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Full Length Research Paper

24-Epibrassinolid in the biometry of acclimatization to salinity in two cultivars of *Vigna unguiculata* (L.) Walp.

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In order to evaluate the influence of application of brassinosteroid phytohormone in mitigating the effects of salt stress at the height, root growth, leaf area, dry mass of leaf and root, stomatal conductance and transpiration, plants of cowpea bean [*Vigna unguiculata* (L.)Walp, cultivars BR3 Guariba and BRS Tracuateua, they were sown in a greenhouse in the absence and presence of brassinosteroid (24-epibrassinolid) in concentrations (0.2 and 0.4 µM), under diferent concentrations of NaCl (50 mM and 100 mM). The highest concentration of NaCl was 100 mM, affects plant growth. This treatment reduced by 46% the length of the BRS Tracuateua root, and 82 and 50% stomatal conductance and transpiration of BR3 Guariba respectively. However, the effects of salinity have been attenuated by supplementation as phytohormone. Under effect of treatment interaction Br of 0.4 µM and NaCl of 100 mM, root length and dry mass of leaves was increased by 87 and 37% in Guariba and Tracuateua cultivars concomitantly compared to those under stress by NaCl to 100 mM. For the same concentration of NaCl and Br 0.2 µM, there increases of 88% in stomatal conductance BR3 Guariba. It is suggested a possible regulation of 24-epibrassinolid on photosynthetic mechanisms of cowpea plants, in order to change made promoted stomatal conductance, which may have induced the greatest uptake and sequestration of CO_2 thereby allowing the growth processes in plants.

Key words: Brassinosteroids, expansion and development, *Vigna unguiculata* (L.) Walp.

INTRODUCTION

The caupi bean [*Vigna unguiculata* (L.) Walp.] belongs to the legume family (Fabaceae), subfamily Papilionoidea (Faboideae). It features rustic genotypes adapted to soils with low fertility and drought, but still express significant

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(production standards, which is an alternative low risk to grain crops in regions with latent edaphoclimatic problems (Correa et al., 2012).

In 2014, world production of this species amounted to approximately 25 million tonnes (FAO, 2016). In the same period, the area harvested in Brazil totaled 3.1 million hectares in the North and Northeast of the country, stand out as the top producers. For the same year, in the state of Pará, the area planted with cowpea amounted to 41,300 ha (IBGE, 2016).

However, the productivity of the culture is affected by various biotic and abiotic factors. Among the abiotic factors, salinity is what promotes the greatest metabolic and nutritional disorders (Hasegawa, 2013). According to Maas (1986), although this species is classified as moderately tolerant to salinity by presenting a threshold of saturation 4.9 dS^{-1}, excess of Na^+ cytosolic affects the activity of enzymes and proteins, which may impair the transport of water and minerals to the plants resulting from the lower osmotic potential of the soil, potentially toxic ions, particularly Na^+ and Cl^- interfere with the assimilation of nutrients and plant growth, in view of the effect of competition by the same transport membrane between these essential ions and essential elements (Aragão et al., 2011; Calvet et al., 2013).

These ions promote decrease in the potential of turgor of the plant, which is essential for plant growth. One of the primary effects of salt stress is the reduction of leaf area, due to the ionic or osmotic effect or both, promoted by the excess of salts (Munns and Tester, 2008; Silveira et al., 2010; Hasegawa, 2013). As a result, there is significant reduction in transpiration and stomatal conductance, which appear as a defense mechanism of plants to lower osmotic potential.

As a result of excess salts occur, we decrease in CO_2 assimilation which reflects in lower photosynthetic rates and synthesis of carbohydrates (Calvet et al., 2013). Accordingly, the carbon skeletons to be used for the growth processes, synthesis of organic compounds and photosynthate translocation, are diverted to the power plant maintenance, promoting decreases in leaf and root dry weight and plant height (Bezerra et al., 2010; Lacerda et al., 2011; Silva et al., 2013).

In this regard, scientific researches indicate that a number of hormones are involved in modulating plant response to stresses such as those induced by NaCl (Sharma et al., 2007). Thus, brassinosteroids are a new class of phytohormones with esteroídicapolioxigenada structure, with pronounced regulatory activity growth, which seems to be related to its role in antioxidative mechanism of plants, as well as their involvement in the metabolism of carbohydrates (Rao et al, 2002; Zullo and Adam, 2002).

Thus, this research aims to evaluate the changes in the growth biochemistry cowpea plants exposed to increasing levels of NaCl, and analyze the modulation mechanisms of brassinosteroid in which gives the acclimatization of this species to salinity.

MATERIALS AND METHODS

The experiment was conducted in a greenhouse belonging to the Rural Federal University of Amazonia-UFRA, Belém, Pará, located at 01° 28'03 "S; 48° 29'18 "W, in the period July-August 2015, the physiological and biochemical analyzes were performed on Biodiversity Studies Laboratory in Higher Plants (EBPs) belonging to the Institute of Agricultural Sciences (ICA) of the university campus.

In this study, we used two cultivars of cowpea bean [*V. unguiculata* (L.) Walp] being BRS Tracuateua and BR3 Guariba, susceptible and tolerant to salt stress, respectively. The seeds were acquired from the Germplasm Bank of the Brazilian Agricultural Research Corporation - EMBRAPA Eastern Amazon.

Seeding and experiment conduction

Five cowpea seeds were sown in each prolipropilenom cup with volumetric capacity of 300 ml, containing sand autoclaved and moistened with distilled water, and each of them was sown a cultivar. Seven days after sowing (DAS), at which time the plants have launched the first pair of leaves, cowpea seedlings were transplanted cups of polypropylene for vessels with 1000 ml capacity, containing in its base 500 ml of nutrient solution Hoagland and Arnon (1950), with ionic strength ¼.

The nutrient solution was replaced daily during the hours of 8:00 and 17:00, according to the need of absorption of seedlings and the reduction solution by evaporation. The pH of the solution was maintained at a range of 5.5 ± 0.5. At 12 DAS when the cowpea plants released third leaf nutrient solution was changed to ½ of its original concentration and at that time, treatment was initiated with NaCl concentrations (50 and 100 mM) levels, brassinosteroids of the (0.2 and 0.4 µM) and control (without brassinosteroids and NaCl). The plants remained for 12 days under the effect of these treatments.

Collection of plants and growth variables

The collection of plants occurred at 24 DAS to 06:00. At that time, was determined biometrics plants: height, stem diameter, root length, number of leaves, leaflets and leaf area. Gas exchange, transpiration and stomatal conductance were also determined even in the greenhouse through a portable porometer to obtain the data evaluated. The different parts of the plant, leaf, stem and root were collected for the determination of dry matter (DDM).

The biometric variables were determined with the aid of a centimeter ruler, determining plant height and root length. Using a digital caliper ZAAS Precision model obtained if the diameter of the neck of the plant height, number of leaves and leaflets was determined by manual counting.

The dry matter was determined by separation of plant leaves, stem and root, and then were taken to dry in air oven of forced air at a temperature of 65 ± 5 ° C for 48h. After drying, the material was weighed on analytical balance, ground in mill to obtain a fine and stored in falcon tubes powder. The leaf area was determined by scanning the leaves with your area designed by Jimage program.

Determination of physiological variables

The stomatal conductance (gs) and transpiration (E) of cowpea

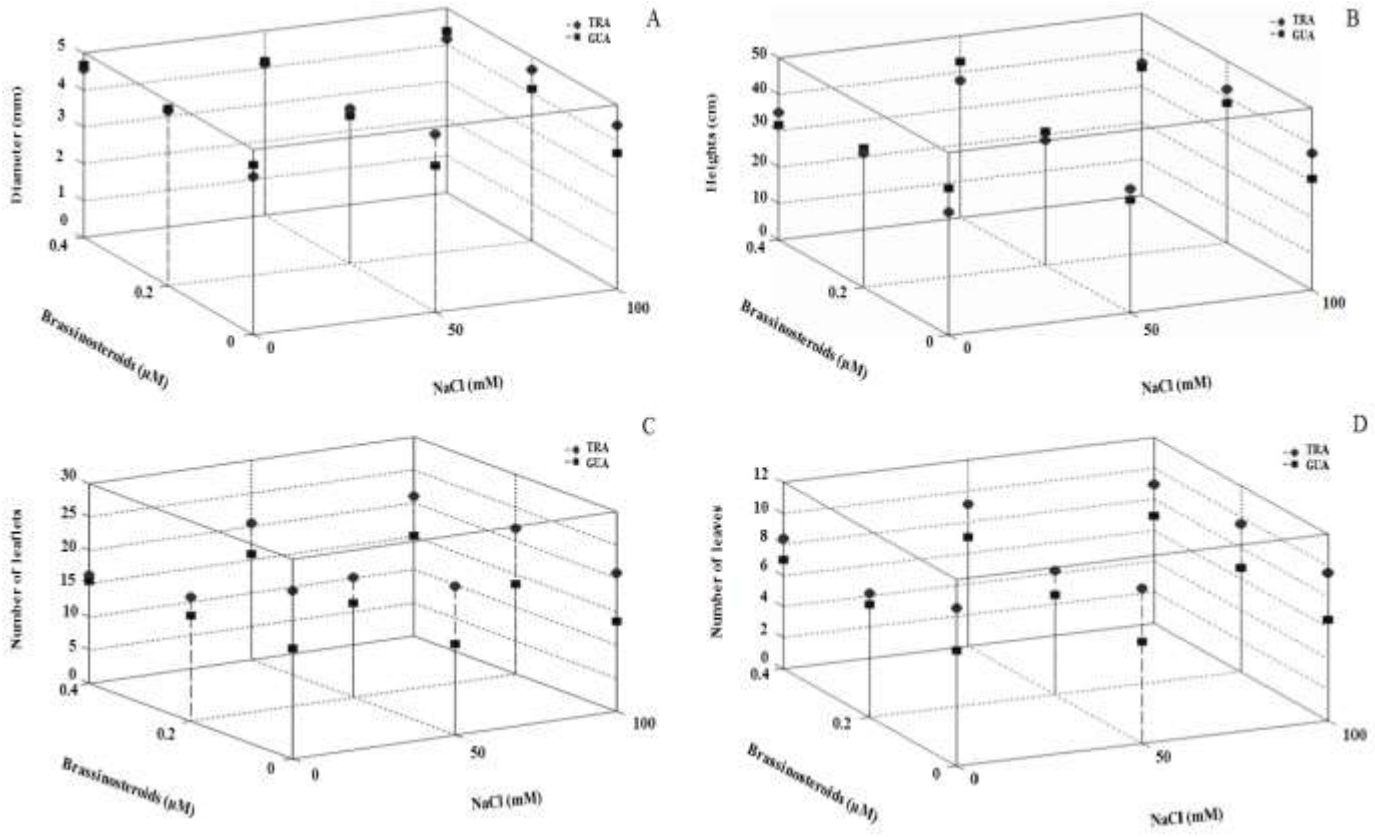

Figure 1. Diameter (A), heights (B), number of leaflets (C) and leaves (D) in cultivars BRS Guariba and BR3 Tracuateua of *Vigna unguiculata* (L.) Walp grown in nutrient solution as a function of increasing concentrations of brassinosteroids (Br) and NaCl.

plants were determinated in the morning on time from 9:00 am to 11 am. To obtain the data used a portable porometer dynamic equilibrium (MOD. Li 1600 liquor, Nebraska, USA). Measurements were performed with fully expanded leaves, selected from the third pair of leaves counted from the apex to the base.

Experimental design and statystical analysis

The design was completely randomized (DIC) in factorial scheme 2 x 3 x 3, two cultivars of cowpea (BRS Guariba Tracuateua and BR3), three levels of brassinosteroids with concentrations (0.2 µM and 0.4 µM) and with three levels of salinity concentrations (50 mM and 100 mM) yielding 18 treatments and 4 replicates, totaling 72 experimental units, each containing two plants per pot. Statistical results were submitted to analysis of variance (ANOVA) by Sisvar program version 5.4, where the averages were compared by Tukey test level of 5% probability.

RESULTS

Stem diameter, Plant height, number of leaflets and leaves

In this study, we sought to evaluate the effect of brassinosteroids in height, stem diameter, number of leaves and leaflets bean plants cowpea subjected to salt stress and treated with phytohormone (Figure 1). Analysis of variance showed that, in effect interaction between the Br concentrations and NaCl, there was a statistically significant difference ($p < 0.05$) for all the variables mentioned except for stem diameter for which it was not observed significant increases ($p > 0.05$) under the effect of such treatment on both cowpea bean cultivars analyzed (Figure 1).

