis

Qualitative analyses were performed through gas chromatography coupled to mass spectrometry (GC-MS) using the Shimadzu GC-2010 model equipped with selective detector for the mass Model QP2010Plus, with the equipment operated under the following conditions: fuzed silica capillary column RTX-5MS (30 m × 0.25 mm × 0.25 μm film thickness), with the following schedule of temperature in the column: 60 to 240°C (3°C/min), temperature of the injector 220°C, helium gas carrier, injection with rate of split (1:100) with injected volume of 1 µL of a solution 1:1000 in hexane. For the mass spectrometer (MS), the following conditions were used: impact energy of 70 V and temperature of the source of ions and the interface at 200°C. A homologous series of n-alkanes ($C_9H_{20}...C_{26}H_{54}$) were injected under the same conditions as for samples. The constituents were identified by comparing their spectra of masses with those from the databases from the Nist and Wiley 229 libraries and also by comparing between their rates of retention calculated using those reported in the literature (Adams, 2007). The quantification of the levels of the compounds, expressed as a percentage based on the standardization of areas, was obtained by using a gaseous chromatograph equipped with a detector flame (DIC), using a diagnostic Shimadzu GC-2010, in the following experimental conditions: a capillary column RTX-5MS (30 m × 0.25 mm × 0.25 μm film thickness); temperature of the injector at 220°C; temperature of the DIC 300°C; programing the column: initial temperature of 60°C with a heating rate of 3°C/min up to 240°C, then increasing to a heating rate of 10°C/min up to 300°C and remaining at this temperature for 10 min; nitrogen drag gas

(1.18 mL min^{-1}); rate of split 1:50; pressure in the column of 115 kPa, and injected volume of 1 µL, diluted in hexane (1:100 v/v). The calculated retention index was performed according to Mühlen (2009).

Pathogenicity of *C. lunata*

The pathogenicity of *C. lunata* was verified according to the methodology of Sá et al. (2011) by testing the inoculations in maize plants. Maize seeds were sown in pots made of polyethylene containing substrate and 10 g of a commercial fertilizer in each pot. Nine pits were made and two seeds were added to each pit. There was no need to perform pruning. When the plants reached two pairs of true leaves, the inoculation could be initiated using a manual spray (capacity 500 mL) containing the conidial suspension of *C. lunata* incubated for 10 days in biochemical oxygen demand (BOD) at 25°C at concentrations of 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 conidia mL^{-1} until the point of outflow on the leaves. Positive controls were prepared using only sterile distilled water with the same volume used for concentrations of conidia. Then, the pots were maintained for 48 h with humid cotton and sealed with a plastic bag to provide a humid chamber. After 48 h of inoculation, the pots were left in a shaded place until the appearance of the first symptoms of leaf spot of *Curvularia*.

Phytotoxicity of essential oil of *L. sidoides*

The experiment for testing the phytotoxicity was carried out under greenhouse conditions (relative humidity of 70 to 80% and temperature at 27 to 33°C) according to the methodology used by Santos et al. (2013). For planting of maize, Traktor (Syngenta®) seeds were used by seeding two seeds in each pit. After sowing, the pots were irrigated daily until the growth of the seedlings reached four definite leaves or till 15 days of planting. Only the manual spray trigger was used for the application of treatments, coupled to a 10-mL test tube containing the solution. Each pot was sprayed with 5 mL of 2.5 to 50 mg mL^{-1} solutions. After 24 h of application, the evaluation was performed according to the scale of phytotoxicity adapted by Freitas et al. (2009).

Effect of *L. sidoides* essential oil on mycelia *C. lunata*

To verify the effect of essential oil on the mycelial growth of the phytopathogen, 100 µL of each of the five solutions (2.5, 5, 7.5, 10, and 50 mg mL^{-1}) was spread on the surface of the culture medium. Then, a disk of mycelium-agar of 6 mm in diameter was placed in the center of the plates and incubated in BOD at 25°C for 10 days according to the methodology of Seixas et al. (2011). The evaluations were carried out by measuring the diameter of the mycelial outlining of two orthogonal axes with each other over the center of the plates, resulting in an arithmetic mean, and measured every 2 days (2, 4, 6, 8, and 10 days). Two controls, one containing Tween 80% (0.03%) in sterile distilled water (positive control) and the other containing methyl thiophanate (fungicide and negative control) at 2 mg mL^{-1}), were tested. All experiments were conducted in triplicate. The regression equations were adjusted for the quantitative factor using a program for preparation of spreadsheets, SigmaPlot 12.0.

Assay of inhibition of conidial germination

This assay was conducted in a completely randomized design with three replications. An aliquot of 50 µl of the conidial suspension of *C. lunata* (10^4 conidia mL^{-1}) and another of 50 µL at different concentrations (0, 0.625, 1.25, 2.5, and 5 mg mL^{-1}, respectively) of *L. sidoides* essential oil containing Tween 80% (0.03%) were placed in each of the containers ("little wells") of 96-well tissue cultures plates, round bottom (TPP®) (Balbi-Peña et al., 2006). They were incubated in a humid chamber in a photoperiod of 12 h for a total time of 24 h. A total of 200 conidia were counted per treatment by observing the germinated and ungerminated conidia under an optical microscope (Aguiar et al., 2014). The data were analyzed using regression analysis.

Effect of *L. sidoides* essential oil on *C. lunata* in maize plants

On each plate containing an inoculum of 7 days of incubation, 10 mL of sterile distilled water was added for the preparation of conidial solutions. The concentration of 10^4 mL^{-1} of conidia was chosen for assessing the biological activities. Then, the vessels were maintained for 48 h with a humid and sealed cotton with a plastic bag to provide a humid chamber. After 48 h of inoculation, the pots were left in a shaded place until the appearance of the first leaf symptoms of *Curvularia* spot (Santos et al., 2013). For evaluating the severity of the disease, a grading scale was used according to Santos et al. (2005), where 0 = healthy plant, 1 = <1% of leaf area sick, 3 = 1 to 5% of the leaf area sick, 5 = 6 to 25% of the leaf area sick, 7 = 26 to 50% of the leaf area sick, and 9 = >50% of the leaf area sick.

Preventive effect of essential oil

To assess the preventive effect of essential oil, a completely randomized experimental design in a factorial design was used with three replicates, where the factors were a type of oil and the following five concentrations of the solutions of oil: 0.625, 1.25, 2.5, 5, and 7.5 mg mL^{-1}. As a positive control, plants sprayed with Tween 80% (0.03%) in sterile distilled water were used, whereas plants sprayed with 2 mg mL^{-1} methyl thiophanate (fungicide) served as the negative control. For each treatment, 5 mL was sprayed on each pot, and after 1 h, it was inoculated with 5 mL of 10^4 mL^{-1} *C. lunata* conidia. The severity of the disease was assessed every 2 days after the inoculation (five assessments in total) (Santos et al., 2013).

Curative effect of essential oil

To evaluate the curative effect of essential oil, a completely randomized experimental design in a factorial design was used with three replicates, where the factors were a type of oil (the same applied for assessing the preventive effect) and the following five concentrations: 0.625, 1.25, 2.5, 5, and 7.5 mg mL^{-1}. The controls were also the same as described for assessing the preventive effect. The maize plants were inoculated with 5 mL of 10^4 mL^{-1} conidial solution, and then the vessels were kept for 48 h with a humid sealed cotton with a plastic bag to provide a moist chamber. After 48 h of inoculation, the pots were left in a shaded place until the appearance of the first leaf symptoms of *Curvularia* spot. For each treatment, 5 mL was sprayed on each pot after checking the onset of the disease, and the severity of the disease was assessed every 2 days (five assessments in total) after the application of the solutions of oil. Using the results obtained in these assessments, the area under the disease progression curve (AUDPC) was calculated, according to Schneider et al. (1976).

Cytotoxicity of *L. sidoides* essential oil on (*Mesocricetus auratus* W.) hamster cells

To verify the cytotoxicity effect of *L. sidoides* essential oil,

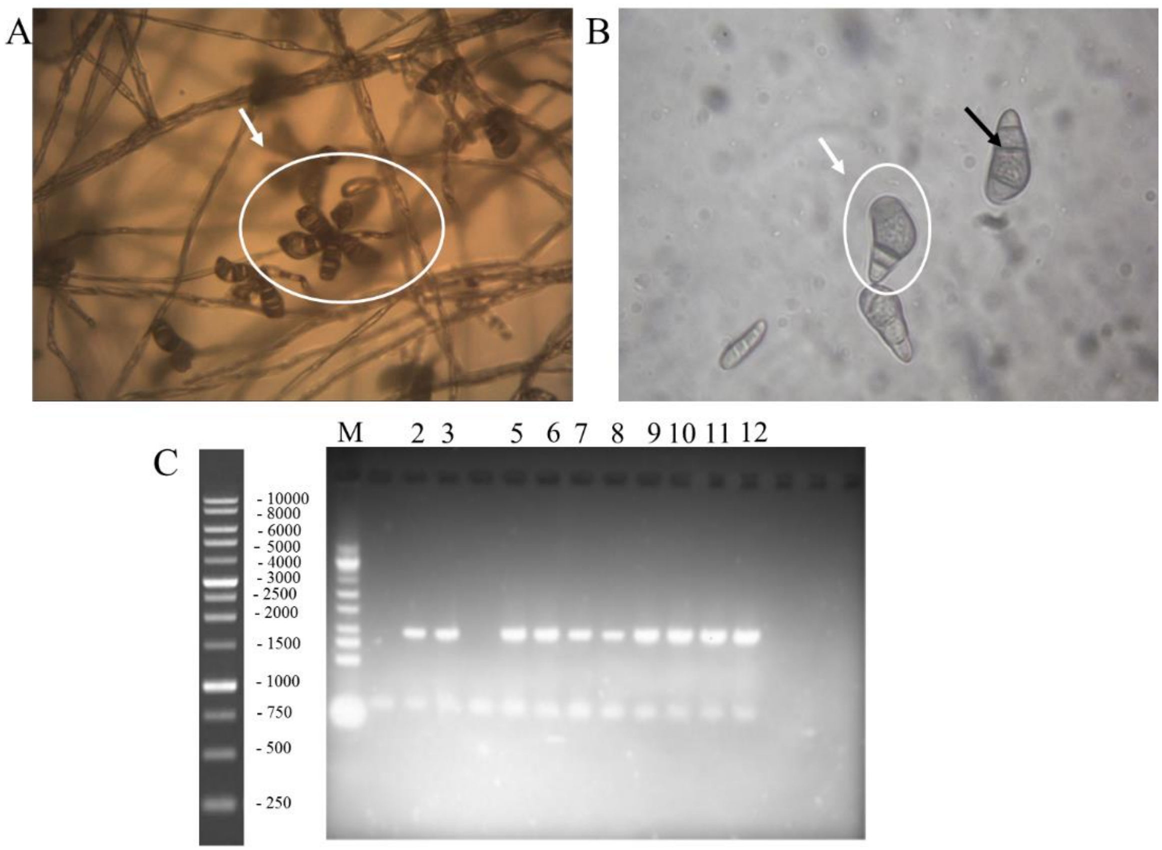

Figure 1. Morphological and molecular characterization of *C. lunata* obtained from maize plant. (A) Morphological characterization of conidiophore of *C. lunata*, white arrow. (B) White arrow indicates conidial morphology and black arrow indicates conidia septate. (C) Molecular characterization by PCR amplification of specific oligonucleotides in lanes 2–3 and 5–12 (≈966 bp), M: 1-kb ladder molecular marker (Invitrogen).

mammalian cells (*M. auratus*), hamster cells (blood, spleen, and liver), were used according to the methodology of Riss et al. (2013). The cells were washed twice in phosphate-buffered saline (PBS), filtrated once (pH 7.2), and centrifuged at 2000 rpm for 10 min/TA. After counting, the cells (1×10^5 cells/mL) were incubated (100 µl) with 50 µl of five concentrations (625, 250, 2500, 5000, and 7500 µg mL^{-1}) of the solution of essential oil of *L. sidoides* control (water + tween 80 to 0.03%) for 48 h in a 96-well plate. After 48 h, the plate was incubated with 20 µl of [3-(4.5-dimetthythiazol-2-yl)-2.5 diphenyltetiazolium (MTT) bromide] at a concentration of 2.5 mg mL^{-1} for 4 h at 37°C. Then, the supernatant was aspired from the wells and added to 200 µL of 0.04 M HCl solution in isopropanol or sodium dodecyl sulfate (SDS) to under the crystals of formazan. The plate was read at a wavelength at 540 nm.

Statistical analysis

Results were expressed as mean ± standard error of mean. Linear regression analysis was performed using SISVAR 4.6 according to Ferreira (2001), and graphs were produced using SigmaPlot 12.0. A p-value less than 0.05 was considered statically significant.