The height of the cultivars BR3 Guariba and BRS Tracuateua was 52 and 64% concomitantly higher plants under stress by 50 mM NaCl, Br 0.4 µM when treated with saline and the same level (Figure 1 B). However, the isolated treatment with the highest concentration of phytohormone (0.4 µM), did not guarantee greater plant height. Under unique effect of this treatment, the height of Guariba and Tracuateua cultivars was 28 and 13% lower, respectively, those under treatment interaction with the highest dosage of the 24-epibrassinolide and the median concentration of NaCl (50 mM).

The number of leaflets was significantly increased ($p<0.01$) by 69 and 32% in BR3 Guariba and BRS Tracuateua, respectively, when under stress 50 mM NaCl, supplemented with the highest concentration of the phytohormone in relation to plants under only saline treatment at 50 mM (Figure 1C). For the number of

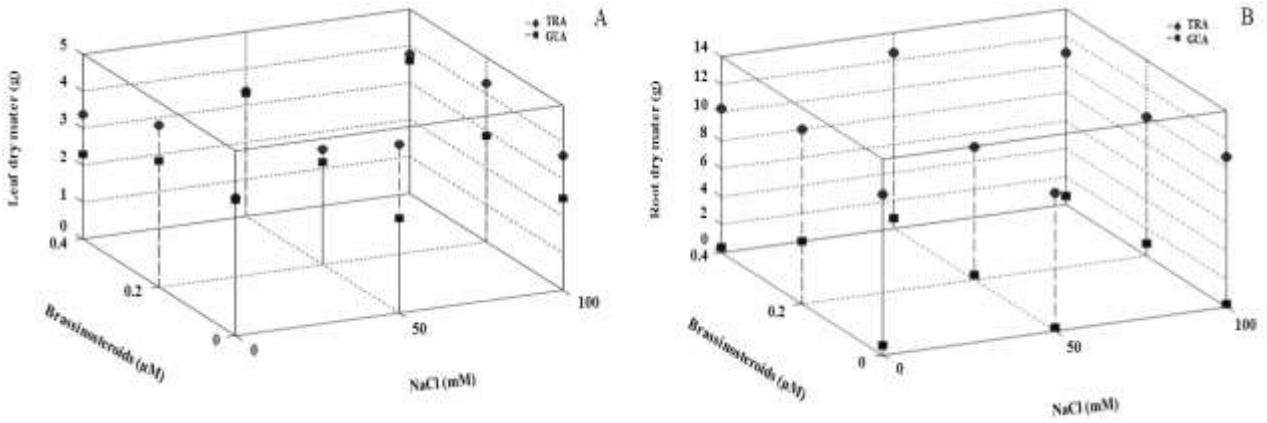

Figure 2. Leaf dry mater (A), Root dry mater (B) in cultivars BRS Guariba and BR3 Tracuateua of *Vigna unguiculata* (L.) Walp grown in nutrient solution as a function of increasing concentrations of brassinosteroids (Br) and NaCl.

sheets, there was a similar increase of 62% in this variable in bean cultivars cowpea under effect of the treatment cited interaction and was no statistical difference ($p>0.05$) between control plants and those supplemented only with Brassinosteóides concentrations of 0.2 µM and 0.4 µM (Figure 1 D).

Dry weight of shoot and root

Salinity promoted significant decrease ($p<0.05$) in the dry matter of shoot and root in cowpea plants. The Guariba and Tracuateua cultivars when treated with 100 mM NaCl, showed a reduction of 49 and 40%, respectively, in dry mass (Figure 2).

Under the same salt treatment, the dry root mass of the growing Guariba kept constant, while that for the BRS Tracuateua, that same organ, there was a 20% decrease in the measured variable (Figure 2B). Thus, the latter cultivar sensitive to excess salts, was considerably affected by incremental increases in NaCl concentration. Only in isolated effect with the brassinosteroid 0.2 µM, there was 75% increase in root dry weight of the plant variety Guariba.

However, concentrations of 24 epibrassinolídeos partially reversed negative effects of salinity on the variables under consideration. Under treatment interaction 0.4 µM and 100 mM NaCl, there was a significant increase ($p <0.05$) of 37% and 33% of dry weight of shoot and root for BRS Tracuateua compared to plants under stress by NaCl to 100 mM (Figure 2A, B). However, for this same cultivar, higher increases in root biomass 66% occurred under the effect of interaction treatment 0.2 µM and 50 mM NaCl as compared to saline treatment plants exclusively to 50 mM; thereby indicating that the lower concentration of the hormone and the lowest salt level ensured higher production biomass at the root.

Root length

In this research, it is evident that the greater salt concentration, significantly reduced ($p <0.01$) in 46% of the root length cultivate Guariba, in relation to their control, indicating that this crop was the most affected by salt stress, in relation to BRS Tracuateua. For the latter variety, the stress 100 mM NaCl, did not cause significant changes in root growth compared to those cultured in the absence of NaCl (Figure 3).

The deleterious effects of salinity were partially reversed by concentrations of 24-epibrassinolídeos. There were significant increases ($p <0.05$) 87 and 25% in root length cultivar howler when maintained under stress by 100 mM NaCl, and treated with Br to 0.4 µM to 0.2 µM concurrently in relation those maintained only under stress with the highest salt concentration used in this research.

Surprisingly, the root growth 62% was observed to grow Tracuateua when treated with the lowest dose of the phytohormone 0.2 µM and higher salt level 100 mM grating those treated with 100mM NaCl. The results of this study show that moderate salt levels NaCl to 50 mM, and the isolated treatment with Br concentrations of 0.2 µM and 0.4 µM, kept the root length of both cultivars with values very close to showing that such treatments did not interfere in biochemical processes essential to the growth and development of this species. Thus, as salt concentration, it proved ineffective in promoting negative changes in root length.

Leaf area

Gradual increases in the concentration of brassinosteroids did not promote significant increases ($p> 0.05$), leaf area of Guariba cultivar compared to the respective control. Conversely, for BRS Tracuateua there

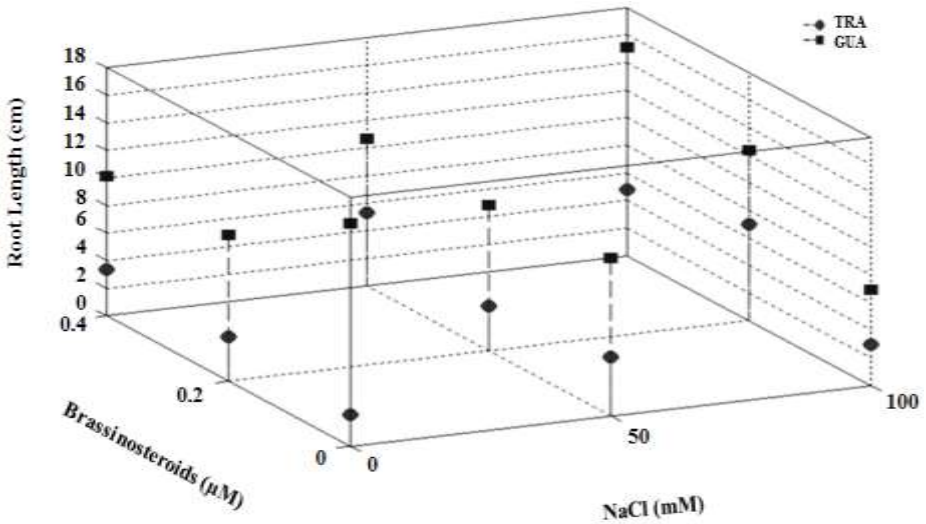

Figure 3. Root length in cultivars BRS Guariba and BR3 Tracuateua of *Vigna unguiculata* (L.) Walp grown in nutrient solution as a function of increasing concentrations of brassinosteroids (Br) and NaCl.

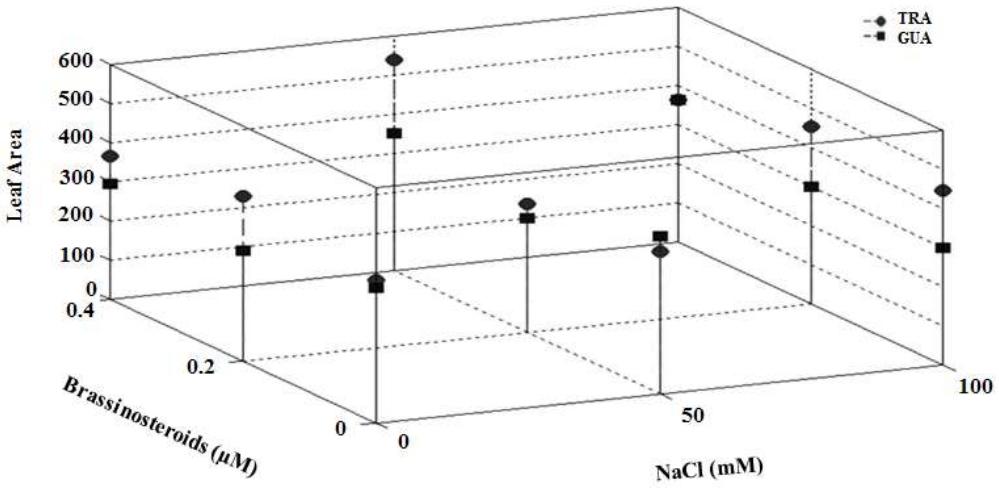

Figure 4. Leaf Area in cultivars BRS Guariba and BR3 Tracuateua of *Vigna unguiculata* (L.) Walp grown in nutrient solution as a function of increasing concentrations of brassinosteroids (Br) and NaCl.

were significant increases (p> 0.01) of 180 and 220% when treated with Br to 0.2 µM and 0.4 µM, respectively, compared to plants cultivated in the absence of this hormone (Figure 4).

However, under 0,4µM treatment interaction effect and 50 mM NaCl leaf area was significantly increased (p <0.01) by 47 and 28% in BR3 Guariba and BRS Tracuateua concurrently from the plants under stress 100 mM NaCl. It is noteworthy that the salt stress did not significantly affect the variable in question, which can be attributed to the time when the plants were subjected to stress.

Isolated concentrations of 50 mM NaCl and treatment of interacting Br 0.2 µM and 50 mM NaCl, induced similar responses in growth variable studied. It is considered in this respect that the low concentration of hormone was not sufficient to promote changes in physiology and biochemistry growth of both cultivars.

Stomatal conductance and transpiration

For physiological variables stomatal conductance and transpiration, there was a significant interaction effect (p

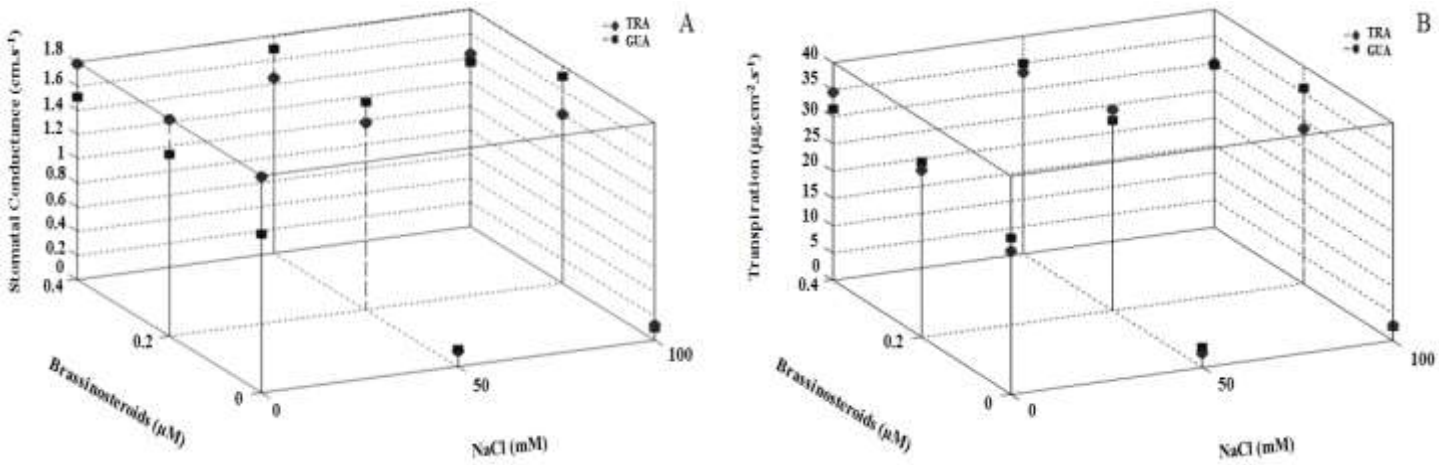

Figure 5. Stomatal conductance (A) and Transpiration (B) in cultivars BRS Guariba and BR3 Tracuateua of *Vigna unguiculata* (L.) Walp grown in nutrient solution as a function of increasing concentrations of brassinosteroids (Br) and NaCl.