RESULTS

The *C. lunata* isolate was morphologically and molecularly characterized (Figure 1a, b, and c). In PDA culture media, it presented thin aspect of its mycelia; the reverse side of the colony had a dark color with the presence of conidia after 7 days of incubation and forms of regular board and with gray pigmentation and the pattern of amplification by PCR with the expected size (≈966 bp) *C. lunata* (Figure 1c).

Typical symptoms of maize leaf burning were recorded after the incubation period of 48 h after inoculation of plants. Among the six concentrations of conidia prepared (10^1 to 10^6 mL^{-1} conidia), there was a higher incidence of leaf spots with the concentration of 10^4 mL^{-1} conidia. However, all the suspensions caused lesions on the leaves of maize plants. The concentration of 10^4 mL^{-1} conidia was chosen for assessing the fungal toxicity and the preventive and curative effects in the later stages. According to Agrios (2005), a pathogen can lose its ability of parasitism in relation to its host if successive subcultures in a culture medium were performed. Therefore, in all the isolation procedures, the fungus was picked only once.

The chromatographic analysis of essential oil of *L. sidoides* revealed its chemical constituents, followed by

Table 1. Chemical constituents of essential oil of *L. sidoides* and their contents expressed as percentage.

[a]NC	Compounds	[b]RT	[c]CRI	%
1	α-Thujene	5.915	927	0.051
2	α-Terpinene	8.680	1018	0.091
3	ρ-Cymene	8.944	1025	1.162
4	γ-Terpinene	10.176	1058	0.250
5	cis-Sabinene hydrate	10.656	1071	0.102
6	4-Terpineol	15.19	1182	0.453
7	Thymol methyl ether	17.264	1230	0.430
8	Thymol	20.075	1294	92.684
9	(E)-caryophyllene	25.369	1419	2.235
10	α-Humulene	26.849	1456	0.134
11	Caryophyllene oxide	31.878	1582	0.617
-	Total (%)	-	-	98.179

[a]NC: Number of compounds; [b]RT: retention time; [c]CRT: calculated retention index.

Table 2. Effect of the essential oil of *L. sidoides* on the mycelial growth (mm) of *C. lunata* at different concentrations.

Treatment	Evaluation times (days of incubation)					Regression equation	R^2
	2	4	6	8	10		
Positive Control	26.21 ± 0.25	44.69 ± 0.76	59.44 ± 1.90	68.96 ± 1.80	80.56 ± 1.80	y = 6.5285x + 15.375	0.98
[a]M. thiophanate	25.27 ± 0.71	42.51 ± 0.18	59.44 ± 0.50	67.4 ± 3.76	78.11 ± 2.87	y = 6.6485x + 16.081	0.98
[b]M. thiophanate	8.59 ± 0.20	9.14 ± 0.89	9.74 ± 1.07	9.94 ± 0.99	10.51 ± 0.90	y = 0.2320x + 08.192	0.98
2.5[f]	22.88 ± 0.10	41.32 ± 0.73	55.74 ± 1.47	68.78 ± 5.27	82.34 ± 7.66	y = 7.3190x + 10.298	0.99
5.0[f]	18.97 ± 0.07	36.15 ± 0.15	50.31 ± 0.25	61.07 ± 0.61	77.76 ± 0.49	y = 7.1250x + 06.102	0.99
7.5[f]	11.89 ± 0.24	22.61 ± 1.00	29.95 ± 1.85	37.03 ± 2.33	47.98 ± 3.55	y = 4.3300x + 03.912	0.99
10.0[f]	7.37 ± 0.86	13.97 ± 1.27	18.68 ± 1.31	20.76 ± 1.76	29.91 ± 1.71	y = 2.5935x + 02.577	0.96
50.0[f g]	-	-	-	-	-	-	-

[d]M. thiophanate (*C. lunata* inoculated BDA + thiophanate Methyl (negative control 1)) and [e]M. thiophanate (*Fusarium* inoculated BDA + thiophanate Methyl (negative control 2)). [f]mg mL^{-1}. 50.0[g] (-) There was no growth at 10 days of evaluation.

their retention times and the rates of retention calculated and charted, and their levels were expressed as percentage (Table 1). The primary constituents included thymol (92.684%), (E)-caryophyllene (2.235%), and ρ-cymene (1.162%) (Table 1).

The effect of essential oil on the mycelium (mm) of *C. lunata* when submitted to essential oil of *L. sidoides* is shown in Table 2 and Figure 2. Fungal growth was observed at concentrations from 2.5 to 10 mg mL^{-1}. As for the concentration 50 mg mL^{-1}, a strong inhibition of *C. lunata* was observed (Table 2). Comparing the mean values of mycelial growth of the pathogen for 2 and 4 days after incubation, it was observed that the essential oil of *L. sidoides* promoted the inhibitory effect at the concentration 5 mg mL^{-1} (Table 2), where it can be seen that in all the concentrations tested, the mean values differ among themselves (Table 2). The fungus exhibited mycelial growth inhibition when submitted to 2.5 and 5 mg mL^{-1} for 2 days and only at 7.5 mg mL^{-1} for 2 and 4 days. The concentration 2.5 mg mL^{-1} was not effective in reducing the mycelial growth over time (Table 2).

This comparison may be more evident if the growth of positive control was examined in relation to the other treatments. It can be inferred that after the 6th day of incubation, the inhibition of the mycelia of the fungus *C. lunata* is not satisfactory, since the third assessment at 5 mg mL^{-1} does not differ from that at 2.5 mg mL^{-1}, and this also does not differ from the positive control (Table 2). The last two assessments (8 and 10 days) confirm that 2.5 and 5 mg mL^{-1} are statistically equivalent to the mycelial growth of the positive control. For the inhibitory effect, the concentration 7.5 mg mL^{-1} can be used, since in all the five assessments, there was a reduced growth of fungal mycelia for the same concentration, and for 10 and 50 mg mL^{-1}, lower quantity of oil will be required. On the other hand, the application of higher concentrations can be more efficient because the pathogen can be inhibited more quickly (Table 2).

For *C. lunata*, the first negative control with methyl thiophanate showed no significant difference in the

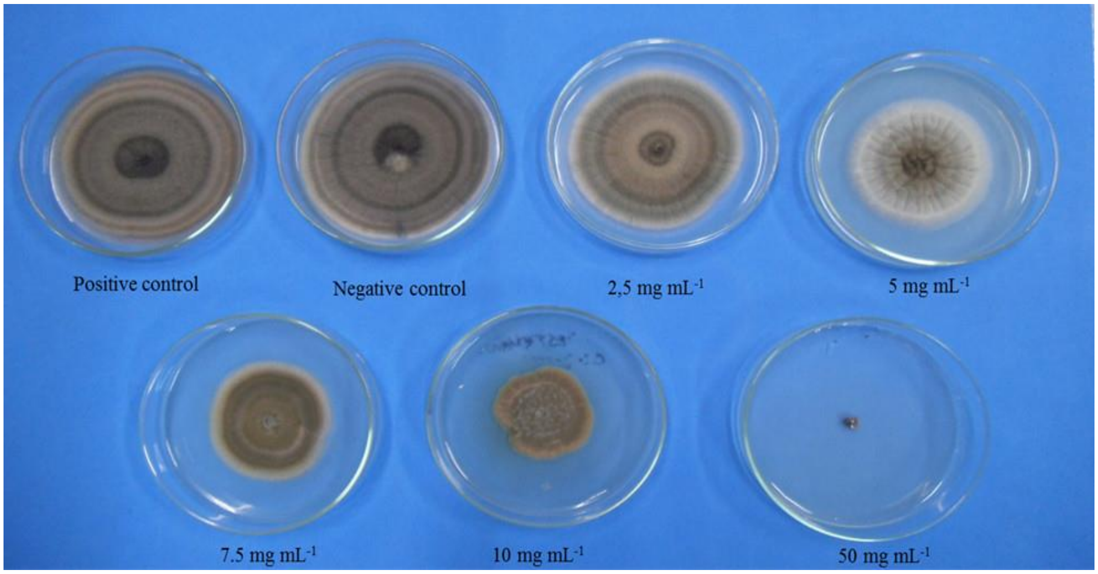

Figure 2. Inhibition of *C. lunata* mycelial growth, obtained from 7-day-old maize plants in PDA culture medium with the addition of five different concentrations (2.5-50 mg mL^{-1}) of the essential oil of *L. sidoides*, positive control, and negative control (methyl thiophanate at 2 mg mL^{-1}).

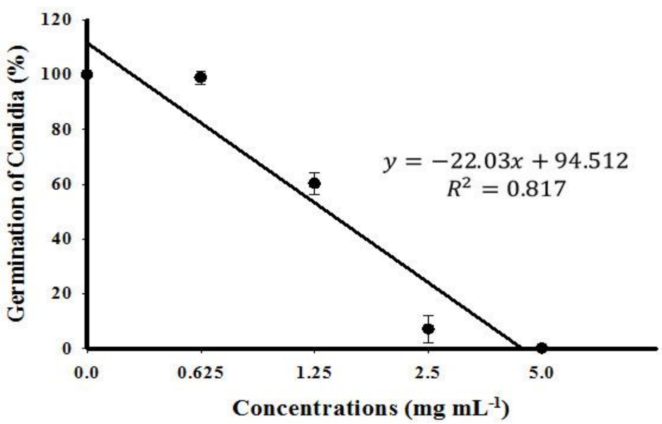

Figure 3. Effect of *L. sidoides* essential oil on the germination of conidia of *C. lunata*. $p<0.05$ indicate statistically significant difference.

positive control, even with the use of the concentration above the recommended dose. On all days of incubation, the fungus exhibited growth with the same measurements.

The effect of the concentrations of the essential oil of *L. sidoides* (0, 0.625, 1.25, 2.5, and 5 mg mL^{-1}) was also found in the conidial germination as shown in Figure 3. There was inhibition of conidial germination at increasing doses of *L. sidoides* essential oil. The two highest concentrations completely inhibited the conidial germination. At the concentration of 2.5 mg mL^{-1}, the conidia germinated in 7% of the total, producing an inhibition of 93%, whereas at the concentrations of 0.625 and 1.25 mg mL^{-1}, inhibitions of 98.83 and 60.17% were observed, respectively (or 1.17 and 39.83% of conidial germination, respectively). The lowest concentration refers to the positive control, which provided a suitable environment for the conidial germination at 100%. Thus, the concentration of 2.5 mg mL^{-1} was the one that showed better results at 24 h of analysis. A negative control with methyl thiophanate (1 mg mL^{-1}) was used, where it was observed that there was also a complete inhibition of conidial germination of *C. lunata*.

The phytotoxicity diseases in maize plants have as their primary symptom the appearance of necrotic spots on the leaves, specifically in the regions where the solution builds up, such as on the edges and in the leaf ribs causing loss of leaf green area (Magalhães et al., 2000). In the phytotoxicity assay, the same concentrations were used in the on mycelia *C. lunata*. Symptoms developed 12 h after the applications of the essential oil in the leaves. It was verified that the concentrations of 10 (10.67% of phytotoxicity) and 50 mg mL^{-1} (69.33% of phytotoxicity) may not be used for tests of disease control in the plant, because they resulted in wilting, followed by subsequent necrosis in some regions. Based on these results, it can be inferred that the solutions of the oil must be used at a concentration below 7.5 mg mL^{-1}, where there were no visible symptoms of phytotoxicity developing from the application of the essential oil studied.