<0.01) between the saline levels and phytohormone concentrations used (Figure 5). The higher salt concentration (100 mM), reduced by 66% and 82% stomatal conductance and 37.5% and 50% perspiration of Guariba and Tracuateua cultivars in relation to their control.

However, the dosages of the hormone, mitigated the salt stress, which was evidenced by the increase in the variables analyzed in both cultivars when under treatment effect interaction with NaCl to 100 mM and level of phytohormone Br to 0.2 µM. Under the effect of this treatment, particularly cultivating guariba presented increments of 88% in stomatal conductance and transpiration of 83% compared to plants maintained only with the highest salt concentration (Figure 5B).

For BRS Tracuateua, there was no statistically significant difference (p> 0.05) in stomatal conductance control plants and those under treatment effect interaction analysis. However, for this cultivar, perspiration was increased by 83% in plants under stress 100 mM NaCl and treated with Br to 0.2 µM.

DISCUSSION

Salinity changes the water balance in the plant, which is assigned to its ionic, or both osmotic effect, which results in less water availability in plant tissues. The immediate consequence of this process is to reduce the potential turgor essential to expansion and cell growth (Munns and Tester, 2008; Hasegawa, 2013).

In this research, it became clear that the high salt concentrations negatively affected the growth variables of cowpea plants, especially in regard to height, biomass and root length, which was most evident in the growing Tracuateua, sensitive salt stress.

According to Silva et al. (2009), NaCl stress affects differently the growth variables, suggesting that its deleterious effects are not evenly distributed among the various organs of plants. In fact, in this study, the roots were considerably more affected by salinity.

From the results the high salt concentrations, have promoted ionic changes, resulting in metabolic and nutritional disorders, reflecting in reduced root growth. According to Aragão, et al., (2011) high levels of Na+ inhibit the absorption of K+ ions, which is involved in the activity of cytosolic enzymes that participate, albeit indirectly, in growth processes in plants.

Thus, the competition Na^+ / K^+ for binding sites on the membrane leads to less absorption of the latter root nutrient salt conditions. Thus, it is considered that something similar has occurred in this study, resulting in less root growth of cowpea.

Some studies suggest that brassinosteroids act stimulating root growth, or still may inhibit it (Núñez, 2008; Borcioni and Negrelle, 2012). In fact, it is noted from this research, the effects of salinity were mitigated by concentrations of 24-epibrassinolídeos being observed increases in dry weight of leaves and roots.

To Araujo et al. (2010), reduction of dry mass production, are mainly associated to the toxic effect of ions such as Na^+ and Cl^- to net carbon fixation and consequent production of assimilates. As adaptation to salt stress mechanisms, some species accumulate organic solutes in the vacuole, to balance the salts pressure in the cytosol which ensures the maintenance of the water status of the plant and the efficiency of the photosynthetic apparatus (Willadino et al., 2011; Hasegawa, 2013).

The higher dry matter production both shoot dry mass and root of Guariba and Tracuateua cultivars under stress and supplemented with the phytohormone, is due to a possible involvement of this in osmorreguladores accumulation as free proline in the vacuole.

This assertion appears to be plausible given that, in works developed by Maciel et al. (2012) and Guedes. - Filho et al, (2013), confirm the osmoregulator role of brassinosteroids, stimulating the accumulation and compartmentalization of inorganic solutes in the vacuole and organic solutes in the cytoplasm. This process maintains the salt concentration inside the cells at low levels and, thus, excess salts not interfere with the hydration of proteins and the biochemical metabolism of plants.

Thus it is believed that stomata and photosynthetic pathways procedures may have been changed by the phytohormone inducing greater uptake of CO_2 from which organic compounds are produced essential to plant growth resulting in higher biomass production.

In this respect, it appears that the osmotic adjustment in plants under stress by NaCl, assigned to the 24-epibrassinolídeos restored growth pathways which could be confirmed by increases in dry weight and height of cowpea bean cultivars studied here.

Authors such as Silva et al. (2007) and Monteiro et al. (2014), show that the increase in the concentration of 24-epibrassinolid in plants sensitive to abiotic stress, induce significant increases in growth variables, thus corroborating with the results found in this research.

Several studies claim that inhibition of growth in height by excess NaCl is due to increased power offset for the maintenance (Garcia et al., 2010; Ashraf and Harris, 2013). The decrease in this variable may reflect the metabolic energy cost associated with the adaptation to salinity and reducing the carbon gain (Garcia et al, 2010; Ashraf and Harris, 2013).

Thus, the increments in time arising from 24 epibrassinolids modulation may be due to its probable regulation in carbon metabolism and nitrogen in plants in favor of the latter, which appears to be consistent in order that this study did increases in conductance stomatal plants under stress and treated with the phytohormone, which probably influenced to higher CO_2 assimilation.

Authors like Larré et al. (2011), claim that the action of the hormone in the highest growth induction can in part be explained, too, for their involvement in the modification in the structure and permeability of cell membranes of plants on stress by NaCl.

It is suggested thereby that the phytohormone modulated such changes with a view to their possible involvement in the antioxidant system, working in greater regulation of the photosynthetic machinery of cowpea plants. The exact understanding of what major routes the phytohormone set initially is not well defined, but it can be inferred from this study, supplementation with brassinosteroids accelerated the synthesis of protein and increased net CO_2 assimilation, these processes are essential to plant growth, which could be observed in both legume cultivars analyzed.

Our results show were consistent in that under hormonal effect, leaf area was significantly increased, which is an important growth parameter because it shows the size of the photosynthetic apparatus, which determines the dry matter accumulation, plant metabolism, the ability photosynthetic potential. Thus, justifiable increments observed in plant height, stem diameter and the biomass production under the effect of 24-epibrassinolide.

Conclusion

The deleterious effects of salinity are minimized by the 24-epibrassinolid in cultivating sensitive to salinity. The increases in growth were more significant variables in plants under stress and 100 mM NaCl supplemented with 0.2 µM BR, which may result from the regulation of phytohormone on carbon and nitrogen metabolism in plants in favor of the latter.

The deleterious effects of salinity on the height and dry matter production of shoots and roots, both BR3 Guariba and BRS Tracuateua were reversed by concentrations of 24-epibrassinolid, assuming the involvement of brassinosteroids on photosynthetic matabolism plant, in view to greater stomatal conductance, which can guarantee the highest CO_2 assimilation in inducing these variables increases.

Conflict of Interests

The authors have not declared any conflict of interests.

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Chemical attributes of Oxisol under different tillage systems in Northeast of Pará

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The different tillage systems interfere with soil chemical attributes mainly due to the site of preparation techniques. The aim of this study was to determine, in two time evaluation periods, the changes in soil chemical attributes affected by three tillage systems in Yellow Oxissol. The experimental design consists of a randomized block design, with split-plot (Soil Tillage systems X Depths samples) with 3 repetitions in two evaluation periods (2009 and 2012). The treatments consisted of three tillage systems, being Conventional Tillage (CT); No-Tillage (NT) and Reforestation with Paricá (RP). The two depths sampled were 0-0.1 m and 0.2-0.3 m. The attributes were evaluated as pH, organic matter, macronutrients levels, exchangeable acidity and micronutrient level. The soil tillage systems significantly affected the soil chemical attributes. In the NT system, the chemical attributes Ca, Mg, MO, P, K, Mn and Zn are concentrated on the most superficial layer of the soil, whereas in the CT there is a distribution of these variables along the topsoil.

Key words: Macronutrients, conventional tillage, no-tillage, reforestation

INTRODUCTION

The tillage system is considered the most important soil management system for the sustainability of Brazilian agroecosystems (Caires et al., 2008; Crusciol et al., 2010). The practice of no-tillage provides the concentration of nutrients in the upper layers of the soil (Reddy et al., 2009; Cunha et al., 2011). In this respect, it is found that the soil mixing may provide the standardization of nutrients in the soil profile. However,

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Table 1. Usage history and localization of the tillage systems in Yellow Oxissol in the Northeast of the State of Pará, Brazil.

Symbol	Historic
CT	Use intensive mechanization, plow and disc harrow graders with disc harrow and leveling grader carried out annually at the time of site preparation for planting of grain (soybean and corn).
NT	Initiated in the harvest 2004/2005 with rice crops sequence (*Oryza sativa* L.) between 2006 and 2007 by the usage of corn crop (*Zea mays* L.) and winter maize from the crop 2008/2009.
RP	In 2004 was implemented the reforestation experiment with paricá (*Schizolobium amazonicum* Huber ex Ducke). Prior to the installation of the experiment, the site was submitted to plowing and harrowing with lime application at a dose of 1.5 t ha^{-1}.

CT: Conventional Tillage, NT: No-Tillage and RP: Reforestation with Paricá.

there are still a few experiments of the evaluation of soil chemical attributes in no-tillage on the long-term.

In this way, the knowledge of the changes caused in the soil chemical attributes by the use of crop rotation and continued no-tillage with the use of occasional plowing, may provide a better understanding of these changes occurring in the soil and may result in more efficient use of nutrients for subsequent crops (Crusciol et al., 2010). Nascente et al. (2014) assessing the changes in chemical attributes affected by soil management in alternate no-tillage with conventional tillage and crop rotation, noted that the no-tillage system with periodic plowing provide smoother distribution of nutrients in the soil profile and crop rotation provides significant increases in soil fertility.

Pereira et al. (2009) assessing the chemical attributes of a dystrophic cohesive Oxissol observed that the tillage systems do not influence the pH in the depths of 0-10, 10-20 and 20-30 cm. They also noted that with the increased sampling depths, the variables pH; OM, K, Ca + Mg and base saturation significantly reduce their values, with an increase to the variables H + Al and aluminum saturation.

The aims of the study were to evaluate the effects over the years of soil management systems in the chemical attributes of an Oxisol.

MATERIALS AND METHODS

The experiment consisted of two chemical analyzes of the soil, with the first chemical evaluation in the year of 2009/2010, while the second in the year of 2011/2012, in Tailândia city, State of Pará, Brazil, Mesoregion Northeast of Pará, on Company property site G. M. Sufredini Ltda. (2° 36' and 3° 24' south latitude and 48° 58' and 48° 33' west longitude).

The regional climate follows the Köppen climate classification, Am typ, it is characterized by having average temperature of the coldest month always above 18ºC featuring two well-defined periods of rainfall: a significantly marked by heavy rains (January to May); and another characterized by a warmer and less rainy season (June to December).

The agricultural and forestry production systems assessed were implemented in earlier times to the data collection, in sites with slopes below 2%, where the natural vegetation (Subperenifólia tropical forest) has been removed in the late 80s. From beginning of the 90s up to the implementation of soil tillage systems studied, the sites were being cultivated with grass (*Brachiaria brizantha* cv. Marandu) pastures under crop-livestock with a low investment management. The preparation of sites for the implementation of the systems evaluated were carried out by means of subsoiling operations, followed by disc harrow and two strides with grid graders to even the land.

During this same period, following the subsoiling operations, limestone has been applied and incorporated with passages of grader bars / graders. Both dosages used in liming as corrections and/or adjustments of fertilization were calculated on the basis of the results of soil analysis. Table 1 shows the land use history of the sites studied.

The soils were classified as dystrophic Yellow Oxissol, medium texture (Embrapa, 2013). The particle size determination, organic carbon content and textural classification in the depths of 0.0-0.1 and 0.2-0.3 m prior to the installation of the experiment (Table 2).

The experimental design consisted of a randomized block design with split-plot (Soil tillage systems x depths sampled) with 3 repetitions in two evaluation periods (2009 and 2012). The treatments were three soil tillage systems: a) Conventional tillage – CT; b) No-tillage – NT; and c) Reforestation with Paricá – RP, in which is not to mobilize the soil annually for the establishment of crops, and the sub-plots samples collected from the depths 0-0.1 and 0.2-0.3 m. Each plot showed dimensions of 10 x 30 m (300 m^2) and were arranged in the north-south direction, with the blocks of a size of 2 ha.

To determine the chemical attributes initially soil samples were collected and properly stored and then sent to a laboratory for chemical analysis. Soil pH was determined potentiometrically using the ratio 1:2.5 (soil:water); exchamgeable calcium (Ca), magnesium (Mg), and aluminum (Al) were extracted with a solution of potassium chloride (KCl) 1 mol L^{-1}; potassium (K) and phosphorus (P) were extracted by Mehlich 1; potential acidity (H + Al) was extracted with a solution of calcium acetate using the methodology proposed by Donagema et al. (2011); and organic matter (OM) was obtained by colorimetry according Raij et al. (2001). The copper, iron, manganese, and zinc levels were determined in aliquots containing Mehlich solution (HCl 0.05 M+H_2SO_4) in soil:extractor relation of 1:5; using atomic absorption spectrophotometry using atomic absorption spectrophotometry for the reading of the samples (Donagema et al., 2011).

The results were submitted to analysis of variance and when significant by the F test at a level of 1 and 5%, the treatment means were compared by the Tukey test at 5% probability. For the procedure of statistical analysis was used the Sisvar software (Ferreira, 2011).

Table 2. Particle size distribution, textural class and organic carbon (OC) soil under conventional tillage (CT), no-tillage (NT) and reforestation with Paricá (RP) in the depths of 0.0-0.1 and 0.2-0.3 m.

Tillage systems	Silt	Clay	OC	Textural class
		(g kg^{-1})		
		0.0-0.1 m		
CT	130	201	22	Sandy clay loam
NT	149	354	23	Sandy clay loam
RP	212	310	27	Sandy clay loam
		0.2-0.3 m		
CT	117	245	16	Sandy clay loam
NT	91	392	16	Sandy clay loam
RP	138	321	20	Sandy clay loam

Particle size analysis was conducted by the pipette method and the organic carbon content by the Walkley-Black method (Embrapa, 1997). The textural classification stated as a textural triangle.

RESULTS AND DISCUSSION

pH, organic matter and macronutrients in soil

It appears that the higher pH values are found in the CP system in both depths and in the years 2009 and 2012 (Table 3). In tillage systems with higher soil mobilization, for example using heavy grade in the soil mixing, there is an increase of pH in depth due to the addition of limestone and crop residues from the soil surface (Falleiro et al., 2003; Fageria, 2009). According to Nascente et al. (2014), in a general way the lower pH soil values are found in the tillage systems with slighter soil tillage, especially in the NT system continuing into deeper soil layers, because Caires et al. (2008) emphasize that in the NT system the practice of liming is carried out on the soil surface without addition, which provides further slow reactions in the soil profile.

Generally speaking, there is no large variation in pH with increase of the depth in the three soil tillage systems, yet the RP was the system that had the lowest pH values in both depths and assessment periods. Falleiro et al. (2003) have obtained different results to the present study, observing higher pH values in the surface soil layer decreasing with depth. Pereira et al. (2009) assessing different tillage systems in Yellow Oxissol obtained pH values of 6.3, 6.31 in the depth of 0.0-0.1 m and 4.61, 4.58 in the depth of 0.2-0.3 m for the CT and NT systems, respectively, being higher than those found in this study in both periods assessed.

The OM content in both depths is higher in the RP system for the year 2009, whereas in the year 2012 the highest values are found in the NT system (Table 3). It is noted further that the higher OM content are in the depth of 0.0-0.1m in the three soil tillage systems. Such a fact may be related to the conditions of the region with hot and humid climate, average temperatures above 30ºC which favor the rapid mineralization of the organic material (D'Andréa et al., 2004).

Results similar to those of the present study were obtained by Bayer and Bertol (1999), Silveira et al. (2000) and Falleiro et al. (2003), in which obtained higher OM content in the surface layer, due to the no soil mixing and residence within crop residue on the surface.

The NT system shows the highest values in surface of OM, P, K, Ca and Mg (Table 3). De Maria et al. (1999) have attributed to the buffering characteristics of OM and/or to the increase of the ionic strength of the soil solution, by the increase in the levels of Ca^{2+}, Mg^{2+} and K^{+} in the surface soil layer, the values pH situated in the range of 5.0 to 6.0, considered ideal as the availability of nutrients to plants.

The soil tillage systems have effects on the OM which change directly and indirectly the chemical, physical and biological attributes of soil (Bayer and Bertol, 1999). The same authors also observed that the degree of soil mixing in the practice of the preparation modifies the manner of distribution of the organic material, where the CT becomes this relatively uniform distribution in the arable layer (0.0-0.2 m). Rossetti et al. (2013) studying the organic matter content in different tillage systems have also observed that the higher content of OM lie in surface layers and that in the CT system, there is a decrease in these levels over time due to the action of microbial decomposition, combined with increasing aeration, soil temperature, fractionation and mixing of plant residues to the soil caused by this management system.

In the NT system, the P content in the layer 0.0-0.1 m were higher at other systems in the year 2009 (5.40 mg dm^{-3}) and 2012 (4.73 mg dm^{-3}). Merely RP showed an increase in depth for the content of P in the year 2012 (Table 3). Among the systems evaluated, in both depths and in the two evaluation periods, the NT showed the highest P content in the soil. This result confirms those founded by other authors (Bayer and Bertol, 1999; De

Table 3. Chemical properties at two depths of a Yellow Oxissol submitted to different soil tillage systems in two evaluation periods, Tailândia-PA.

Tillage systems	pH (H_2O)		OM (g Kg^{-1})		P (mg dm^{-3})		K (mg dm^{-3})		Ca ---- ($cmol_c$ dm^{-3}) ----		Mg		Tillage systems	pH (H_2O)		OM (g Kg^{-1})		P (mg dm^{-3})		K (mg dm^{-3})		Ca --- ($cmol_c$ dm^{-3}) ---		Mg	
0.0-0.1m																								
2009													**2012**											
CT	6.0	aA*	37.9	cA	4.83	abA	38.0	bA	3.04	bA	0.76	abA	CT	5.5	aB	30.9	cA	3.19	bA	26.0	bB	3.36	bA	0.59	bB
NT	5.6	bA	39.8	bA	5.40	aA	47.0	aA	3.99	aA	0.89	aA	NT	5.6	aA	58.4	aA	4.73	aA	63.0	aA	4.12	aA	0.83	aA
RP	5.0	cA	47.1	aA	4.16	bA	31.0	cB	2.96	bA	0.57	bA	RP	5.1	bA	47.8	bA	2.41	cB	28.0	bA	2.13	cA	0.53	bA
0.2-0.3m																								
CT	5.9	aA	27.8	bB	3.18	bB	35.0	aB	2.99	bA	0.66	bA	CT	5.9	aA	26.5	cB	3.41	abA	36.0	aA	4.02	aA	0.78	aA
NT	5.7	aA	28.4	bB	4.86	aB	35.0	aB	3.96	aA	0.79	aA	NT	5.6	bA	48.8	aB	3.65	aB	36.0	aB	3.98	aA	0.76	aA
RP	5.2	bA	34.5	aB	3.10	bB	34.0	aA	3.02	bA	0.68	bA	RP	5.1	cA	39.9	bB	3.18	bA	26.0	bA	3.03	bA	0.58	bA

CT: Conventional tillage, NT: No-tillage and RP: Reforestation with Paricá. *Averages followed by the same letter not significantly different by the Tukey test 5%. Lowercase letters compare the systems within the same depth, and capital letters, the depths within the same system.

Maria et al., 1999; Falleiro et al., 2003; Nascente et al., 2014), in which observed greater accumulation of P in the surface layer of the NT system. Probably because the P is an immobile nutrient in soil and the fertilization being carried out annually 0.05-0.08 m deep into the planting furrow, occurs a natural tendency of this nutrient remaining concentrated in the surface layer, with few changes in the deepest layers (Fageria, 2009).

The K content was higher in the surface layer of the NT system, being 47 and 63 $cmol_c$ dm^{-3} for the years 2009 and 2012, respectively. It was the only system that has increased its content over the years (Table 3). These results confirm those obtained by Bayer and Bertol (1999), De Maria et al. (1999), Almeida et al. (2005), and Pereira et al. (2009). In the depth of 0.2-0.3 m, there was no statistical difference between the tillage systems for both evaluation periods, although just the decrease in RP showed K content through time. Pereira et al. (2009) have observed that the K content in the depth of 0.2-0.3 m was higher in minimum tillage systems and no-tillage (36.29 and 35.08 mg dm^{-3}), compared to the conventional tillage (26.41 mg dm^{-3}).

Nascente et al. (2014) emphasized that systems that have biomass on the surface provide increments of this nutrient in the superficial layer, whereas in other soil managements with tillage, the nutrient is distributed in a greater volume of soil.

The level K can descend down the soil profile, reaching relatively high values in the deepest layers, due to this nutrient being extremely mobile in soil (Crusciol et al., 2010).

The levels Ca and Mg were higher in the NT system in both depths and two evaluation periods (Table 3). These results confirm those obtained by Silveira et al. (2000) in experiments comparing the attributes of the soil in the NT and CT system (plowing and harrowing). Almeida et al. (2005), Pereira et al. (2009), and Nascente et al. (2014), observed that the levels of Ca+Mg were higher in the surface layer of the soils analyzed, whereas Falleiro et al. (2003) attribute to this fact of not using soil tillage and the recycling of nutrients by plants in NT systems and minimum tillage.

It can be observed a tendency of higher values of this nutrient in managements with depth of tillage in some soils of 0.0-0.1 m. In the layer 0.2-0.3 m, we observed an inverse a tendency with higher values in treatments with more soil tillage (Table 3).

Aluminium and micronutrients in the soil

The exchangeable soil acidity (Al^{3+}) shows in both depths, insignificant levels, as can be observed by the weak acidity demonstrated by the values of pH. The exchangeable acidity is null in CT system in depth of 0.0-0.1m and in the NT and RP system at a depth of 0.2-0.3m, in both periods evaluated. At a depth of 0.0-0.1m, the null value presented by CT system can be explained by the higher pH

Table 4. Exchangeable acidity and micronutrient levels in two depths of a Yellow Oxissol submitted to different soil management systems in two evaluation periods, Tailândia-PA.

Tillage systems	Al		Cu		Fe		Mn		Zn		Tillage systems	Al		Cu		Fe		Mn		Zn	
	($cmol_c\ dm^{-3}$)		---------- ($mg\ dm^{-3}$) ----------									($cmol_c\ dm^{-3}$)		---------- ($mg\ dm^{-3}$) ----------							
0.0-0.1 m																					
2009											**2012**										
CT	0.00	aA*	0.21	aB	127.9	aA	11.0	bA	8.1	bA	CT	0.00	aA	0.21	bA	133.4	aA	11.3	cA	9.9	cA
NT	0.10	aA	0.21	aA	97.9	cB	20.8	aA	12.8	aA	NT	0.00	aA	0.27	aA	100.0	cB	20.1	aA	12.9	bA
RP	0.15	aA	0.23	aB	111.3	bB	11.7	bA	12.4	aB	RP	0.10	aA	0.28	aB	115.5	bB	13.5	bA	14.3	aA
0.2-0.3 m																					
CT	0.00	aA	0.27	bA	109.2	bB	10.0	bA	7.7	bA	CT	0.00	aA	0.16	cB	101.6	cB	7.2	cB	6.0	bB
NT	0.00	aA	0.20	bA	139.6	aA	8.4	cB	7.9	bB	NT	0.00	aA	0.28	bA	150.0	aA	9.4	bB	5.2	bB
RP	0.00	aA	0.54	aA	119.9	bA	12.8	aA	14.2	aA	RP	0.00	aA	0.63	aA	133.1	bA	13.8	aA	15.1	aA

CT: Conventional tillage, NT: No-tillage and RP: Reforestation with Paricá. *Averages followed by the same letter not significantly different by the Tukey test 5%. Lowercase letters compare the systems within the same depth, and capital letters, the depths within the same system.

reached by this treatment (Table 4). It confirms the results of Pereira et al. (2009) in which observed reduction in exchangeable acidity with increasing of pH. However, there was no statistical difference for this variable in any of the assessed factors, differing from the results of Pereira et al. (2009) founded lower values of H+Al in the soil surface layers, however with the CT and NT systems presenting superior to minimum tillage system. Almeida et al. (2005) observed that the levels of H+Al were significantly higher in no-till system than in conventional tillage.