Regarding the preventive effect of *L. sidoides* essential oil under the progression of the disease spot of *Curvularia* (Figure 4, black column), the plants treated with the fungicide methyl thiophanate showed symptoms of the disease in smaller areas than those with all other

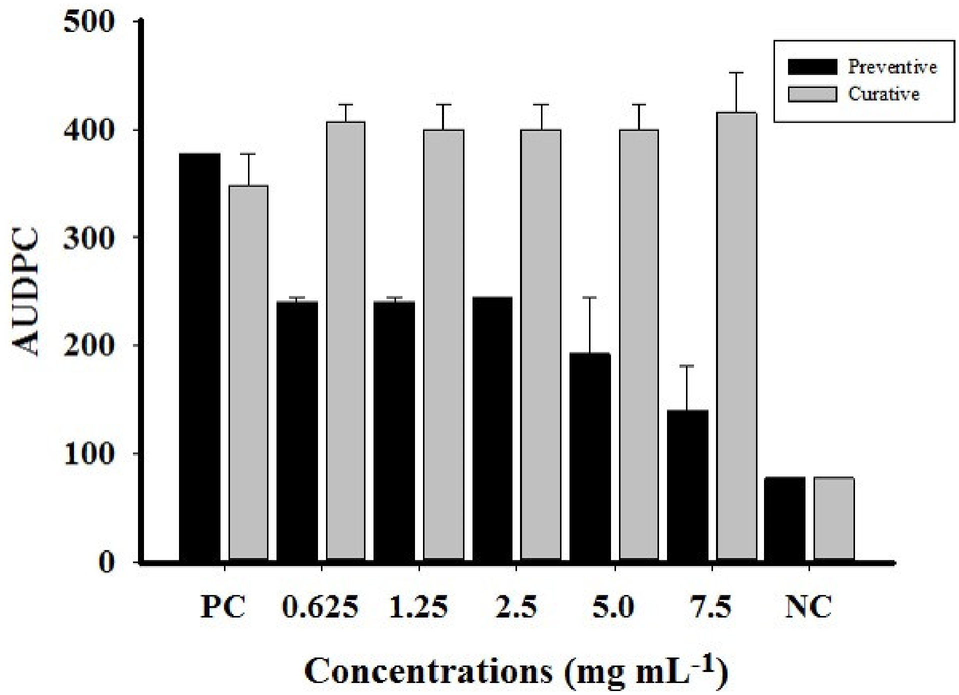

Figure 4. *In vivo* preventive (black column) and curative (gray column) effect of *L. sidoides* oil essential oil at five different concentrations and under positive (PC) and negative thiophanate methyl (NC) controls.

treatments. However, it is obvious that there is a large difference between the progression of the disease in the positive control (Tween 80% (0.03%) in sterile distilled water or 1 = 0.0 mg mL^{-1}) and that with other treatments. The black columns in Figure 4 indicate the approximate area values for the concentrations 0.625, 1.25, and 2.5 mg mL^{-1}. At the concentration 5 mg mL^{-1}, a decline in the progression of the disease was observed and when the leaf area with disease at the concentration of 7.5 mg mL^{-1} is observed (Figure 4), one may say that the value is very close to the negative control.

In cytotoxicity assay, *L. sidoides* essential oil at concentrations of 0.625, 1.25, 2.5, 5.0, and 7.5 mg mL^{-1} was not toxic to any of the cells tested (Figure 5). It was observed that the values of optical density were very close to all substances, where the control used was sterile distilled water. The amount of formazan, measured by spectrophotometry, was directly proportional to the number of viable cells. Shortly, the cells were metabolically active, both in the presence of five concentrations of the essential oil and in the presence of sterile distilled water.

DISCUSSION

Different values were reported by Veras et al. (2014), where they obtained 84.9% concentration of thymol and 5.33% of ρ-cymene in essential oil from *L. sidoides*. Fontenelle et al. (2007) also found much lower values of 59.65% of thymol. Botelho et al. (2007) analyzed the essential oil of *L. sidoides* and reported a lower concentration (56.7%) of the major compound thymol. These monoterpenes, according to Shettigar et al. (2015) can be found in several species of *Lippia*.

It was observed in the present study that this plant has a great potential to be used in the alternative control of phytopathogenic fungi, since it is possible to obtain high concentrations of the major compound. Thymol has been recognized as a compound that has antimicrobial, insecticidal, leishmanicidal, larvicidal, acaricidal, and anti-inflammatory activities (Santos et al., 2015; Carvalho et al., 2013).

Cavalcanti et al. (2004) found a concentration of 80.8% of thymol in the essential oil from plants of this species. The strong effect of thymol from the essential oil of *Lippia* was also verified by Fontenelle et al. (2007) on the growth of the fungal species *Microsporum canis*, *Malassezia pachydermatis*, and *Candida* species with inhibition of 100% of the mycelia, evidencing the fungistatic activity of the compound thymol. Furthermore, the essential oil and thymol compound have a reducing effect on the CFU in biofilms of *Enterococcus faecalis in vitro* (time of maturation 72 h), with an exposure time of 30 and 60 min at concentrations of 2.5 and 10%.

These results show that the concentration 50 mg mL^{-1} inhibited the mycelial growth at 100% (Figure 2). Similarly, Souza Júnior et al. (2009) showed the inhibition

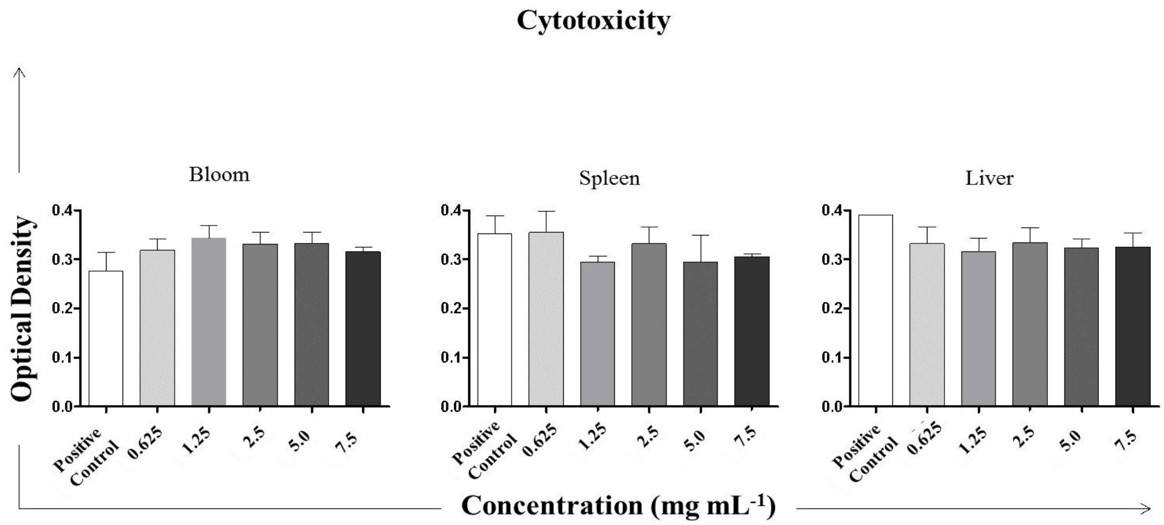

Figure 5. Cytotoxicity test *in vitro* of the different concentrations of *L. sidoides* essential oil on blood, spleen and liver cells of *M. auratus* (hamster) by MTT. $p<0.05$ indicate statistically non-significant difference.

of mycelial growth of the phytopathogenic fungus *Colletotrichum gloeosporioides*, causing the disease anthracnose in the yellow passion fruit, by 100% at concentrations of 1, 3, 5, and 10 mg mL^{-1} using the essential oil of *L. sidoides* when compared with that in the positive control (0 mg mL^{-1}).

Based on this result, it is concluded that the active principle used was not effective in controlling this pathogen. On the other hand, in the second negative control, mycelial inhibition of *Fusarium* genus was observed when subjected to the same fungicide and at the same concentration. Concentration above the recommended dose (2 mg mL^{-1}) inhibited, at the end of 10 days, growth with a percentage of inhibition of 88.32% (Table 2).

A strong inhibition of the formation of germ tube of the conidia was also observed. This sharp reduction is due to effect of the contact components, induced by essential oils, on the development of the mycelia. It can be inferred that the fungitoxic substance may have acted on the transduction signals involved in the change from the vegetative stage to the reproduction stage (Kumar et al., 2014).

Regarding the curative effect (Figure 4, gray column), it was observed that *L. sidoides* essential oil showed no significant results. All five concentrations used practically had the same area. In addition, the areas of these treatments were higher than those with the treatment with positive control (PC). Analyzing the column NC (negative control), where there was the presence of the fungicide methyl thiophanate, the progression of the disease was less, and comparing it with the black column NC for the preventive effect (for the same negative control), there was no difference. It can be suggested that it is indifferent to apply the fungicide before or after the occurrence of the disease, because the effect will be the same. However, for *L. sidoides* essential oil, it was observed that the same can be used as an alternative control, but only by applying the treatments in a preventive manner, that is, prior to the occurrence and progression of the disease.

There are no reports in the scientific literature on the preventive and curative effects regarding any essential oil on the disease spot of *Curvularia*. However, Vigo et al. (2009) showed good results for the effect of the dye of *L. sidoides* on the occurrence of symptoms (AUDPC of the common bacterial blight) on leaves of string beans Bragança cultivar inoculated with *Xanthomonas axonopodis pv*. *phaseoli*, in three application times of the dye (5 days before, 5 days before + 5 days, 5 days after the inoculation of bacteria). The dye had an effect in controlling the disease, with concentrations of 5, 10, and 20% providing the lowest values of AUDPC. Nagy et al. (2014) reported the preventive and curative effects of the essential oil of cinnamon (*Cinnamomum verum*). The concentration 0.2% was protective and curative against *Venturia inaequalis* when applied 24 h before, 1 h before, and 24 after the inoculation.

Determining and understanding the mechanisms of action of thymol is a great need with regard to pathogenic fungi. It is known that due to the presence of the group -OH in the molecular structure, this major compound of *L. sidoides* essential oil has the ability to connect to the amine groups and hydroxylamine of proteins present in cell membranes. Consequently, there is a release of cell contents through changes in the permeability of the fungal membranes (Juven et al., 1994).

Regarding the preventive effect, the conidia were inoculated 1 h after the treatment with the essential oil. This means that the compounds in the substance studied might have a greater contact with the fungal conidia in the imminence of the formation of the germ tube and the subsequent development of hyphae. It can be just that thymol altered the permeability of the cytoplasmic membrane of *C. lunata*, resulting in the inhibition of the biosynthesis of ergosterol content in a manner similar to that of fluconazole (Ahmad et al., 2011), as observed in the membranes of conidia of *Candida* species.

It can also be said that the essential oil of *L. sidoides* probably has a protective mechanism of action, that is, the toxic substances are absorbed by the fungus cell, usually through the germ tube (Nene and Thapliyal, 1979). Thus, when the inoculum (conidia) is deposited in the susceptible tissues and germinates, the germ tube comes into contact with the toxic compound (in this case, the essential oil), absorbing it, which later determines, through biochemical mechanisms, the death of protoplasm. The residual action aims to avoid penetration, thus preventing the infection that would occur in the future. Therefore, essential oils that leave residues act by preventing or decreasing the rate of penetration of the pathogen into host tissues, thereby reducing the number of penetrations or future lesions.

The fungicide used, methyl thiophanate, is considered to be a systemic type, which exhibits a longer protective action due to its ability to be redistributed within organs treated with acropetal and basipetal translocation (Marsh, 1977). In addition, it does not require germination of conidia as the type of protector. Therefore, it was effective in both the preventive and curative effects of the progression of the disease. Probably, the curative effect of *L. sidoides* essential oil was not verified due to nonpenetration of the oil into the cell wall of the fungus already inoculated into the plant.

In the present study, the cytotoxic effect of different concentrations of *L. sidoides* essential oil on blood, spleen, and liver cells of *M. auratus* (hamster) was also verified. This was necessary to verify if the concentrations used in the maize plants bioassay could be toxic to animals cells, since a compound considered as an alternative control should not cause harm to the people who manipulate it.

Therefore, the cell viability test through MTT assay is a method that is based on the ability of metabolically viable cells to reduce the salt of MTT. MTT is a compound soluble in water, which in solution features a pale coloration, being easily incorporated by viable cells. The cells reduce this compound in their mitochondria using the succinic dehydrogenase enzyme. Dehydrogenases are associated with NADPH and NADH. When reduced, MTT is converted into crystals of formazan, a dark-blue compound, not soluble in water and stored in the cytoplasm (Mosmann, 1983).

Essential oils can gain a broader focus on this issue and are being demonstrated as potential tools to help prevent diseases, including their antimicrobial activity. In this study, the antifungal activity of the essential oil of the plant species *L. sidoides* Cham. (Rosemary pepper) was determined, which indicated that this plant has a great antifungal potential against *C. lunata*.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Full Length Research Paper

Effects of mineral salts on forage seeds germination

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The objective of this study was to evaluate seed germination of three different forage species subjected to saline stress. The seeds remained in salt for 0 to 24 h and were then deposited in field beds. After 15 days, the germination was evaluated from its emergence. The effect of NaCl on germination was evaluated for the following treatments: a) 0 h (control); b) permanence in NaCl for 6 h; c) permanence in NaCl for 12 h; d) permanence in NaCl for 18 h; e) permanence in NaCl for 24 h. Five treatments with five replicates were applied to each forage species. The experimental design was completely randomized where the treatment was represented by the moment of observation, being 0, 6, 12, 18 and 24 h. For this experiment, differences ($P < 0.05$) were observed among treatments for the three forage species. For black oat and calopogonium, the different moments of salt stress in which their seeds were treated did not affect their germination. Perennial soybean suffered a decrease in germination as its seeds were exposed to salt for 6 h resulting in only 1.6% of germinated seeds. Saline stress did not affect the germination of black oat and calopogonium, both had higher values as compared to perennial soybean, in exposure time of 24 h. It is concluded that in the experimental conditions, black oat and calopogonium can be mixed with the mineral salt, without negative effect on its viability.

Key words: Seed dormancy, saline stress, mineral salt, environmental sustainability.

INTRODUCTION

In tropical regions, grasses are traditionally the most exploited because they have a forage production potential 2-3 times higher than leguminous (Valente et al., 2016). However, in the last few years, the interest of researchers in pastures has increased, aiming at the use of tropical forage legumes in animal feed, both in the form of hay and in the form of exclusive pastures and/or intercropping and more recently in the form of protein bank (fodder bank). Basically, this interest is due to the high nutritional value of these plants, which is symbiosis with bacterium of the genus *Rhizobium* that fixes atmospheric nitrogen (Pupo, 1979).

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Table 1. Agronomic characteristics of forages, black oats, calopogonium and perennial soybean.

Agronomic characteristics	Black oat	Calopogonium	Perennial soybeans
Germination (%)	75	60	60
Purity (%)	95	85	95
Cultural Value (%)	71.25	51	57
Green mass production (t/ha)	30 - 60	20 - 30	20 - 30
Fixation of nitrogen (kg/ha)	----------	60 - 80	60 - 80
Weight of 1,000 seeds (g)	20 - 23	12 - 13	5 - 6
Sowing density (kg/ha)	65 - 80	8 - 10	7 - 8

The cultivation of forage species that present dormant seeds becomes a problem due to delay in the germination of these seeds, which slows down the development of the seedlings and is susceptible to fungal attacks, which can cause both production and economic losses (Santos et al., 2004; Valente et al., 2017). For it to germinate, the seed must be alive and not dormant, and the ability of a seed to germinate under a broad limit of conditions defined as the manifestation of its vigor depends, among other factors, on the environmental conditions found at the place where it was sown. Periodic droughts, for example, can be found in the field, and the seed should be vigorous to be competitive (Perez and Tabelini, 1995). In addition to moisture, some external factors such as ambient temperature, oxygen, available nutrients, pH and salinity may exert influence on this process.

Among the methods of planting forage fodder in already established pastures, the rational dispersion of the seeds using the animals themselves as propagating agents has been increasingly explored by reducing costs (Silva, 2008; Deminicis, 2009). In this way, the seeds could be mixed or offered next to the mineral salt of the animals, without the salt negatively affecting the germination.

The objective of this study was to evaluate the seed germination of 3 different forage species, black oats (*Avena strigosa Schreb*), calopogonium (*Calopogonium mucunoides*) and perennial soybean (*Neonotonia wightii*) subjected to saline stress.

MATERIALS AND METHODS

Study location

The experiments were conducted in the dairy cattle, horticulture and chemical laboratory sectors of the Fazenda Escola da Etec Benedito Storani, in Jundiaí city, State of São Paulo, Brazil (23°11'S and 46°53'W) in an altitude of 761 m.

Seed source and agronomic characteristics of black oat, calopogonium and perennial soybean forage

Seeds of 3 species of forage were used, black oats (*A. strigosa Schreb*), perennial soybean (*N. wightii*) and calopogonium (*C. mucunoides*). These seeds were acquired through donations from the company, SEMENTES PIRAI LTDA, and the agronomic characteristics are described in Table 1.

Treatments

The effect of NaCl on germination was evaluated for the following treatments: a) 0 h (control); b) permanence in NaCl for 6 h; c) permanence in NaCl for 12 h; d) permanence in NaCl for 18 h; e) permanence in NaCl for 24 h. Five treatments with 5 replicates were evaluated for each forage species. The experimental design was completely randomized, where the treatment in this case was represented by the time of observation which is 0, 6, 12, 18 and 24 h, where 5 replicates were adopted.

The quantity of seeds and salt followed the one adopted by Deminicis (2009). Thus, each sample evaluated consisted of 10 g of seeds and 90 g of NaCl. After observing all treatment times, 50 seeds of each sample were separated and immersed in boiling water (100°C) for 20 min and seeded in beds. The germination readings occurred after 15 days of sowing.

Statistical analysis

The obtained data were analyzed through an inferential statistical analysis. A comparison was made between the different reading moments, and between species for each moment.

First, in order to assess normality, the Shapiro-Wilk test was conducted at 0.05 significance level. In all cases, no adherence to the normal distribution was observed ($P > 0.05$), which directed the choice by non-parametric tests. The comparison of independent variables was performed using the Kruskal Wallis test ($\alpha = 0.05$), while the comparison between the study moments was conducted according to the Friedman test ($\alpha = 0.05$). The logistic regression was used to obtain the equation of the germination % as a function of the residence period to the salt and the respective R^2.

RESULTS AND DISCUSSION

Analysis of the variance indicated that there was a difference ($P > 0.05$) among treatments for the 3 forage species (Table 2). For the forage species, black oat and calopogonium, the different moments of salt stress in which their seeds were submitted did not affect their germination. Unlike the perennial soybean species, when their seeds were exposed to salt, a decrease in their % germination could be observed (1.6) (Table 2). The saline

Table 2. Average seed germination % for each species subjected to different periods of saline stress after 15 days of sowing.

Treatments (h)	Forages			Total	SE	P value
	Black oat	Calopogonium	Perennial soybean			
0	20^{aAB}	30^{aA}	12.4^{aB}	20.5	3.95	0.0314
6	24^{aA}	24^{aA}	1.6^{bB}	16.3	5.78	0.009
12	36.4^{aA}	27.2^{aA}	8^{abB}	23.5	6.48	0.007
18	28.4^{aA}	29.2^{aA}	6.4^{abB}	21	5.78	0.011
24	27.2^{aA}	20.4^{aA}	9.2^{abB}	18.7	4.06	0.047

*Means in the same column, followed by the same lowercase letters do not differ from each other by the Kruskal-Wallis test at 0.05 of significance. **Means in the same line, followed by the same capital letters do not differ from each other by the Kruskal-Wallis test at 0.05 of significance. SE- Standard error of the mean.

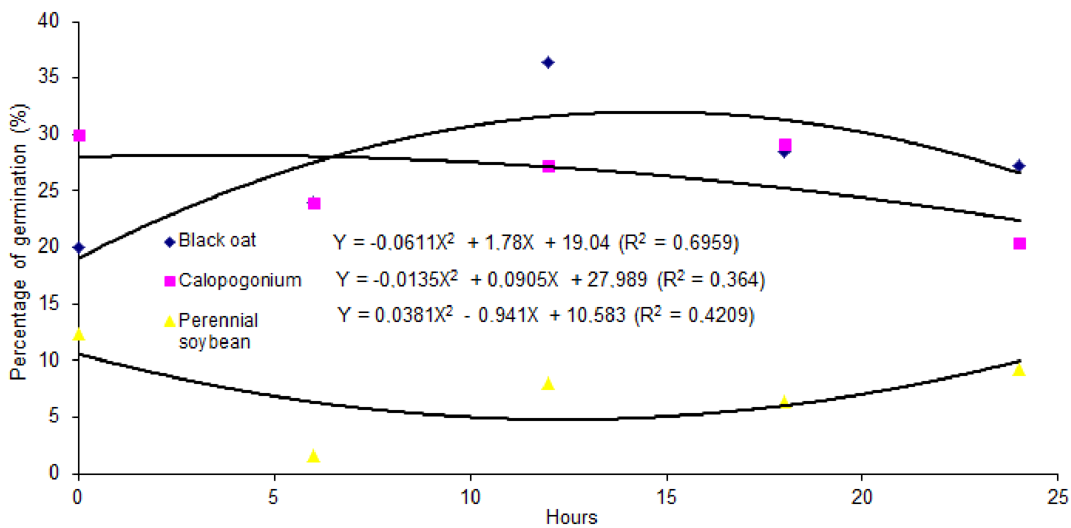

Figure 1. Regression curves for % germination of black oats (*Avena strigosa Schreb*), calopogonium (*Calopogonium mucunoides*) and perennial soybean (*Neonotonia wightii*), as a function of salt permanence intervals.

stress did not affect the germination of black oat and calopogonium, where both had higher values ($P > 0.05$) as compared to the perennial soybean. Considering the above, NaCl can be used as a vehicle in the administration of black oat and calopogonium seeds by cattle, without affecting the quality of the seeds, at 24 h exposure time.

This result was also obtained by Deminicis (2009) who observed that the species that suffered the greatest damage due to salt permanence was perennial soybean, where the % germination was reduced by almost half, as compared to the permanence values of 6 (65.5%) and 24 h (33.0%). In the same work, it was observed that saline stress, up to 18 h, did not present a risk in the germination of perennial soybean seeds, showing that after this period, failures in seed protection and failure in repair mechanisms begin to compromise the activity of seeds and plant development. The regression analysis of the equations that describe the behavior of the number of germinated seeds as a function of salt permanence intervals is shown in Figure 1.

In relation to the treatment with black oats, R^2 was 0.695, indicating that the period of time predicts the percentage of germination in 69.5%. The treatments with calopogonium and perennial soybean had lower determination coefficients, being 0.364 and 0.420. Although, black oats had a higher correlation when compared with the other species, it was observed that there was an increase in the % germination as a function of time of exposure to saline stress. This fact was not observed in the other two species, and it could be assumed that this factor (saline stress) was not the only one that influenced the germination process of the same species, according to Jeller and Perez (2001). Saline stress interferes more strongly with germination speed of the seed as compared to germinability.

Naturally, the high content of salts in the soil, especially NaCl, can inhibit germination, initially due to osmotic effects and, in some cases, to toxic effects whose magnitude depends on the degree of tolerance and/or resistance to salinity, not only on the species as well as the salt type (Ferreira and Rebouças, 1992).

Conclusions

For the forage species, black oat and calopogonium, the different moments of saline stress in which their seeds were subjected did not affect their germination. Different from the perennial soybean species, from the moment their seeds were exposed to salt, a decrease in their percentage of germination can be observed. The saline stress did not affect the germination of black oat and calopogonium where both had higher values as compared to perennial soybean, in the exposure time of 24 h. Seeds of forages mixed with salt, and offered to cattle, can pass through the digestive tract and germinate in pastures; however, there are species that are more sensitive to this stress, which may impair its viability and establishment in a pasture.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Environmental conditions of the interior of the tropical forest and regeneration of tree species

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This study aimed to analyze the interactions between environmental variables and the occurrence of tree species in the understory of an Atlantic forest fragment in Southeastern Brazil. The vegetation data collection was carried out in ten locations, with trees having diameter at breast height (DBH) larger than 5 cm and with distinct physiographic characteristics, orientation and slope. The following variables were collected at each site: sum of the soil bases, soil moisture, leaf area index (LAI) and canopy transmissivity of photosynthetically active radiation (PAR). The interaction between environmental variables and vegetation was investigated employing the canonical correspondence analysis (CCA), using data from the natural regeneration (TNR) of forty-six species and the environmental variables of the locations studied. The Monte Carlo test showed that the correlation between TNR values for the species and environmental variables was statistically significant. The occurrence of four primary species groups was identified through this analysis. Considering the availability of solar radiation, one species group was observed to have occurred under the open canopy with high transmissivity of PAR, and another in locations with high LAI values and low PAR transmissivity. When soil variables were considered, we were able to distinguish two groups; a group of species showed preference for sites with fertile soil and high water content and another group that was more predominant in sites with drier soil, usually on steeper slopes.

Key words: Brazilian Atlantic forest, environmental heterogeneity, photosynthetically active radiation, leaf area index.

INTRODUCTION

Investigation of the behavior of forest species aiming to understand the ecology of these species is an important and current research issue, which have been studied by several researchers. Considering the current level of degradation of the forest ecosystems, a better understanding of, for example, the light or shade tolerances of a species, in high and low fertility soils could be very useful in the recuperation of degraded

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forest ecosystems. Studies seeking to indicate the best species for the recuperation of degraded environments are frequently performed; however, often the greatest challenge in these studies lies in the lack of information on the relationship between the species and the environment.

Considering these issues, phytosociology is one of the most important tools to study forest ecosystems. With this tool it is possible to describe the structure of the forest, and, therefore, make inferences about the relationships that exist among species. However, most phytosociology studies aiming to explain species' succession do not take environmental variables into consideration. Nevertheless, it is well known that these variables are essential to explain the presence or absence of certain species in different environments.

To better understand the process of succession in a forest, it is very important to establish a relationship between the environmental variables and the structural and floristic patterns of the vegetation (Higuchi et al., 2012; Wortley et al., 2013; Barthelemy et al., 2015). Previous studies have investigated mainly edaphic conditions, physiographic factors, and microclimatic characteristics of tropical rainforests.