The NT and RP systems present an increase in levels of Cu, Fe and Zn through time in both depths (Table 4). There were significant differences between the tillage systems at both depths sampled for the level of Cu in the soil, except for the depth of 0.0-0.1m in the year 2009. The RP system reached the highest levels of Cu at a depth of 0.2-0.3m in the two years evaluated.

The highest levels of Zn were found in the RP system in both depths and periods assessed (Table 4), a different result as obtained by Nascente et al. (2014), which shows a tendency that the higher values of Zn are related to the increased soil tillage, in the case of this study it would be the CT system.

One can explain the low levels of Zn in the CT system in both depth and periods evaluated, by the fact of Zn is very influenced by pH, that by increasing provides a reduction in their levels of Zn in soil, as occurred in this study, where the pH showed the highest values in the CT system.

The highest levels of Fe were found in the NT system in a depth of 0.2-0.3m and lowest levels in the soil surface layer, featuring in both cases an increase in function of time. These results differ from those described by Nascente et al. (2014), in which founded no difference between the tillage systems and the levels of Fe in the evaluated depths (0.0-0.1 and 0.1-0.3m). Oxissol are characterized by large amounts of this nutrient and rare cases of agricultural management to alter the balance of this nutrient in the soil (Fageria et al., 2002).

The NT system presents the highest values on the surface Mn (20.8 mg dm^{-3} in 2009 and 20.1 mg dm^{-3} in 2012), according to Table 4, confirming the results obtained by Nascente et al. (2014). According Fageria et al. (2002), low values of pH provide increase in the level of that nutrient, because with the soil acidification Mn the nutrient is made available first and in larger quantities than the other micronutrients.

Nascente et al. (2014) claimed that the higher initial levels of OM, P, K, Cu, Mn and Zn through time in the soil may be an indication of sustainability in the NT system, due to no soil tillage, the nutrients tend to be concentrated in the upper layers (Silveira et al., 2000).

When analyzing the soil chemical properties in both periods evaluated (2009 and 2012), some changes occur in nearly all soil properties (Tables 3 and 4). Thus, it appears that systems with little soil tillage (NT and RP) with the use of crop rotation (NT) were possible to provide similar

values of pH without the use of limestone (Nascente et al., 2014). A possible explanation for this result would be a significant increase in the amounts of organic material, which has a buffering capacity and can contribute to the changing of values pH. In certain cases the presence of plant residues on the surface of the soil characteristic of NT and RP systems can even give increase in values of pH. According to Franchini et al. (2000) in the NT system, the presence of plant residues on the surface can provide increase in the levels of pH and levels of Ca and Mg exchangeable in even deeper soil layers at the expense of exchangeable Al.

Conclusions

Over the years, the soil tillage systems significantly interfere in the dynamics of soil chemical properties. It may be noted that the system NT chemical attributes Ca, Mg, OM, P, K, Mn and Zn are concentrated on the most superficial layer of the soil, whereas in CT there is a uniformity of distribution of concentration of macro and micronutrients in the layer 0.0-0.3m of soil due to tillage promoted at the time of preparation of the site.

Conflict of Interests

The authors have not declared any conflict of interests.

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Legumes as green manure for common bean cultivated in two growing seasons at southeast Brazil

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The use of legumes in pre-cultivation on the common bean has the possibility of providing atmospheric N to the soil, making it available to this crop, and may cover part of its N demand and increase grain yield. The objective of present study was to evaluate the effect of hyacinth bean and jack bean as green manures on the production of common bean grown in two seasons. Cover crops were evaluated for fixed N_2, dry matter yield, nitrogen (N) and carbon (C) concentrations, C:N ratio and N accumulation in the shoot. The jack bean accumulated higher biomass and more total N than hyacinth bean and spontaneous vegetation (control). However, both legume species, when used as green manure, resulted in an increase in the N concentration of common bean. Compared to the spontaneous vegetation, hyacinth bean residue increased yield of common bean by 32% and jack bean residue increased the bean yield by 46%. These yields were recorded when common bean was cultivated a few weeks after residues incorporation into the soil and about seven months later, thus showing a flexibility to family farmers for making their decisions on the best cropping season.

Key words: *Phaseolus vulgaris*, *Dolichos lablab*, *Canavalia ensiformis*, family farming, symbiotic nitrogen fixation.

INTRODUCTION

In Brazil, family farmers produce a majority of the common bean (*Phaseolus vulgaris* L.) crop. Family farming is predominant in the Atlantic Forest region of Minas Gerais state, Brazil. In this region, common bean is either cropped in small mono-crop areas or intercropped with coffee. The yield of these systems is about 650–850 kg ha^{-1} (Didonet et al., 2009). While it is desirable to increase yields, the course of action followed to achieve this should be based on biological processes, be less dependent on industrial inputs, and also be consistent with the traditional practices followed by family farmers.

The common bean absorbs N at varying rates through the cropping cycle, with a higher demand between the

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beginning of flowering until pod formation (Fageria et al., 2008). Despite being a legume, common beans can benefit from biological nitrogen fixation (BNF) to achieve higher yields (Pinto et al., 2007; Cardoso et al., 2012) and, it is necessary to provide N to the crop through other sources.

Green manuring with legumes in pre-cultivation has the potential to increase soil fertility and incorporate N in production systems of family farmers (Fageria and Baligar, 2005; Nyambati et al., 2006). Their use for common bean production can prove economical as compared to that of mineral nitrogen (N) fertilizers (Teixeira et al., 2006; Pietsch et al., 2007), while improving soil properties and increasing crop productivity in a sustainable way (Teixeira et al., 2006; Whitbread et al., 2011). There are reports of the use of legumes as green manure for the common bean crop. However, its effectiveness varies considerably owing to a number of factors, such as chemical, physical, and biological soil properties; the legume species used; local growing conditions; and the residual effect of N fixed by legumes, as well as interactions of these and other factors (Roldán et al., 2007; Whitbread et al., 2011).

In the Minas Gerais Atlantic Forest Zone, family farmers grow rain-fed common beans in two seasons. In the "water season", common bean is grown at the beginning of the rainy season, from October to January. In the "dry season", common bean is grown at the end of the rainy season, from March to May. While the choice of using a green manuring approach should allow the family farmers to cultivate in these two possible planting seasons it should also allow them some flexibility in making decisions about their production systems (Coelli and Flemming, 2004). If the legumes are sown in early summer (November to January), the common bean crop can be grown either from February–March to May, immediately after the green manure is cut, or several months later, from October to January. When grown later, there could be a great time-difference between the cutting of legumes and the next common bean crop.

The hyacinth bean (*Lablab purpureus* L.) and jack bean (*Canavalia ensiformis* (L) DC.) are among the most efficient legumes in biomass production and for N supply in tropical soils (Fageria et al., 2013). Both species are already cultivated by some family farmers and can be easily grown at Minas Gerais Atlantic Forest Zone. The hyacinth bean grows well in a variety of environmental conditions and is tolerant to low soil fertility (Maass et al., 2010; Whitbread et al., 2011; Guretzki and Papenbrock, 2013). The jack bean has early maturity, is erect, and shows semi-determinate growth, in addition to being adaptable to various climatic conditions, ranging from the adverse arid and semi-arid regions to the hot and humid climate of the regions with tropical forests (Teodoro et al., 2011). Few studies have focused on the use of these legumes as green manure for common bean crop, and therefore, the objective of this study was to evaluate the effect of hyacinth bean and jack bean as green manure on the production of common bean grown in two seasons.

MATERIALS AND METHODS

The experiment was conducted from November 2012 to January 2014 at an experimental field of Fitotecnia Department at Universidade Federal de Viçosa (20° 45' S; 42° 51' W, 651 m Mean Sea Level). The climate, according to Köppen classification is Cwa, with a mean annual temperature of 19°C and a mean annual rainfall of 1300 mm. Local weather data collected during the experiment are presented in Figure 1. The experiment was set up as a randomized complete block and split-plot design. The main plot was sub-divided to represent the two growing seasons ("dry" and "water") and these were further divided to represent the three green manure (hyacinth bean, jack bean, and spontaneous vegetation), with five replicates of each. These sub-plots consisted of five rows of 6 m length, spaced at 0.50 m. The sampling area was represented by three central rows, disregarding 1 m from each end.

The soil of the area is classified as Cambisol and had the following characteristics at a depth of 0–20 cm: pH in H_2O = 5.34; available P = 23.2 mg kg^{-1}; exchangeable K = 112 mg kg^{-1}; exchangeable Ca = 2.39 $cmol_c$ kg^{-1}; exchangeable Mg = 0.25 $cmol_c$ kg^{-1}; exchangeable H+AL = 4.8 $cmol_c$ kg^{-1}; BS: base saturation = 37.9%; organic matter = 23.9 g kg^{-1}; remaining phosphorus (Rem. P) = 40 mg kg^{-1}.

Legumes as green manures

The hyacinth bean and jack bean legumes used as green manure were grown in sub-plots with ten replicates (five for each common bean cropping season). Was also grown ten replicates of a control, represented by spontaneous vegetation, comprising of marmalade grass (*Brachiaria plantaginea*), sorghum (*Sorghum arundinaceum*), and pigweed (*Amaranthus spinosus*).

The green manures were sown in November 2012, without fertilization, at a density of 9 and 12 seeds m^{-1} for jack bean and hyacinth bean, respectively. 132 days after sowing, at the beginning of the jack bean pod formation stage and early flowering for hyacinth bean, the aboveground biomass of all plots were cut and incorporated at a depth of 0–20 cm into the soil on the same day using a rotary tiller. Fresh biomass hyacinth bean was 25.68 kg ha^{-1}, fresh biomass jack bean was 47.73 kg ha^{-1} and fresh biomass spontaneous vegetation was 20.00 kg ha^{-1}. Upon cutting, the aboveground biomass green manure was sampled in an area of 2.5 m^2 per plot.

Subsequently, a sub-sample was taken, which was weighed and dried in an oven with forced air circulation at 65 °C until constant mass. These values were then converted to kg ha^{-1}.

After drying, the material was ground and samples taken to the laboratory to determine the total N concentration by the Kjeldahl method (Bremner and Mulvaney, 1982). From the multiplication of the dry biomass by their respective N concentrations, the aboveground N accumulation was estimated [N concentrations (g k-1)*dry matter yield/1000], and the transformed values in kg ha^{-1}.

The contribution of BNF to the N content of the green manure were estimated by the technique of natural abundance of ^{15}N ($\delta^{15}N$, Boddey et al., 1994), with the aid of a mass spectrometer, Finnigan MAT Delta Plus model, in the stable isotope laboratory of John M Day, Embrapa Agrobiologia). The percentage of nitrogen contributed by BNF was estimated by the equation:

$$\% \text{ N-BNF} = 100 \times \frac{\delta^{15}\text{N Spontaneous vegetation} - \delta^{15}\text{N Legumes}}{^{15}\text{N Spontaneous vegetation} - \beta}$$

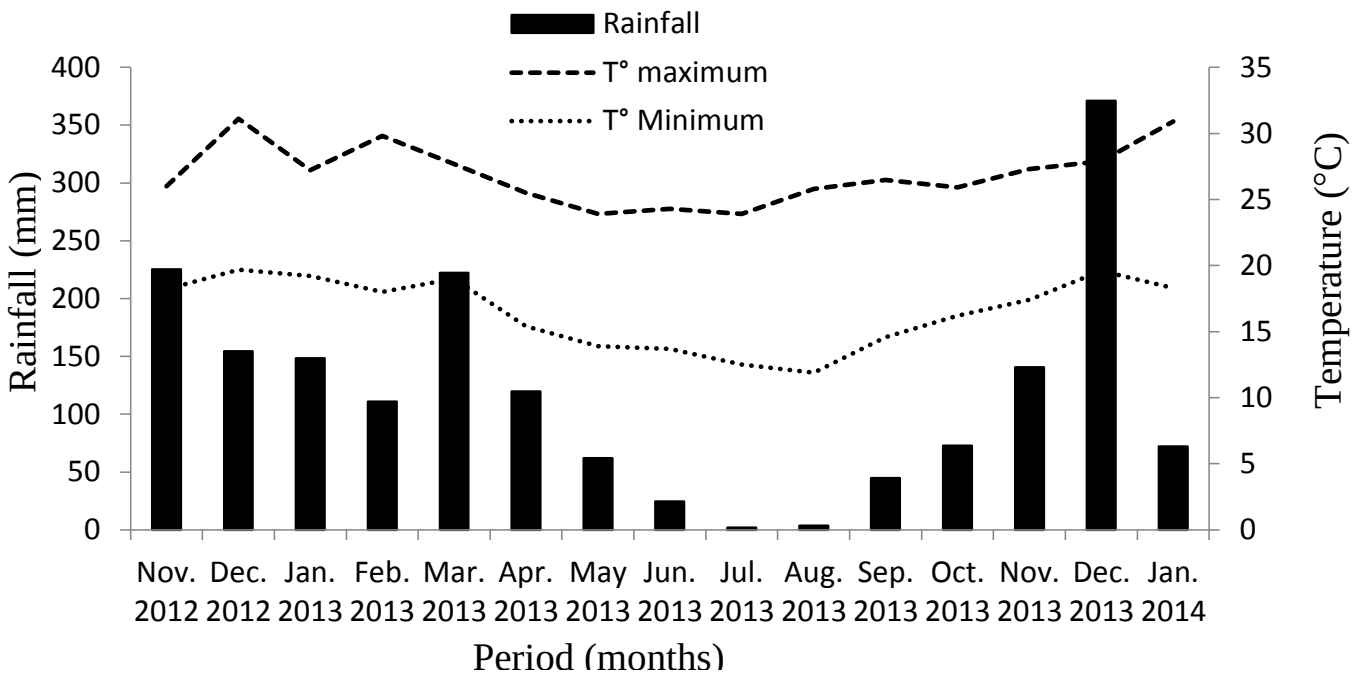

Figure 1. Monthly values of rainfall and maximum and minimum temperature (°C) during experimental period.

where; $\delta^{15}N$ spontaneous vegetation is the value of $\delta^{15}N$ obtained from plants that spontaneously grow in the region and that do not fix nitrogen and β represents the value of isotopic discrimination when $\delta^{15}N = 0$ and is equal to -1.1.

The total carbon concentration (TC), in shoots of legumes, was also analyzed using the Finnigan MAT mass spectrometer.

Common bean cultivation

In April 2013, for "dry" season, fourteen days after green manure incorporation into the soil, one-half of amended plots were sown with common beans, cultivar Pérola. For the "water" cropping season, common beans were sown in October 2013, 6 months and 25 days after incorporation of green manure into the soil, in half size plots. Common beans were sown at a density of 15 seeds m^{-1} and a phosphorus (P) fertilizer (40 kg P_2O_5 ha^{-1}) was applied at planting time.

When more than 50% of the common bean plants had at least one open flower (that is, the R6 growth stage), 30 mature leaves from the mid portion of plants were randomly collected from each plot. These samples were then used to determine the total N content by the Kjeldahl method (Bremner and Mulvaney, 1982).

Quantification of common bean production was carried out by manually harvesting the pods of the three central rows. After drying (to 13% moisture content), the pod grains were weighed and values converted to kg ha^{-1}. The average number of pods per plant was estimated by the ratio between the total number of pods and the total number of plants. We also evaluated the average number of seeds per pod, obtained as the ratio between the total number of seeds and the total number of pods, and the average number of seeds per plant obtained as the ratio between the total seed number and the total number of plants. The weight of 100 seeds was also determined by taking four sub-samples of 100 seeds per replicate of each treatment, and the weight was measured using a 0.001 g precision balance.

Statistical analysis was undertaken using the Statistical Software SISVAR (Ferreira, 2014). Data were subjected to analysis of variance by F test and means were compared by Tukey's test at 5% probability.

RESULTS AND DISCUSSION

Legumes as green manures

The content and amount of N-BNF provided by jack bean was higher than the hyacinth bean (Table 1). The greater supply of N-BNF by jack bean is mainly due to its higher biomass yield rather than its content of N-BNF. A high content of N-BNF in jack bean (76%) was also reported earlier (Ambrosano et al., 2013). The N-BNF content is influenced by several aspects, such as the efficiency of strains of N_2 fixing bacteria, soil fertility, water status, and phenological stage of the legume, which could explain the lower content obtained in the present study.

High yield of common bean (2436 kg ha^{-1}) was obtained with 120 kg ha^{-1} of N supplied by mineral fertilizers, in Brazil (Moreira et al., 2013); thus, the amount of N-BNF accumulated in legumes (Table 1) are able to supply significant amounts of the nutrient to the common bean crop cultivated in succession depending on mineralization rate. In this sense, the use of legumes with high potential for BNF and mass accumulation, as green manure, may reduce or even waive the use of synthetic N fertilizers for common beans.

The legume species showed different biomass accumulation, N concentration, and accumulation of N and C:N ratio after 132 days of cultivation (Table 1).

Table 1. Percentage and accumulation of N-BNF, dry matter yield, N and C concentrations, C:N ratio and N total accumulation in aboveground hyacinth bean, jack bean and spontaneous vegetation, after 132 days of cultivation.

Treatment	N-BNF (%)	N-BNF (kg ha^{-1})	N (g kg^{-1})	C (g kg^{-1})	C:N	Dry matter (kg ha^{-1})	N total accumulation (kg ha^{-1})
Hyacinth bean	49	63.7	27^{a}	453^{a}	17^{c}	4830^{b}	130^{b}
Jack bean	58	147	22^{b}	456^{a}	21^{b}	11490^{a}	253^{a}
Spontaneous vegetation	----	----	9.1^{c}	465^{a}	51^{a}	4780^{b}	44.5^{c}

Means followed by the same letter in the column do not differ by Tukey test ($p \geq 0.05$).

Table 2. Leaf N content, yield components and grain yield of common beans cultivated in amended soil with hyacinth beans, jack bean and spontaneous vegetation. (Data are the average of two growing seasons).

Treatment	N concentration (1) (g kg^{-1})	Number pod/plant	Number seed/pod	Weight of 100 seeds (g)	Grain yield (kg ha^{-1})
Hyacinth bean	38^{a}	8.7^{a}	5.2^{ns}	23^{ns}	2128^{a}
Jack bean	39^{a}	9.3^{a}	5.2^{ns}	24^{ns}	2355^{a}
Spontaneous vegetation	29^{b}	6.1^{b}	5.1^{ns}	24^{ns}	1616^{b}

(1) N concentration in leaf common bean at flowering stage. In each column, means followed by the same letter do not differ by Tukey test ($p \geq 0.05$). ns = F value not significant for $p \geq 0.05$.

Legumes and spontaneous vegetation had similar C concentration. The jack bean accumulated more biomass than hyacinth bean and spontaneous vegetation, which were similar to each other. This result can be attributed not only to the adaptability of the jack bean to the soil and climatic conditions of the region, but also its fast vegetative growth, while hyacinth bean presented a longer life cycle and slower initial growth, resulting in less biomass accumulation in the same culture time period. This result reinforces the importance of the choice of legume species for green manuring. The biomass production of the spontaneous vegetation was similar to the hyacinth bean, but presented a higher C:N ratio and a smaller amount of accumulated N (Table 1). The spontaneous species present in the experimental field were not capable of BNF, which probably influenced the result.

The N concentrations of hyacinth bean and jack bean were higher than those of spontaneous vegetation. However, the N content of hyacinth bean was 23% higher than that of jack bean, resulting in a lower C:N ratio. It is noteworthy that at 132 days after cultivation, hyacinth bean was phenologically younger than jack bean, therefore showing a lower biomass productivity and higher N content. The jack bean accumulated more N (kg ha^{-1}) compared to hyacinth bean and spontaneous vegetation, due to its greater biomass productivity. The amount of N accumulated by the jack bean crop was nearly the double of hyacinth bean, but both species had accumulated higher amounts of N than spontaneous vegetation. Thus, these legumes are important N sources for the agroecosystem through their BNF capacity.

N transfer from crop residues to common bean

There was no significant interaction between the effects of growing season ("dry" and "water") and cover crops over any of the evaluated characteristics of the common bean crop (Table 2). Specifically, present results demonstrated no effect on the N content in leaves of common bean, number of pods per plant, number of seeds per pod, weight of 100 seeds and grain yield (Table 2). The absence of any effect due to the season can be attributed partially to the adequate climatic conditions during the “dry” and “water” cultivation periods. However, a fraction of N in the soil in both seasons ("dry" and "water") originated from hyacinth bean and jack bean residues was available for the common bean, both immediately after legume cutting, during the “dry” cultivation, as well as 6 months and 25 days after legume cutting, during the “water” cultivation.

The cover crops had significant effects on the leaf N concentration of common bean, number of pods per plant and grain yield, in the two growing seasons (Table 2). Leaf N in the common bean was high, with values just above the optimum range for the crop, ranging from 30 to 35 g kg^{-1} (Fontes, 2011). Therefore, present results indicated that apparently crop residues increased the N content in the soil, in a form readily available for uptake by the common bean, when compared to the spontaneous vegetation. The improved soil N content, either few weeks after harvesting the cover crops and after a period of almost seven months, can primarily be due to the chemical composition legume residue. This regulates the organic N mineralization rates over time

(Trinsoutrot et al., 2000). It is likely that the common bean in the "dry" season may have benefited from the mineralization of the more labile N forms, such as organic molecules of low molecular weight (Agehara and Warncke, 2005). On the other hand, common bean cultivated during the "water" season may have benefited from the slower mineralization of organic compounds of higher molecular weight and higher recalcitrance (e.g., lignin), probably resulting in N immobilization after the first weeks after soil amendment (Marschner et al., 2008), also favored by the climatic condition with mild temperature and low rainfall (Havlin et al., 2005) (Figure 1).

Although the hyacinth bean and jack bean residues did not affect differently the number of seeds per pod or the weight of 100 seeds, the number of pods per plant and grain yield were higher when compared to spontaneous vegetation residues (Table 2). The number of seeds per pod is a highly inheritable genetic characteristic and is mostly not influenced by environmental conditions (Andrade et al., 1998). On average, nine pods per plant in both cropping seasons were counted, which is similar to pod number obtained in common bean fertilized with mineral N (Crusciol et al., 2007). The increase in the number of pods per plant is justified by the possible higher N contribution from hyacinth bean and jack bean residues, which in turn causes an increase in grain yield. Some authors stated that legume green manure may provide an N input ≥ 110 kg N ha^{-1} which can result in common bean yields similar to those obtained using mineral N fertilizers (Tonitto et al., 2006).

Compared to the spontaneous vegetation, the yield of common bean in hyacinth bean amended soil increased by 32%, and under jack bean residue increased by 46%. Yields obtained in the present study in amended soils with these legumes were high, considering that the mean yield in Brazil for common bean is 650-850 kg ha^{-1} (Didonet et al., 2009). However, even in the grass amended soil the yield of common bean was higher than the national value. But the most important is that the increase in bean productivity was achieved without application of a synthetic N fertilizer. In this sense, present results contributed to improving food production under the control of family farmers using biological processes based on crop diversification and rotation.

Conclusions

i) Among the evaluated green manure, jack bean was the legume that resulted in higher common bean biomass production, accumulated more N in its tissue, by contributing to the highest amount of N-BNF to the system.
ii) Both legumes as green manure promoted increases in leaf N content of common bean and in the number of pods produced per plant, also increasing the yield of the common bean.
iii) At the Atlantic Forest Zone of Minas Gerais, southeastern Brazil, common bean can be performed well either in the "dry" season, immediately after incorporating the legume residues or later in the "water" season.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Field evaluation of cowpea varieties for adaptation to the forest/savanna transition agroecology of Osun state, Nigeria

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Cowpea (*Vigna unguiculata* [L.] Walp) is an important food legume in tropical and sub-tropical Africa. It is grown for food, fodder, a cover crop and consumed as dried seeds rich in carbohydrates, proteins and vitamins. Production of cowpea has not been practiced on a large scale in the forest/savanna transition agro ecology of Nigeria due to high relative humidity, diseases and insect damage. In 2012 and 2013 cropping seasons, twelve cowpea varieties were assessed on the field for their adaptation to the forest/savanna transition agroecology of Osun State Nigeria. Three foliar diseases (cowpea bacterial blight disease, cowpea mosaic diseaseand cowpea leaf spot disease) and level of insect damage on leaves were observed under natural infections. At sixty days after sowing (60DAS), all cowpea varieties tested showed varying degrees of infections by cowpea bacterial blight disease, cowpea mosaic disease, cowpea leaf spot disease and insect damage. Number of flowers produced per plant per week was not significantly different (P≤0.01) among the varieties. Variety sample 5 showed the least level of infection by the three foliar diseases while the number of pods produced per plant per week was highest in TVX 3236 and least in Dan Borno (88 DAS). Insect damage was least in sample 6 and TVX 3236 but highest in sample 7. Proper management of these three important foliar diseases will enhance the production of some of the promising varieties in the forest/savanna transition agroecology of Osun State.