The occurrence small-scale topographic variations leads to environmental differences among sites due to changes in the availability of solar radiation, hydrologic regime of the soil, and the availability of nutrients. Several studies have demonstrated that physiographic factors influence the distribution of species in tropical rainforests (Rodrigues et al., 2007; Higuchi et al., 2008; Silva et al., 2009; Gasper et al., 2015).

However, topography differences are not always enough to explain the distribution pattern of species in the rainforest. Some environmental variables are not entirely dependent on the topography, as for example the availability of solar radiation in the interior of the rainforest, which is also influenced by the transmissivity of solar radiation through the canopy, which is in turn controlled by the leaf area index. However, despite the fact that solar radiation is one of the most important environmental variables governing the rainforest species distribution pattern, studies on the ecology of tree species in tropical rainforests which take this environmental variable into consideration are practically nonexistent due to the difficulty of measuring this variable in field conditions.

The objective of this work was to study the relationship between the occurrence of tree species in the initial phase of their development and environmental variables measured at different sites in a fragment of the Atlantic Forest in Southeastern Brazil.

MATERIALS AND METHODS

Description of the area of study and database

This study was conducted at the Mata do Paraíso Research Station, located in the municipality of Viçosa, MG, Brazil (latitude = 20°45' South, longitude = 42°55' West and an average altitude of 690 m above sea level). The natural vegetation in the region of study is classified as semi-deciduous seasonal forest, and a portion of the tree species lose leaves in the winter. This type of vegetation is conditioned by the seasonal behavior of the climate, which is characterized by a hot summer with heavy rain, followed by a dry winter with a sharp drop in temperature. The percentage of deciduous trees, at Mata do Paraíso Research Station, varies between 20 and 50%.

According to Koppën classification, the regional climate is classified as *Cwb*, with poorly distributed rainfall throughout the year, with a rainy summer and a dry winter. The total annual precipitation average is 1,221 mm and the annual average temperature is 19.4°C. During the winter, the long-term average (from 1961 to 1990) monthly minimum temperature reaches10.1°C and, in the summer, the average monthly maximum temperature is 30°C in February, according to the Brazilian National Institute of Meteorology (INMET).

Vegetation data and estimation of natural regeneration

To study natural regeneration, Higuchi et al. (2006) identified ten sites at the area Mata do Paraíso Research Station, based on physiographic characteristics, such as orientation and slope. Permanent plots were allocated in the selected sites, where phytosociological surveys of natural regeneration (individual trees with DBH < 5 cm) have been carried out since 1992. Following the methodology proposed by Garcia et al. (2011), an index of total natural regeneration (TNR) was calculated for each species.

Environmental measurements

The average slope values and sum of soil bases (between 0 - 10 cm of depth) of each location studied was determined by Pezzopane et al. (2002). The average values of transmissivity of photosynthetically active radiation and leaf area index were also obtained by Pezzopane et al. (2002), who carried out measurements in the four seasons of the year to conduct and characterize different conditions of radiation flux density, sun position and phonological stages of the vegetation. Soil samples were taken in 0 - 10 cm layer to determine soil moisture, using the gravimetric method, in the end of the dry season. Table 1 shows the summary of the environmental variables measured in this study.

Environmental and vegetation interaction

A multivariate analysis was used to investigate the relationship between environmental variables and the distribution of species within the forest. The canonical correspondence analysis (CCA) is a type of multivariate analysis that allows ordering species, characteristics of the location of occurrence and environmental variables concomitantly (Oliveira-Filho et al., 2007). The CCA identifies the higher correlation between variables and the distribution of the species for each ordination axis. This approach has the advantage in relation to the other methods of multivariate analysis to provide a significance test for the correlations between variables. Thus, the results of one canonical correspondence analysis can be used, for instance, to identify the preferred sites for a specific tree species, as shown by Oliveira-Filho et al. (1994, 2007), Meira-Neto et al. (2005), Carvalho et al. (2007), Ferreira-Junior et al. (2007), Geihl and Jerenknow (2008), Venturoli et al. (2010) and Marcuzzo et al. (2013).

In this study, the environmental and vegetation interaction were studied employing the canonical correspondence analysis (CCA), using data of total natural regeneration of the species (TNR) and

Table 1. Slope (SC), sum of bases (SB), soil moisture (SM), transmissivity of photosynthetically active radiation (t) and leaf area index (LAI) at the ten locations studied at theMata do Paraíso Research Station, in Viçosa, MG, Brazil.

Local	SC (%)	SB ($cmol_c.dm^{-3}$)	SM ($g.100\ g^{-1}$)	t (%)	LAI
1	40	1.5	21.4	8.9	3.6
2	21	1.3	19.1	6.0	4.5
3	43	0.2	23.5	2.7	4.9
4	80	0.1	18.4	9.3	3.6
5	3	6.1	43.0	1.7	5.2
6	51	4.9	21.5	1.8	5.0
7	45	0.5	26.2	1.6	5.2
8	20	0.3	24.1	3.7	4.2
9	14	1.6	23.0	2.8	5.1
10	45	0.1	22.4	2.5	4.3

Source: Pezzopane et al. (2002).

Table 2. List of the species sampled in the natural regeneration stage at Mata do Paraíso Research Station, in Viçosa, MG, Brazil.

Species	Species
Allophylus edulis Radlk.	*Machaerium triste* Vogel
Anadenanthera peregrina (L.) Speg.	*Maprounea guianensis* Aubl.
*Annona sylvatica*A. St.-Hil.	*Matayba elaeagnoide s*Radlk.
*Apuleia leiocarpa*Vogel J. F. Macbr.	*Myrcia fallax* (Rich.) DC.
Bauhinia forticata Link	*Nectandra oppositifolia* Nees & Mart.
Brosimum guianensis (Aubl.) Huber	*Nectandra rígida* (Kurth) Nees
*Casearia aculeata*Jacq.	*Nectandra saligna* Nees
*Citharexylum myrianthum*Cham.	*Octeacorymbosa* (Meisn.) Mez
Copaifera langsdorffii Desf.	*Picramnia glazioviana* Engler
*Croton floribundus*Spreng.	*Piptadenia gonoacantha* (Mart.) J.F. Macbr
Cupania sp.	*Prunus sellowii* Koehne
Dalbergia nigra (Vell.) Allemao ex Benth.	*Pseudopiptadenia contorta* (DC.)
Erythroxylum pelleterianum A. St.-Hil.	*Psychotria conjungens* Müll. Arg.
Eugenia brasiliensis Lam.	*Psychotria sessilis* Vell.
Euterpe edulis Mart.	*Rheedia gardneriana* Plach. &Triana
Guapira opposite (Vell.) Reitz	*Siparuna guianensis*Aubl.
Guarea Guidonia (L.) Sleumer	*Sorocea bonplandii* (Baill.) Lanj. &Wess. Bôer
Inga edulis Mart.	*Sparattosperma leucanthum* (Vell.) K. Schum.
Jacaranda macrantha Cham.	*Swartzia myrtifolia* Sm.
Landenbergia hexandra (Pohl Klotzsch	*Vismia guianensis* (Aubl.) Pers.
Luehea grandiflora Mart. &Zucc.	*Vitex sellowiana* Cham.
Machaerium nyctitans(Vell.) Benth.	*Xylopia sericea*A St.-Hil.
Machaerium stipitatum (DC.) Vogel	*Zanthoxylum riedelianum* Engl.

environmental variables of the studied locations. The Monte Carlo Test was applied to evaluate correlation between the values of TNR and the environmental variables at 1% level of probability.

Among the sampled species found in the plot, we selected those that showed a number of individuals greater than or equal to eight. Thereafter, a new species selection was performed eliminating those that showed only one individual per sampling unit, following the criteria proposed by Higuchi et al. (2006). Among the 128 species sampled in the botanical survey, 46 were selected for the study of environmental interaction × species, corresponding to 36% of the total species (Table 2).

RESULTS AND DISCUSSION

The matrix of correlations between the environmental

Table 3. Correlation matrix of the first two ordination axes and the environmental variables (EV), obtained via canonical correspondence analysis, of the data collected in Mata do Paraíso Research Station, in Viçosa, MG.

Correlation	Species axis 1	Species axis 2	VA axis 1	VA axis 2	SB	SC	SM	LAI	T
Species axis 1	1.000								
Species axis 2		1.000							
EV axis1	0.974		1.000						
EV axis2		0.970		1.000					
SB	0.786	0.600	0.766	0.582	1.000				
SC	0.029	-0.720	0.028	-0.699	-0.411	1.000			
SM	0.590	0.432	0.575	0.419	0.658	-0.593	1.000		
LAI	0.595	-0.021	0.580	-0.021	0.471	-0.484	-0.522	1.000	
T	-0.584	0.144	0.656	0.139	-0.350	0.431	-0.499	-0.885	1.000

SB, SC, SM, LAI and t, correspond, respectively, to sum of bases ($cmol_c dm^{-3}$), terrain slope (%), soil moisture ($g100\ g^{-1}$), Leaf area index, and transmissivity of photosynthetically active radiation (%).

variables and the first two ordination axes generated by CCA is shown in Table 3. The correlations between environmental variables (EV) and species were high (0.974 and 0.970), showing that the environmental variables used in the study explained satisfactorily the first two ordination axes.

The eigenvalues measured from the body of one axis, for the first and second axes of ordination, were 0.395 and 0.302, respectively, which are considered low values. The first two axes were responsible for 43% of the total variance. According to Martins et al. (2008), in this type of study, low eigenvalues are expected to occur due to the complexity of the interaction among the factors that determine the floristic and structure of the vegetation. In this study, the Monte Carlo test, applied to both axes, showed that the correlation between the RNT values of the species and the environmental variables were statistically significant (P<0.01).

The soil moisture showed positive correlation with the sum of bases and the leaf area index, and was negatively correlated with the slope and transmissivity of PAR (Table 3). These results are expected and can be explained by the topography since gentle slopes tend to show elevated fertility and soil water content, which is in agreement with results presented by Botrel et al. (2001) and Machado et al. (2008). Moreover, the transmissivity of PAR and LAI govern the availability of energy for the evaporation of soil water; therefore, the lower the attenuation of solar radiation (lower LAI), the lower is the soil water content, as a consequence of greater evaporation on the soil surface.

A significant correlation between the leaf area index and transmissivity of PAR is also expected since the LAI determines the amount of solar radiation that penetrates the plant canopy, as shown in the work of Wirth et al. (2001), Marques-Filho et al. (2005), Keeling and Phillips (2007) and Russo et al. (2012).

The separation of the sampling units or of the species in the ordination space can be analyzed graphically, in which the environmental variables are represented as vectors with size proportional to the influence of each variable, with the location or the plotted species next to the vector that exerts the most influence upon them.

Analyzing the ordination diagram of the locations studied (Figure 1), it is possible to see the formation of basically three groups. The first group, formed by locations 1, 2, 8 and 9, is under the influence of the transmissivity of PAR. These locations have the greatest transmissivity values of solar radiation and, consequently, the lowest LAI. Location 4 also showed high transmissivity of PAR, but it is not a part of the group, probably due to its steep slope. The second group, formed by locations 3, 4, 7 and 10 is affected by the terrain slope. Due to the steep slope at these locations, the soil water content and the sum of bases show low values.

The third group, formed by locations 5 and 6, is affected by the sum of bases, soil water content and LAI. Both locations showed elevated values for the sum of bases, soil water content, and LAI, but the slope of location 6 is relatively elevated, greater even than at locations 3, 7 and 10, which are affected by slope, as previously stated. However, location 6 has a slope in a terrain with a concave shape that could lead to greater water retention in the soil and better preservation of soil nutrients, which is in agreement with the results found by Ferreira-Junior et al. (2007) and Soares et al. (2015). In addition, the vegetation succession stage appears to be more advanced in location 6, resulting in a larger deposition of organic material in the soil, which may have interfered in the results from the soil sampling, carried out from 0 to 10 cm.