Key words: Cowpea, adaptation, foliar diseases, forest/savanna transition agroecology.

INTRODUCTION

Cowpea (*Vigna unguiculata* [L.] Walp) is an important grain legume in the tropics and is cultivated for grain, leaves and green pods (Quin, 1997; Awurun and Enyiukwu, 2013). It is also a source of quality protein as well as a cash crop for most West and Central African farmers (Langyintuo et al., 2003). It serves as a significant dietary compliment in developing countries of Africa, Latin America and Asia (Philips et al., 2003).

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Nigeria is the highest producer of the crop (IITA, 2001) and it can be grown under various production systems such as rain-fed, irrigated, and areas with low rainfall (Singh et al., 1997). One of the major constraints to cowpea production is the effect of pests and diseases induced by different groups of pathogenic organisms (fungi, bacteria, viruses, nematodes) and parasitic flowering plants thereby causing seed rots, seedling mortality, stem and root decay, foliar diseases and deterioration of seed quality (Hampton et al., 1997; Agbicodo et al., 2010; Adegbite and Amusa 2008).

Cowpea bacterial blight is caused by *Xanthomonas campestis* p.v. Viginicola (Burkholder). Dye is an important disease of cowpea (Nandini and Shripad, 2015; Emechebe and Florini, 1997). Cowpea mosaic virus causes mosaic, distortion and mottling of leaves of cowpea resulting in significant yield loss. The virus has the potential for both seed and aphid transmission (Aliyu et al., 2012). Leaf spot and insect damage can also cause total yield loss. Another important pest of cowpea is flower thrips (*Megalurothrips sjostedti* (trybom). It attacks buds, racemes and flowers causing premature abortions of these reproductive organs. Excessive moisture during rainy years may also cause yield reduction, disease outbreaks or both (Muleba et al., 1991).

Cowpea is one of the most widely adapted and versatile cultivated grain legumes (Gibbon and Pain, 1985). A vast number of high-yielding and or disease tolerant varieties have been released by research institutes in Nigeria such as Institute of Agricultural Research, Zaria (IAR). The performances of these varieties may be location dependent and virulence of pathogens may also be influenced by the environment. In addition, majority of cowpea varieties consumed in South West Nigeria are produced in savanna zone of Nigeria (FAO, 1999). The study was undertaken to assess the adaptation of some cowpea varieties released by the Institute of Agricultural Research Samaru to the forest/savanna agroecology of Osun state in South West Nigeria in terms of foliar diseases and insect damage.

MATERIALS AND METHODS

The experiment was conducted during the cropping season of 2012 and 2013 at the Bowen University Teaching and Research Farm, Iwo, Osun State Nigeria. The crops were established during the late planting season of 2012 and early planting season of 2013. The soils are friable, deep and rich. The soil pH is slightly acidic with a sufficient amount of soil organic matter and soil organic carbon (Salami and Sangoyomi, 2013). The mean annual temperature ranges from between 21 and 32°C. There are two rainfall peaks in June and September with dry spell in August which produces the bimodal rainfall pattern in southwestern Nigeria. The dry season runs from early November to the end of March or early April, while the wet season is from end of March or early April to about middle of November. The average annual rainfall is 1279 mm. Relative humidity is high and ranged between 60 and 90% at 16.00 h (Adeyolanu et al., 2015). Twelve cowpea varieties were planted in 5 × 4 m plots with 1m space between rows and 30 cm within rows in four replicates and two seeds were planted per hole. A local variety that is popularly grown in the region was also planted as a check. Imidazolinone-based pre-emergence herbicide (Bush fire - Jubaili Company, China) was sprayed onto the plots immediately after sowing and subsequently. Insecticide (Lambda-Cylalothrin 2.5 EC insecticide; Marshall - FMC Chemicals Corporation, USA) was sprayed at two-week interval to control cowpea aphids. Data on incidence and severity of three foliar diseases (bacterial blight disease, cowpea mosaic virus disease, leaf spot) and insect damage were taken separately at 7 days interval from 60 to 88 days after sowing. A disease rating scale of 1 to 10 of which; 1 means no symptom and 10 means highly susceptible (death of plant) was used to estimate disease severity (Brinkerhoff, 1977). The scale was used to measure 20 plants per row of the 3 middle rows in each replicate. Data were also taken for number of flowers/pods produced per plant per week. Data were analysed using SAS software (SAS, 2006). Means for the pooled data were compared using the Duncan Multiple Range test.

RESULTS AND DISCUSSION

The eleven cultivars and a local variety used as a check were all affected by cowpea bacterial blight. The severity was generally low in all the varieties at 60 and 67 DAS but least in sample 4 at 60 DAS (1.25) and highest in Dan Borno (2.50), there were no significant differences among all varieties tested at 67 DAS however; sample 7, 9 and 10 had the least disease severity rating. At the end of the assessment period (88 DAS), disease severity was lower in sample 5 and 6 (Table 1). Results on the effects of cowpea mosaic disease on the 12 varieties were not significantly different from each other at 60 and 67 DAS but, TVX 3236 had the least rate of infection for the two time periods (1.5 and 2.06, respectively). The local check had the highest severity at 60 DAS (2.50) while sample 2, 8 and 10 had the highest severity rating at 67 DAS (3.00) (Table 2). At 88 days after sowing, there were no significant differences between the varieties and the local check, although, the highest disease severity was observed in samples 7 and 8 (6.75) and least in sample 5 and the local variety (Table 2).

Table 3 shows the effect of cowpea leaf spot on the 12 cowpea varieties. Sample 5 was significantly lower than sample 7 and Dan Borno but not different from all other varieties at 60 DAS (Table 3). Cowpea leaf spot severities were low in all varieties except in sample 7 that had 4.38 (Less than 26 to 50% damage). At 88 days after sowing, sample 7 had the highest level of cowpea leaf spot damage (6.11) while the least was recorded in sample 5 (4.29).

Production of flowers and or pods per week increased consistently throughout the period of assessment (Table 4a and b). Sixty days after sowing, sample 2 gave the highest number of flowers while the local check gave the least and at 88 DAS, sample 2 and TVX 3236 had the highest number of flowers per plant per week (4.0). Production of pods per week was highest in TVX 3232 at 88 DAS and lowest in Dan Borno (2.50) sample 2, 4, 5, 6,

Table 1. Cowpea varieties in response to cowpea bacterial blight disease.

Variety	60 DAS	67 DAS	74 DAS	81 DAS	88 DAS
Sampea 1	2.00^{a-c}	2.21^{b}	2.76^{a}	3.58^{a}	4.75^{ab}
Sampea 2	1.75^{a-c}	2.07^{b}	3.12^{a}	4.56^{a}	5.70^{ab}
Sampea 4	1.25^{c}	2.49^{b}	3.12^{a}	3.99^{a}	4.83^{ab}
Sampea 5	2.00^{a-c}	2.50^{b}	3.11^{a}	4.14^{a}	4.08^{b}
Sampea 6	2.00^{a-c}	2.25^{b}	2.89^{a}	3.84^{a}	4.10^{b}
Sampea 7	2.00^{a-c}	2.00^{b}	3.14^{a}	4.55^{a}	6.38^{a}
Sampea 8	1.99^{a-c}	2.50^{b}	3.24^{a}	4.75^{a}	4.95^{ab}
Sampea 9	1.50^{bc}	2.00^{b}	2.80^{a}	3.62^{a}	4.45^{ab}
Sampea 10	1.75^{a-c}	2.00^{b}	3.43^{a}	4.40^{a}	4.53^{ab}
TVX 3236	1.50^{bc}	2.15^{b}	3.21^{a}	4.19^{a}	4.50^{ab}
Dam Borno	2.50^{a}	2.75^{b}	3.56^{a}	4.52^{a}	4.50^{a}
Local	2.25^{ab}	2.50^{b}	2.25^{a}	4.39^{a}	5.35^{ab}

DAS = Days after sowing. Means followed by the same letters are not significantly different from each other.

Table 2. Cowpea varieties in response to cowpea mosaic disease.

Variety	60 DAS	67 DAS	74 DAS	81 DAS	88 DAS
Sampea 1	2.00^{ab}	2.92^{a}	3.67^{ab}	4.01^{bc}	5.00^{a}
Sampea 2	2.23^{ab}	3.00^{a}	4.06^{a}	5.47^{ab}	5.50^{a}
Sampea 4	1.75^{ab}	2.43^{a}	3.96^{ab}	4.52^{a-c}	5.50^{a}
Sampea 5	2.25^{ab}	2.50^{a}	3.46^{ab}	4.03^{a-c}	4.50^{a}
Sampea 6	2.25^{ab}	2.50^{a}	3.71^{ab}	4.82^{a-c}	5.00^{a}
Sampea 7	2.25^{ab}	2.95^{a}	4.02^{ab}	5.56^{ab}	6.75^{a}
Sampea 8	2.25^{ab}	3.00^{a}	3.92^{ab}	5.99^{a}	6.75^{a}
Sampea 9	1.75^{ab}	2.25^{a}	2.70^{b}	3.16^{c}	4.50^{a}
Sampea 10	2.25^{ab}	3.00^{a}	3.79^{ab}	4.41^{a-c}	5.67^{a}
TVX 3236	1.50^{ab}	2.06^{a}	4.21^{a}	4.86^{a-c}	5.25^{a}
Dam Borno	2.25^{ab}	2.47^{a}	3.32^{ab}	4.58^{a-c}	4.75^{a}
Local	2.50^{ab}	2.50^{a}	3.5^{ab}	4.50^{a-c}	4.50^{a}

DAS = Days after sowing. Means followed by the same letters are not significantly different from each other.

Table 3. Cowpea varieties in response to cowpea leaf spot disease.

Variety	60 DAS	67 DAS	74 DAS	81 DAS	88 DAS
Sampea 1	1.75^{ab}	2.17^{b}	2.64^{b}	3.50^{cd}	4.50^{a}
Sampea 2	1.81^{ab}	2.56^{ab}	3.87^{ab}	5.14^{ab}	5.75^{a}
Sampea 4	2.00^{ab}	2.79^{ab}	3.21^{ab}	4.05^{a-d}	4.35^{a}
Sampea 5	1.50^{b}	2.25^{b}	2.81^{b}	3.97^{b-d}	4.25^{a}
Sampea 6	2.00^{ab}	2.50^{ab}	3.27^{ab}	4.57^{a-c}	4.97^{a}
Sampea 7	2.50^{a}	3.25^{a}	4.38^{a}	5.30^{a}	6.11^{a}
Sampea 8	2.25^{ab}	2.49^{ab}	3.15^{ab}	4.14^{a-d}	6.06^{a}
Sampea 9	2.25^{ab}	2.25^{b}	2.81^{b}	3.22^{d}	4.29^{a}
Sampea 10	1.75^{ab}	2.50^{ab}	3.32^{ab}	5.01^{ab}	5.25^{a}
TVX 3236	1.75^{ab}	2.45^{ab}	3.55^{ab}	3.84^{b-d}	4.78^{a}
Dam Borno	2.50^{a}	2.50^{ab}	3.68^{ab}	4.67^{ab}	4.88^{a}
Local	2.25^{ab}	3.00^{ab}	3.06^{b}	3.62^{cd}	4.50^{a}

DAS = Days after sowing. Means followed by the same letters are not significantly different from each other.

Table 4a. Cowpea varieties in response to number of flower produced per plant per week.