The ordination diagram to investigate the relationship between species and environmental variables is shown in Figure 2. The species that appear on the left side show a more consistent relationship with the transmissivity of PAR, which is associated with axis 1. The species located on the right side of the diagram are associated

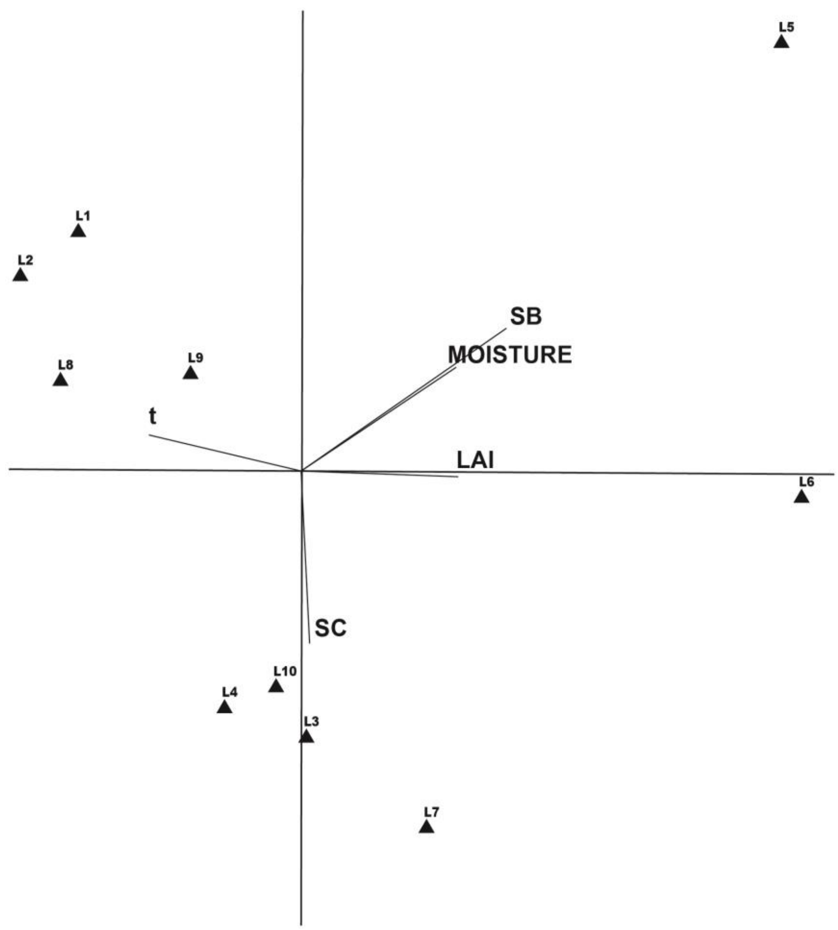

Figure 1. Ordination diagram produced using canonical correspondence analysis, showing the distribution of 10 locations and five environmental variables in Mata do Paraíso Research Station, in Viçosa-MG. Where SB, SC, MOISTURE, LAI and t, correspond, respectively, to sum of bases ($cmol_c\ dm^{-3}$), terrain slope (%), soil moisture ($g\ 100\ g^{-1}$), leaf area index, and transmissivity of photosynthetically active radiation (%).

with the sum of bases, soil water content and LAI. Another species group that appears on the bottom part of the diagram shows the most consistent relationship with average slope of the terrain, associated with axis 2. A noteworthy feature in this diagram is that the further away from the origin of the diagram a species is located, the stronger its relationship is with the environmental variables associated with the closest axis.

Among the species, having occurrences that are strongly correlated to transmissivity of PAR, are: *Croton floribundus, Sparattosperma leucanthum, Machaerium nyctitans, Xylopia sericea, Dalbergia nigra*, *Anadenanthera peregrina* and *Piptadenia gonoacantha.* These species occur more readily in the locations with an open canopy, as shown in the phytosociological study conducted by Garcia et al. (2011) in the same plots. The occurrence of the following species: *Citharexylum myrianthum*, *Vitex sellowiana, Jacaranda macrantha, Casearia aculeata, Erythroxylum pelleterianum, Machaerium stipitatum, Bauhinia forticata* and *Annona sylvatica* also showed relationship with the transmissivity of PAR, but less consistently, as shown by their closer proximity to the origin of the diagram. The grouping observed in the diagram appears to be consistent, since these species are considered primary and secondary succession species in the works of Higuchi et al. (2006), Garcia et al. (2011) and Martins et al. (2008).

On the opposite side of the vector of transmissivity of solar radiation, that is, species affected by LAI, the following species: *Picramnia glazioviana, Swartzia myrtifolia, Sorocea bonplandii, Psychotria conjungens, Nectandra oppositifolia*, *Cupania* sp. and *Inga edulis* can be found. These species occur in locations with low transmissivity of PAR (high LAI), and are amongst those with the highest RNT in locations 5 and 6, which showed a closed canopy with high LAI values. The species that compose this group require shady conditions; at least in the initial life stage, the majority of them was classified as

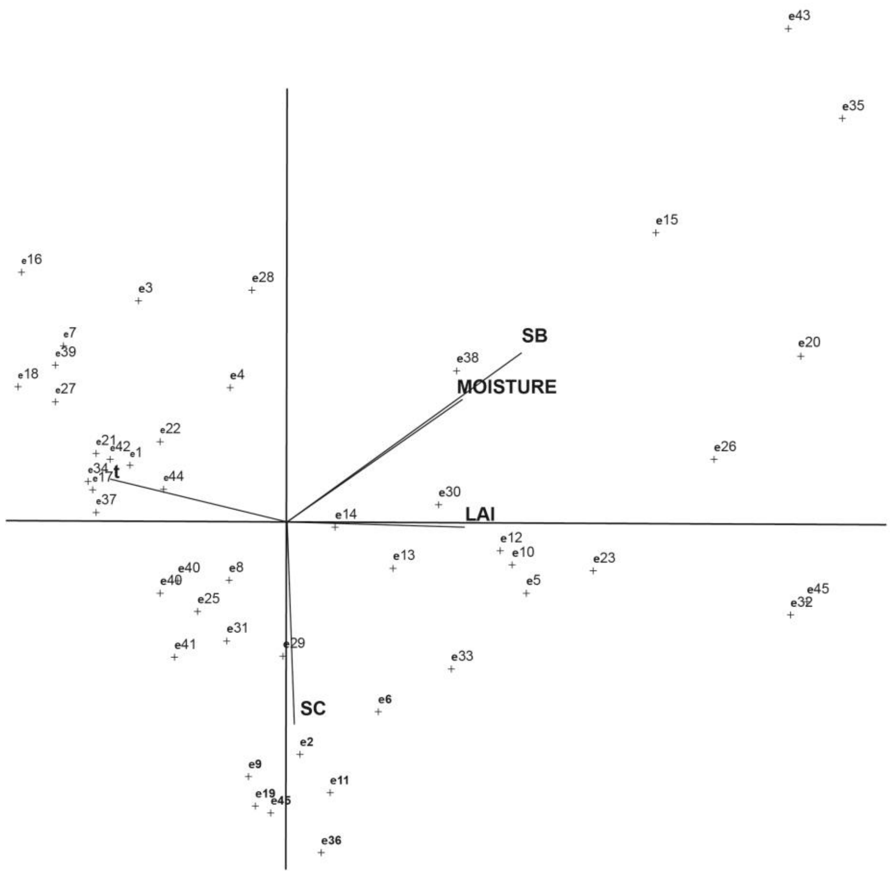

Figure 2. Ordination diagram produced using canonical correspondence analysis, showing the distribution of 46 species and five environmental variables sampled in Mata do Paraíso Research Station, in Viçosa-MG. Where SB, SC, MOISTURE, LAI and t, correspond, respectively, to sum of bases ($cmol_c.dm^{-3}$), terrain slope (%), soil moisture ($g.100g^{-1}$), leaf area index, and transmissivity of photosynthetically active radiation(%). e1-*Luehea grandiflora,* e2-*Pseudopiptadenia contorta,* e3-*Anadenanthera peregrina,* e4-*Annona sylvatica,* e5-*Psychotria conjungens,* e6-*Rheedia gardneriana,* e7-*Machaerium nyctitans,* e8-*Psychotria sessilis,* e9-*Zanthoxylum riedelianum,* e10-*Cupania sp,* e11-*Matayba elaeagnoides,* e12-*Nectandra oppositifolia,* e13-*Nectandra rígida,* e14-*Octea corymbosa,* e15-*Nectandra saligna,* e16-*Croton floribundus,* e17-*Jacaranda macrantha,* e18-*Sparattosperma leucanthum,*e19-*Copaifera langsdorffii,* e20- *Guarea Guidonia,* e21-*Casearia aculeata,* e22-*Machaerium stipitatum,* e23-*Sorocea bonplandii,* e24-*Siparuna guianensis,* e25-*Apuleia leiocarpa,* e26-*Inga edulis,* e27-*Dalbergia nigra,* e28-*Piptadenia gonoacantha,* e29-*Eugenia brasiliensis,* e30-*Maprounea guianensis,* e31-*Myrcia fallax,* e32-*Swartzia myrtifolia,* e33-*Guapira opposita,* e34-*Vitex sellowiana,* e35-*Euterpe edulis,* e36-*Landenbergia hexandra,* e37-*Citharexylum myrianthum,* e38-*Prunus sellowii,* e39-*Xylopia sericea,* e40-*Vismia guianensis,* e41-*Machaerium triste,* e42-*Erythroxylum pelleterianum,* e43-*Allophylus edulis,* e44-*Bauhinia forticata,* e45-*Picramnia glazioviana,* e46-*Brosimum guianensis.*

late secondary by Higuchi et al. (2006), Garcia et al. (2011) and Martins et al. (2008).

The species group formed by *Allophylus edulis, Euterpe edulis, Nectandra saligna, Guarea guidonia* and *Prunu ssellowii* was strongly affected by the sum of bases and soil moisture. The extension of the vectors in the opposite direction shows the group of species formed by *Machaerium triste, Myrcia fallax*, *Apuleia leiocarpa, Vismia guianensis, Siparuna guianensis* and *Psychotria sessilis,* which develop in areas with low soil water content. To the following species: *Landenbergia hexandra, Brosimum guianensis, Copaifera langsdorffii, Zanthoxylum riedelianum, Pseudopiptadenia contorta, Rheedia gardneriana* and *Eugenia brasiliensis,* can be added to this group, since they show a more consistent relationship with the terrain slope, which is negatively correlated to soil moisture.

The results of species environment interaction obtained through the use of this methodology are in agreement with previous results found in literature. For example, the strong positive relationship with the moisture content of the soil in the case of *Euterpe edulis* and *Allophylus edulis* is in agreement with results found by Rondon-Neto et al. (2002) that considers these species to be selective hygrophytes. On the other hand, the preference of *Apuleia leiocarpa* for drier soil is in agreement with Garcia et al. (2011), which consider this to be a species from high ground, and usually shows lower water content in the soil.

Ecophysiological studies performed by Pezzopane et al. (2002) demonstrated that the species *C. floribundus* shows high photosynthetic potential, behavior typical of full sun plants, and that the species *S. bonplandii,* under the same incidence values of solar radiation, showed lower photosynthetic rate values, showing typical behavior of shade plants. In this work, *C. floribundus* is classified as a species that occurs beneath a more open canopy and the opposite was observed for *S. bonplandii.*

The results obtained in this study indicate the environmental preferences for the species with the greatest natural regeneration index in the studied forest fragment, which can enhance the understanding of the ecology of the species of the Atlantic Forest. This knowledge could be of great use in the elaboration of projects, for example, of forest management or even in the definition of species and design of degraded area regeneration projects and establishment of ecological corridors.

Conclusions

Results of the study showed that it was possible to identify the occurrence of four primary species groups. Considering the availability of solar radiation, two species groups were observed; one species group that occurs under the open canopy with high transmissivity of solar radiation and another that occurs in locations with high LAI, that is, under closed canopy. Two species groups were observed considering soil characteristics; one species group showed preference for environments with fertile soil and high water content while another group showed preference for environments with drier soil, predominantly on steeper slopes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Agronomic efficiency of *Bradyrhizobium* pre-inoculation in association with chemical treatment of soybean seeds

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The competitiveness of Brazilian soybean in the international market is highly dependent on biological nitrogen fixation, whose efficiency is related to factors that affect the survival of the bacteria, such as the chemicals used in the treatment of seeds. The study objective was to evaluate *Bradyrhizobium* pre-inoculation (10 days before sowing) of soybean seeds treated with fungicides and insecticide compared to the standard inoculation performed on the planting day. Four experiments were conducted in two distinct ecosystems, two in Brazilian savanna (Cerrado) and two in Cerrado/forest transition areas of Roraima state. In each ecosystem, the experiments were performed in areas without and with soybean cultivation history. The treatments were in: 1- Control without inoculation; 2- inoculation on sowing day in untreated seeds; 3- treatment with carbendazim; 4- treatment with pyraclostrobin + methyl thiophanate + fipronil; 5- treatment with fludioxonil + metalaxyl-M. Groups 3, 4, and 5 were pre-inoculated 10 days before sowing. Viable cells in the seeds were recovered on the sowing day. Nodule number and dry mass, root, and aerial part dry mass were evaluated 35 days after emergence. Grain yield was evaluated at harvest. The number of viable cells was negatively affected by seed treatment. For all evaluated variables, treatments with pre-inoculation plus fungicides/insecticide were similar to the standard sowing-day inoculation. Pre-inoculation performed 10 days before planting, along with seed treatment with fungicides/insecticides, positively affected soybean crop productivity and could be used without compromising the nitrogen fixation.

Key words: *Glycine max* (L.) Merr., biological nitrogen fixation, Amazon.

INTRODUCTION

Soybean (*Glycine max* L. Merrill) is a plant of the Fabaceae family very important to agricultural, with significant economic and social impact and it is the main cultivated crop in Brazil (Cattelan and Dall'Agnol, 2018).

Brazil soybean production for 2017/18 is estimated at 112 million metric tons and harvested area is estimated at 35 million hectares, thus, Brazil and United States of America are currently the world's largest producers (USDA, 2018).

Nitrogen is the most required element by soybean and it can be supplied by the use of nitrogen fertilizers and by the biological nitrogen fixation (Hungria et al., 2007). Most of nitrogen requirements to cultivation of soybean in Brazil is provide by inoculation with selected nitrogen-fixing bacteria, *Bradyrhizobium japonicum, Bradyrhizobium elkanii* and *Bradyrhizobium diazoefficiens* (Hungria and Mendes, 2015). These bacteria can covert atmospheric nitrogen to ammonia and the inoculation with *Bradyrhizobium* is an indispensable technology in Brazilian soybean production. This technology is used in practically all soybean-producing regions in Brazil and generates an economy of over 15 billion US dollars that would have to be spent with nitrogen fertilizer each season (Hungria and Mendes, 2015; Araújo et al., 2017). It makes Brazilian soybean highly competitive in the international market (Hungria et al., 2005).

The effectiveness of symbiosis between *Bradyrhizobium* and soybean plants depends on the inoculation process carried out by the producers on the farm. For example, seed treatment with fungicides may kill the bacteria in the moment of inoculation and compromise productivity (Zilli et al., 2009; Costa et al., 2013). Given this widespread use of inoculation, Brazilian researchers have sought alternatives to maximize the efficiency of biological nitrogen fixation (BNF), through inoculation methods, compatibilization of fungicides, micronutrients, and other methods (Campo and Hungria, 1999).

The treatment of seeds with fungicides, in addition to controlling important pathogens that are transmitted via seeds, is an efficient practice to ensure adequate populations of plants under soil-climatic conditions unfavorable to the germination and emergence of soybean (Balardin et al., 2011). Water deficiency, in particular, slows the germination and emergence process and lengthens seed exposure to soil fungi (*Rhizoctonia solani*, *Pythium* spp., *Fusarium* spp., *Aspergillus* spp., and others), that may cause seedling decay or death (Embrapa, 2011; Henning et al., 1997).

However, fungicides can result in mortalities of up to 60% after 2 h in contact with *Bradyrhizobium* cells and 95% after 24 hr (Campo et al., 2009). Thus, compatibilizing the application of fungicides and inoculants is necessary to guarantee a higher bacterial population in the seeds and consequently increase nodulation in the roots, thereby increasing biological nitrogen fixation efficiency and crop productivity.

Although inoculation with nitrogen-fixing bacteria is used in most Brazilian soybean crops, seed inoculation at the time of sowing is often described as an activity that reduces planting efficiency due to time spent on inoculation operations. This makes some producers to not use inoculation (Campo and Hungria, 2006). Pre-inoculation may enable faster sowing and increase the use of inoculation, particularly by producers in areas with limited planting time. Pre-inoculation of seed is an alternative method to increase the planting efficiency and new technologies are being developed to inoculant companies in Brazil (Araújo et al., 2017).

Thus, new pre-inoculants that are compatible with fungicide and/or insecticide treatment of seeds can enable faster sowing and increase the use of inoculation. The objective of this study was to compare pre-inoculation of fungicide and insecticide-treated soybean seeds 10 days before sowing with the standard inoculation performed on the sowing day.

MATERIALS AND METHODS

Field experiments

Four experiments were conducted. Two experiments were conducted at the Água Boa Experimental Field (CEAB) (02°40'10.7" N, 60°50'55.8" W) in the municipality of Boa Vista, which is in the Brazilian savanna (Cerrado) ecoregion and has dystrophic Yellow Argisol. One CEAB experiment was conducted in an area without soybean and one in an area with at least two years of soybean cultivation.

Two experiments were conducted at the Serra da Prata Experimental Field (CESP) (02°23'25.3" N, 060°58'59.8" W) in the municipality of Mucajaí, which is in a Cerrado/forest transition ecoregion and has dystrophic Yellow Latosol. One CESP experiment was conducted in an area without soybean and the other in an area with at least two years of soybean cultivation. Both experimental fields belonged to Embrapa Roraima. Chemical characterization of the soil at 0 to 20 cm depth was performed for each area before the beginning of the experiments (Table 1), following the procedures described in Embrapa (1999).

Soil correction was performed two months before soybean planting. Liming was performed with dolomitic limestone. The CEAB area received 1500 kg ha^{-1}, and the CESP area received 2000 kg ha^{-1}, applied by scattering and incorporated via plowing and two harrows. The soil in undisturbed areas with no soybean history at both CEAB and CESP was corrected with 100 kg ha^{-1} P_2O_5 in the form of single superphosphate, 60 kg ha^{-1} K_2O in the form of KCl, and 50 kg ha^{-1} of FTE BR12 Nutriplant (7.1% Ca, 5.7% S, 1.8% B, 0.8% Cu, 2.0% Mn, 0.1% Mo, and 9.0% Zn) as a source of micronutrients.

In addition to correcting the fertilization, a planting fertilization was conducted in the sowing furrow according to that recommended for the region (Embrapa, 2005). It used 100 kg ha^{-1}

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Table 1. Chemical and particle size analysis of soil samples (0-20 cm of depth) of the experiments of soybean pre-inoculation in Roraima, Brazil.

Area	Chemical characteristics							Particle size		
	pH H_2O	Ca	Mg	K	Al	P (Melich)	OM*	Clay	Silt	Sand
		cmolc dm^{-3}				mg dm^{-3}	g kg^{-1}	g kg^{-1}		
CEAB – native Cerrado	5.0	0.22	1.16	0.05	0.29	1.68	12.0	277.7	95.3	627.0
CEAB – cultivated Cerrado	5.2	0.90	0.40	0.05	2.20	17.00	17.0	189.0	71.0	740.0
CESP – native Cerrado/forest transition	4.1	0.13	0.66	0.06	0.92	2.34	33.0	193.6	140.5	666.0
CESP – cultivated Cerrado/forest transition	6.1	6.23	2.16	0.90	0.00	8.15	28.0	200.6	143.5	656.0

*Organic matter.

Table 2. Dose of inoculant and fungicides/insecticides utilized in the experiments of pre-inoculation with soybean in Brazil.

Treatments	Dose of products (mL kg^{-1} of seeds)	Area without cultivation	Cultivated area
		Dose of inoculant (mL kg^{-1} of seed)	
Control (without inoculation)	0	0	0
Standard inoculant	0	4	2
Pre-inoculation + carbendazim	1	6	3
Pre-inoculation + pyraclostrobin + methyl thiophanate + fipronil	2	6	3
Pre-inoculation + Fludioxonil + Metalaxyl-M	1	6	3

of P_2O_5 in the form of single superphosphate and 90 kg ha^{-1} of K_2O in the form of potassium chloride, the latter being applied 50% at planting and 50% at 35 days after germination (Embrapa, 2005). The experimental design for the four experiments was in randomized blocks with five treatments and four replicates. The treatments tested were:

(1) Control without inoculation
(2) Inoculation of untreated seeds with standard liquid inoculant on the day of planting
(3) Pre-inoculation of carbendazim (500 g L^{-1}; Derosal® 500 sc, Bayer S.A.,Brazil) fungicide-treated seeds with liquid inoculant 10 days before planting.
(4) Pre-inoculation of pyraclostrobin (25 g L^{-1}) + methyl thiophanate (225 g L^{-1}) fungicide-treated and fipronil (250 g L^{-1}; Standak Top®, BASF, Brazil) insecticide-treated seeds with liquid inoculant 10 days before planting.
(5) Pre-inoculation of fludioxonil (25 g L^{-1}) + metalaxyl-M (10 g L^{-1}; Maxim XL, Syngenta, Brazil) fungicide-treated seeds with liquid inoculant 10 days before planting.

These fungicides and insecticide were chosen because they are widely used in soybean cultivation in Brazil. Each experimental plot measured 5 m x 5 m, with 10 furrows spaced 0.50 m apart, with a spacing of 1.0 m between plots. The soybean cultivar used was BRS Tracajá, recommended for the state of Roraima, Brazil, with a stand of 14 plants per linear meter (Gianluppi et al., 2001).

At 35 days after germination (at flowering), 10 plants were sampled from each experimental plot (from the central area of second line of each plot). First, the shoots parts were collected and stored in Kraft paper bags. Then, with the aid of a straight blade, the roots were removed along with their nodules, which were stored in plastic bags until arrival in the laboratory. The number of nodules (NN), nodule dry mass (NDM), root dry mass (RDM), shoots dry mass (SDM), and total dry mass (TDM) were evaluated in the soil microbial laboratory of Embrapa Roraima. All dry mass variables were determined by drying the material at 65°C for 72 h.

At 118 days, when the physiological maturation of the soybean was reached, the grains were harvested. For evaluation purposes, the six central rows of each plot were collected, always leaving one meter of border at the head of the rows. Grain yield was corrected to 13% moisture and is expressed in kg ha^{-1}.

Seed treatment

Ten days prior to planting, seed treatment and inoculation of groups 3, 4, and 5 were performed. Seed treatment and pre-inoculation were performed according to manufacturer recommendations (Table 2). For areas with no history of soybean cultivation, the inoculant dose was twice that recommended for cultivated areas, due to the absence of an population of *Bradyrhizobium* that could establish symbiosis with soybean, following the manufacturer's instructions. The seeds were packed in a polyethylene bag with a capacity three times the volume of the seeds. First, the fungicide was added, leaving an equivalent air volume to that occupied by the seeds, closing the bag and mixing with rotary movements to homogenize the distribution of the products. The treated seeds were transferred to a tray to dry for 2 h. The same procedure was used for seed inoculation. After drying, these were packed in a Kraft paper bag and stored at 20 to 30°C for 10 days. The liquid inoculants used in the treatments were Rizoliq® (standard inoculant, lot B01038203) and Rizoliq Top® (pre-inoculant, lot T040239), both supplied by Rizobacter of Brazil. These bacterial inoculants had SEMIA 5079 and SEMIA 5080 (*Bradyrhizobium japonicum*) with declared concentrations of 5.0 x 10^9 (standard inoculant) and 6.0 x 10^9 (pre-inoculant) colony-forming units (CFUs) per mL. On the tenth day after pre-inoculation, field-planting of all treatments was conducted. Inoculation of group 2 (standard inoculant without seed treatment) was conducted on the day of planting according to the manufacturer's recommendation.

Viable cell recovery testing

For all treatments, after field planting, pre-inoculated seed samples were taken to the soil microbiology laboratory of Embrapa Roraima for viable cell recovery testing, following the specifications listed in the Regulatory Instructions No. 30, December 12, 2010 (Brazil, 2010). Samples of 100 seeds (considering the weight of 100 seeds of cultivar BRS Tracajá), were placed in sterile Erlenmeyer flasks with a capacity of 250 to 300 mL, containing 100 mL of physiological solution with Tween 80 (polyoxyethylene sorbitan monolaurate) (8.5 g of NaCl in 1.0 L of distilled water, added to 0.4 mL stock solution of Tween 80). This represented the 10° dilution. The Erlenmeyer flask was placed on an orbital shaker for 15 min at 150 rpm. A 1-mL aliquot of dilution 10° was withdrawn, placed in a sterile flask with 9 mL of physiological solution (0.85% NaCl), yielding the 10^{-1} dilution. This procedure was repeated until the 10^{-7} dilution was obtained. Samples of the 10^{-3} to 10^{-7} dilutions (0.1 mL) were plated in triplicate, by means of the spreading technique, in Petri dishes containing Yeast Extract Mannitol medium with Congo red (0.05%) (Fred and Waksman, 1928). The plates were incubated at 28°C for seven days, and the number of CFUs was estimated (between 30 and 300 colonies). The number of bacteria recovered from the seeds was obtained by the following formula: No. of recovered cells / seed = f x N, where f = dilution factor and N = average number of colonies of the three plates for the selected dilution. The dilution factor is given by the reciprocal of the dilution in the plate multiplied by 10, in the case of inoculation of 0.1 mL (Brazil, 2010).

Statistical analysis

All the evaluated variables were subjected to the tests of normality and homogeneity of the error variance. When these requirements were met and showed significance in the analysis of variance for the F test, the means of the treatments were compared by the Tukey test at the 5% significance level using the Sisvar version 4.3 program (Ferreira, 2011). The data were analyzed jointly to verify the effects of treatments (inoculations), places (Cerrado and Cerrado/forest transition), and interactions between treatments and places in areas with and without a history of soybean cultivation.

RESULTS AND DISCUSSION

No cells were recovered in the control treatment without inoculation, indicating that the seeds were free of *Bradyrhizobium* cells (Table 3). Seeds treated on the day of planting with the standard inoculant showed a higher number of cells per seed than pre-inoculated seeds treated with fungicides and insecticide, with 4.7 × 10^6 CFU seeds^{-1} (seeds intended for areas with no soybean history) and 1.2 × 10^6 CFU seeds^{-1} (seeds intended for areas already grown with soybean) recovered, respectively. These values are in accordance with the recommended inoculant dosage for soybean, which should result in 1.2 × 10^6 cells per seed (Hungria et al., 2007).

However, all products used for seed treatment drastically reduced the number of cells recovered 10 days after inoculation. The pre-inoculated group that received the pyraclostrobin + methyl thiophanate + fipronil showed the lowest number of cells recovered per seed for the two ecosystems (Table 3). These results differ from those obtained by Alcântara Neto et al. (2014), in which the product pyraclostrobin + methyl thiophanate + fipronil had the lowest deleterious effect on cell recovery in the seeds. However, those authors only evaluated for a period up to 48 h. Similar results were observed by Costa et al. (2013), they observed a drop in the concentration of cells after four days of inoculation when using seed treatment products, using similar methods, but with different active principles.

Several studies have demonstrated a reduction in the number of cells recovered in soybean seeds treated with different fungicides and inoculated with standard inoculants (Campo and Hungria, 2000; Campo et al., 2003; Campo et al., 2009; Costa et al., 2013; Alcântara Neto et al. al., 2014). This is the first result demonstrating the recovery of cells in soybean seeds after 10 days of inoculation with three different products to seed treatment used in Brazil, demonstrating that there is a drastic reduction but not total mortality of the cells after this period using an inoculant specific to pre-inoculation. A recent study of soybean pre-inoculation of seeds treated with pyraclostrobin + methyl thiophanate + fipronil and using a bacterial protector showed that after 35 days of inoculation it was possible to recover 1.13 × 10^{-3} CFU seed^{-1} (Araújo et al., 2017). Therefore in pre-inoculation of soybean seeds treated with fungicides/insecticides, specific inoculants with a kind of cell protection must be used.

As for the results of the four experiments under field conditions, the combined data analysis showed a significant interaction between inoculation treatments X places only for the number of nodules in areas with no history soybean cultivation (Table 4). For the experiment in the Cerrado area in the CEAB, the treatment of seeds even with a drastic reduction in the number of cells was sufficient to induce nodulation, with results similar to the standard inoculant on the day of planting. For the Cerrado/forest transition area, the environment was a determining factor for the reduction of nodulation, since all the treatments showed a low number of nodules (Table 4).

For the other variables evaluated, the pre-inoculated and seed treatments did not differ from the standard inoculant treatment performed on the day of planting, and both were superior to the group that did not receive inoculation (Table 5). Although there were no significant differences, all the pre-inoculated treatments showed a

Table 3. Cell concentration of *Bradyrhizobium* in recovery testing in soybean seed treated with different fungicides/ insecticides and pre-inoculated by 10 days before planting.

Treatments	Area without cultivation	Cultivated area
	CFU seed^{-1*}	
Control (without inoculation)	0[2]	0
Standard inoculant	4.7×10^6 A[1]	1.20×10^6 A
Pre-inoculation + carbendazim	1.6×10^5 B	7.0×10^4 B
Pre-inoculation + pyraclostrobin + methyl thiophanate + fipronil	3.0×10^4 C	1.0×10^4 C
Pre-inoculation + Fludioxonil + Metalaxyl-M	3.1×10^5 B	4.6×10^4 BC
CV (%)	3.48	5.64

[1]Means followed of the same letter in the columns do not differ by the Tukey test (5% probability). *Applied base 10 logarithm for data transformation and subsequente analysis. [2]Disregard for purposes of analysis the treatment without inoculation. * CV: Coefficient of variation.

Table 4. Deployment of double interaction of nodule number (NN) from joint analysis of the experiments of pre-inoculation + seed treatments in areas of Cerrado and Cerrado/ forest without history of soybean cultivation.

Treatments of inoculation	NN (n plant^{-1})	
	Areas[1]	
	CEAB – Cerrado	CESP –Cerrado/forest transition
Control (without inoculation)	16.6 B	0
Standard inoculant	48.5 A	4.3
Pre-inoculation + carbendazim	54.7 A	6.9
Pre-inoculation + pyraclostrobin + methyl thiophanate + fipronil	50.9 A	6.9
Pre-inoculation + Fludioxonil + Metalaxyl-M	59.3 A	7.0
CV (%)*	28.43	

[1]Means followed of the same letter in the columns do not differ by the Tukey test (5% probability). * CV: Coefficient of variation.

trend of grain yield higher than the treatment using the standard inoculant. On the other hand, the control, which did not receive seed treatment or standard inoculation, was significantly lower in the number of nodules, nodule dry mass, root dry mass, shoot dry mass, total dry mass, and grain yield (Table 5). These results reinforce the importance of soybean inoculation in areas that were not previously cultivated in Brazil.

We also observed a statistically significant difference between the places (Cerrado and Cerrado/forest transition) (Table 5). The Cerrado area (CEAB) showed more than three times the nodule dry mass as the Cerrado/forest transition areas. The nodule dry mass has a direct correlation with N content (Döbereiner, 1966) and consequently with shoot dry mass and grain yield. The shoots dry mass of plants cultivated in the Cerrado showed an average of 1.9 grams plant^{-1} more than the Cerrado/forest transition area and a yield of grain of 1461.9 kg ha^{-1} higher (Table 5).

In areas where soybean had already been cultivated, there was no significant interaction between treatments and places. In these areas, shoot dry mass, total dry mass, and grain yield differed between treatments (Table 6). All the treatments inoculated showed superior results to the control that received neither inoculation nor addition of nitrogen.

These results reinforce the need to carry out re-inoculation in areas that have previously been cultivated with soybeans, especially in the Roraima Cerrado, where the *Bradyrhizobium* population in the dry season is drastically reduced (Zilli et al., 2013). In the areas without soybean cultivation, the pre-inoculation with seed treatment did not affect the evaluated variables, as they were similar to the treatment with standard inoculant applied on the day of planting (Table 6). Regarding the places, there was also a significant effect on shoot dry mass, total dry mass, and grain yield, being also higher in the Cerrado areas (Table 6). The deleterious effects of fungicide/insecticide application on nodulation and soybean yield have been inconsistent. Some studies have demonstrated a reduction in nodulation in inoculated and fungicide-treated plants in areas with no soybean cultivation history where there is no established *Bradyrhizobium* population (Zilli et al., 2009; Costa et al., 2013).

In soil without a history of soybean cultivation, Costa et al. (2013) demonstrated a reduction of up to 50% in nodulation of plants inoculated and treated with different

Table 5. Nodule dry mass (NDM), root dry mass (RDM), shoot dry mass (SDM), total dry mass (TDM) and grain yield from joint analysis of the experiments of pre-inoculation + seed treatments in areas of Cerrado and Cerrado/ forest without history of soybean cultivation.

Factors[1]	**NDM**	**RDM**	**SDM**	**TDM**	**Grain yield**
Treatments	**mg plant^{-1}**	**-------g plant^{-1}-------**			**kg ha^{-1}**
Control (without inoculation)	140.1 [b]	0.75 [b]	3.77 [b]	4.51 [b]	1784.6 [b]
Standard inoculant	270.7 [a]	1.12 [a]	5.35 [a]	6.48 [a]	3139.6 [a]
Pre-inoculation + carbendazim	243.6 [a]	1.19 [a]	5.09 [a]	6.28 [a]	3375.2 [a]
Pre-inoculation + pyraclostrobin + methyl thiophanate + fipronil	281.7 [a]	1.25 [a]	5.74 [a]	6.99 [a]	3609.3 [a]
Pre-inoculation + Fludioxonil + Metalaxyl-M	320.7 [a]	1.07 [a]	5.27 [a]	6.34 [a]	3187.6[a]
Areas					
CEAB	387.5[a]	1.35[a]	5.99[a]	7.34 a	3750.2 [a]
CESP	115.3[b]	0.80[b]	4.09[b]	4.90 [b]	2288.3 [b]
CV(%)*	43.9	28.4	26.3	26.2	14.3

[1]Means followed of the same letter in the columns do not differ by the Tukey test (5% probability). * CV: Coefficient of variation.

Table 6. Nodule number (NN), nodule dry mass (NDM), root dry mass (RDM), shoot dry mass (SDM), total dry mass (TDM) and grain yield from joint analysis of the experiments of pre-inoculation + seed treatments in areas of Cerrado and Cerrado/ forest already cultivated with soybean.

Factors[1]	**NN**	**NDM**	**RDM**	**SDM**	**TDM**	**Grain yield**
Treatments	**n plant^{-1}**	**mg plant^{-1}**	**-------g plant^{-1}-------**			**kg ha^{-1}**
Control (without inoculation)	46.6	390.0	1.48	6.30 [b]	7.78 [b]	2886.4 [b]
Standard inoculant	51.1	432.8	1.68	7.24 [a]	8.93 [a]	3399.0 [a]
Pre-inoculation + carbendazim	51.0	427.6	1.90	8.09 [a]	9.98 [a]	3354.5 [a]
Pre-inoculation + pyraclostrobin + methyl thiophanate + fipronil	53.2	437.3	1.48	7.86 [a]	9.51 [a]	3322.7 [a]
Pre-inoculation + Fludioxonil + Metalaxyl-M	50.8	430.9	1.47	7.34 [a]	8.81 [a]	3274.1 [a]
Areas						
CEAB	48.1	514.0 a	1.65	7.80 [a]	9.45 [a]	3900.7 [a]
CESP	53.0	333.4 b	1.62	6.93 [b]	8.55 [b]	2594.0 [b]
CV(%)*	9.9	7.7	17.6	13.5	13.1	10.3

[1]Means followed of the same letter in the columns do not differ by the Tukey test (5% probability). * CV: Coefficient of variation.

fungicides. When cultivated in an area where there was an established population of bacteria, that is, areas that had already been cultivated with soybeans, there was no reduction in nodulation in soybean plants grown under greenhouse conditions (Bueno et al., 2003; Costa et al., 2013). Campo et al. (2009) observed a decrease in nodulation and productivity of plants that were treated with fungicides, and these effects were higher in sandy soils. Treatment with pyraclostrobin + methyl thiophanate + fipronil did not affect nodulation compared to control without seed treatment (Alcântara Neto et al., 2014).

Concerning pre-inoculation, there were no negative effects on the development of soybean for up to five days in the Cerrado of Roraima. However, pre-inoculation together with the treatment of seeds with fungicides (carboxin + thiram) reduced the nodulation, nodule dry matter, shoot dry matter, and grain yield (Zilli et al., 2010). Analogously, Zilli et al. (2009) observed a negative effect of the application of the fungicide based on carbendazim + thiram, with a significant reduction (approximately 50%) of the nodulation and more than 20% (approximately 700 kg ha^{-1}) of grain yield in the group inoculated with the strain SEMIA 587.

The negative results of the treatment of inoculated seeds on the day of planting (Bueno et al., 2003; Campo and Hungria, 2000; Campo et al., 2009; Zilli et al., 2009) with inoculation plus seed treatments (Zilli et al., 2010) were both obtained with standard inoculants. However, in this study, a new inoculant developed for the pre-inoculation was evaluated, and despite the reduction in the number of cells recovered in the seeds, these were sufficient to promote the symbiotic efficiency without affecting the productivity. A recent study of pre-inoculation using a bacterial protector in seeds also found a reduction of *Bradyrhizobium* cells, but the nitrogen fixation and productivity in the field were not affected in areas with and without history of soybean cultivation (Araújo et al., 2017). It is a new technology will be a valuable tool to soybean producers in Brazil and worldwide.

Pre-inoculation along with the fungicides and insecticide promoted nodulation, plant development, and grain yield similar to the standard inoculant applied on the day of planting without seed treatment. In the two environments where the evaluations were conducted (Cerrado and Cerrado/forest transition), and in both native and soybean-cultivated areas, a similar response was observed both for the treatments inoculated with the control treatment and for the treatments where standard inoculant and pre-inoculant were applied.

Conclusions

Application of the inoculant to pre-inoculation in seeds treated with fungicides and insecticides reduces the number of cells recovered per seed. Pre-inoculation performed 10 days before planting, along with seed treatment, positively affects soybean crop productivity. The two places tested, a without soybean cultivation and an area already cultivated with soybean, presented highly similar responses.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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