Variety	60 DAS	67 DAS	74 DAS	81 DAS	88 DAS
Sampea 1	1.25^{ab}	1.34^{bc}	2.23^{ab}	2.36^{b}	3.00^{a}
Sampea 2	1.83^{a}	2.00^{ab}	2.49^{ab}	2.58^{ab}	4.00^{a}
Sampea 4	1.25^{ab}	1.75^{a-c}	2.81^{ab}	3.36^{ab}	3.00^{a}
Sampea 5	1.00^{b}	1.75^{a-c}	1.90^{b}	2.93^{ab}	3.45^{a}
Sampea 6	1.50^{ab}	2.50^{a}	1.75^{b}	2.13^{b}	3.25^{a}
Sampea 7	1.25^{ab}	2.50^{a}	4.10^{a}	4.06^{a}	3.25^{a}
Sampea 8	1.50^{ab}	2.00^{ab}	2.45^{ab}	2.81^{ab}	3.25^{a}
Sampea 9	1.50^{ab}	2.00^{ab}	2.30^{ab}	2.25^{b}	3.50^{a}
Sampea 10	1.50^{ab}	2.00^{ab}	2.70^{ab}	2.73^{ab}	3.50^{a}
TVX 3236	1.25^{ab}	2.00^{ab}	2.55^{ab}	2.73^{ab}	4.00^{a}
Dan Borno	1.25^{ab}	1.25^{bc}	1.52^{b}	2.17^{b}	3.25^{a}
Local	1.00^{b}	1.00^{c}	1.25^{b}	2.15^{b}	3.25^{a}

DAS = Days after sowing. Means followed by the same letters are not significantly different from each other.

Table 4b. Cowpea varieties in response to number of pods produced per plant per week.

Variety	60 DAS	67 DAS	74 DAS	81 DAS	88 DAS
Sampea 1	1.60^{b}	1.50^{b}	2.25^{bc}	2.70^{de}	3.25^{de}
Sampea 2	1.25^{b}	1.50^{b}	3.03^{bc}	5.25^{bc}	6.00^{bc}
Sampea 4	1.25^{b}	2.20^{b}	2.92^{bc}	4.87^{bcd}	5.75^{b-d}
Sampea 5	1.25^{b}	1.25^{b}	2.87^{bc}	3.72^{b-e}	5.50^{b-d}
Sampea 6	1.25^{b}	1.25^{b}	1.83^{bc}	2.11^{e}	3.50^{c-e}
Sampea 7	3.00^{a}	4.25^{a}	11.88^{a}	7.92^{a}	5.50^{b-d}
Sampea 8	1.50^{a}	3.00^{ab}	5.78^{b}	4.16^{b-e}	6.50^{ab}
Sampea 9	1.25^{b}	1.75^{b}	2.37^{bc}	2.93^{c-e}	3.25^{de}
Sampea 10	1.00^{b}	2.25^{b}	5.79^{b}	5.40^{bc}	5.00^{b-e}
TVX 3236	1.00^{b}	2.00^{b}	4.98^{bc}	5.92^{ab}	8.50^{a}
Dan Borno	1.00^{b}	1.00^{b}	1.27^{c}	2.12^{e}	2.50^{e}
Local	1.00^{b}	1.50^{b}	2.00^{bc}	2.17^{e}	3.75^{c-e}

DAS = Days after sowing. Means followed by the same letters are not significantly different from each other.

7, 8 and 10 produced more pods per week per plant than the locally cultivated variety in the region (Table 4b). Insect damage was mild in all varieties at 60 DAS but increased thereafter (Table 5). Sample 7 had the highest level of insect damage consistently throughout the assessment period while sample 2, 6, 10 TVX 3236, Dan Borno and the local check did not differ in their responses to insect damage.

Major foliar diseases of cowpea observed in the forest/savanna agroecology of Osun state include bacterial blight disease, cowpea mosaic virus disease, and leaf spots. Their severities increased over time and at the end of production 40 to 50% leaf area have been damaged. These diseases have been reported to cause severe yield losses in the humid agro ecologies of Nigeria (Adegbite and Amusa, 2008; Emechebe and Florini, 1997) and more research efforts should be geared towards their detection and management especially before visible appearance of symptoms. Planting of cowpea varieties that show some levels of tolerance to the diseases would be more attractive to farmers as means of ameliorating the effects of diseases on cowpea on the field. Variety sample 5 showed the least level of severity to cowpea bacterial blight, cowpea mosaic disease and leaf spot. Samples 6, 8 and 9 would also be good compliments to cowpea production as they performed better than the local check in terms of cowpea bacterial disease which is the major production constraint (Nandini and Shripad, 2015). The low severities of insect damage and higher number of flowers and pod production per week per plant in TVX 3236 are a good indication of adaptability to the forest/savanna transition Agroecology of Osun State. The performance of any variety that shows some closeness to the local check that is adapted and grown by farmers in a particular locality is an indication of possibilities of adaptation. Improved cultivation of many cowpea varieties in the south west Nigeria will enhance food security and sustenance.

Table 5. Cowpea varieties in response to Insect damage.

Variety	60 DAS	67 DAS	74 DAS	81 DAS	88 DAS
Sampea 1	2.01^{bc}	2.25^{bc}	3.09^{b}	4.11^{bc}	5.00^{bc}
Sampea 2	2.11^{a-c}	2.50^{a-c}	4.12^{ab}	5.38^{ab}	4.50^{c}
Sampea 4	2.00^{bc}	2.75^{a-c}	4.52^{ab}	4.25^{bc}	6.50^{ab}
Sampea 5	2.25^{a-c}	2.50^{a-c}	3.66^{b}	3.97^{c}	5.06^{bc}
Sampea 6	2.50^{ab}	2.50^{a-c}	3.19^{b}	3.86^{c}	4.00^{c}
Sampea 7	2.75^{a}	3.25^{a}	5.61^{a}	6.28^{a}	7.50^{a}
Sampea 8	2.46^{a-c}	3.00^{ab}	3.86^{b}	5.41^{ab}	6.37^{ab}
Sampea 9	1.75^{c}	2.75^{a-c}	3.03^{b}	3.66^{c}	5.00^{bc}
Sampea 10	1.75^{c}	2.00^{c}	3.16^{b}	3.99^{c}	4.06^{c}
TVX 3236	2.00^{bc}	2.22^{bc}	3.62^{b}	3.97^{c}	4.00^{c}
Dan Borno	2.75^{a}	2.75^{a-c}	3.71^{b}	4.27^{bc}	4.50^{c}
Local	2.50^{ab}	3.00^{ab}	3.75^{b}	4.19^{bc}	4.50^{c}

DAS = Days after sowing. Means followed by the same letters are not significantly different from each other.

Conclusion

The study suggests the prospects of growing some of the cowpea varieties released by Institute of Agricultural Research (IAR) and the International Institute of Tropical Agriculture (IITA) in the savanna/forest transition agroecology of Osun state, Nigeria. In terms of foliar disease infections, samples 5, 6, 8 and 9 performed better than the local variety (check) in that order while TVX 3236 was the most resistant to insect pest damage. Further improvement work can be done on these identified varieties to improve their suitability to the savanna/forest transition agroecology of Osun State.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Use of cattle waste on the farm

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Lack of fertile soil is among the main problems in world agricultural production areas, mainly in vegetable crop production areas. In this sense, we aimed to evaluate the the production of chili (*Capsicum annuum* L.) using cow urine as an organic fertilizer. The survey was conducted from June to September 2014 at the School State of Primary and Secondary Nossa Senhora da Conceição, Belém de Brejo do Cruz – PB, Brazil. The experimental design was completely randomized (DIC), in 5 x 2 factorial scheme of 40 plants, with seven repetitions. There was a total of 10 treatments. The effects of 5 cow urine doses were studied: 0, 25, 50, 75 and 100 ml, applied to soil combined with fertilizers: A1 = wood powder + cattle manure + sand washed (1:1:1) and A2 = cattle manure + sand washed (2:1)). There was a significant effect at 1% probability in F test for cow urine doses in all variables. Except the fruit length and number of unmarketable fruits, organic fertilizers statistically influenced the level of p <0.01 in all variables. There was no significant response to the interaction doses × organic fertilizers. 100 ml of cow urine provided good results in the production of chili. The organic manure fertilizer bovine + washed sand is great for chili culture. it is not recommended to incorporate wood powder organic fertilizers.

Key words: Agroecology, *Capsicum annuum* L., cow urine.

INTRODUCTION

Chili culture is adapted to tropical climate, requiring high temperature. Its cultivation requires good chemical and physical characteristics of soil, developing well in organic production. Good productivity can be obtained by combining with mineral organic fertilizer (Albuquerque et al., 2012). Proper management of agricultural activities such as fertilization is essential for this practice, so that producers can use it throughout the nation in a rational and economical way.

Organic agriculture is a sustainable alternative for small

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Table 1. Chemical attributes of cow urine used in chili experiment. Belém do Brejo do Cruz - PB, 2014.

Specifications	Value
pH	-
ECw (dS m^{-1})	-
NUTRIENTS	(g L^{-1})
Nitrogen (%)	2.80
Phosphorus (mg / dm^3)	4.80
Potassium (cmol $_c$ L^{-1})	10.00
Calcium (cmol $_c$ L^{-1})	0.30
Magnesium (cmol $_c$ L^{-1})	0.40
Sodium (cmol $_c$.dm^{-3})	-
Sulphur (cmol $_c$.dm^{-3})	-

and large producers; as its use has no greater sustainability due to preservation of natural resources found on farms, as well as reduced application of chemicals. In this regard, organic fertilizers provide physical improvement to soil, which are storage, aeration, improved internal structure and soil drainage, reducing sudden changes in temperature of the soil. They also affect the biological processes of soil and infiltration of nutrients in plant (Trani et al., 2013).

Cow urine is considered an organic fertilizer as a byproduct of livestock activity; it is available in most rural properties. It is rich in minerals, example Nitrogen and Potassium. Thus it provides nutrients and other essential elements for healthy plant growth. Their use presents no health risk to those who handle them. Another advantage with their use is the possibility of joining the livestock farming horticulture, allowing a reduction in spending on crop production as it reduces the costs of fertilizers.

The use of organic fertilizers with suitable characteristics for plant species leads to reduction of cultivation time and consumption of inputs such as chemical fertilizers, pesticides and labor-intensive. Among the most organic fertilizers used and the ones thar are easily acquired and rich in various nutrients, N, P and K stand out.

In this sense, we aimed to evaluate chili production *(Capsicum annuum* L.) under the application of cow urine functioning as organic fertilizer.

MATERIALS AND METHODS

The survey was conducted from June to September 2014 at the School State of Primary and Secondary Nossa Senhora da Conceição, Belém de Brejo do Cruz – PB, Brazil. Its geographical coordinates are: 60 28'12 "South, 370 20'32" west of Greenwich longitude with an elevation of 176 m. The climate of the city, according to Köppen classification, is BSWh type.

The experimental design was completely randomized (DIC), in 5 x 2 factorial scheme of 40 plants, with seven repetitions. There was a total of 10 treatments. The effects of 5 cow urine doses were studied: (0, 25, 50, 75 and 100 ml); they were applied together in the soil with fertilizers: (A1 = wood powder + cattle manure + sand washed (1:1:1) and A2 = cattle manure + sand washed (2:1)). The planting was done in pots with 5 L capacity.

The water used for irrigation showed electrical conductivity of 0.9 dS m^{-1}. The water analysis was carried out by the Irrigation and Salinity Laboratory (LIS) of the Center for Technology and Natural Resources of the Federal University of Campina Grande - UFCG. It had the following chemical characteristics: pH = 7.50; Ca = 2.45 (cmol $_c$/dm^3). Mg = 1.26 (cmol $_c$/dm^3); Na = 3.50 (cmol $_c$/dm^3); K = 0.03 (cmol $_c$/dm^3); Chloride = 3.20 (cmol $_c$/dm^3); Carbonate = 0.45 (cmol $_c$/dm^3); Bicarbonate = 3.35 (cmol $_c$/dm^3); RAS = 2.58 (mmol $_c$ $L^{-1})^{1/2}$.

The cow urine used in the experiment was collected from lactating cows, dairy herd of Agrotécnica Cajueiro School - EAC municipality of Catolé do Rocha-PB. To obtain the nutrient fertilizer solution cow urine was diluted to a concentration of 1% applied to the soil.

Treatments with cow urine began 21 days after emergence (DAE), then an interval of eight days between applications; making it 6 applications. Chemical analysis of cow urine is given in Table 1. Chemical analysis of the manure comprised the following attributes: pH = 7.75; P = 56.15 mg.dm³; K = 23.46 mg.dm³; Ca = 7.70 cmol $_c$ .dm $^{-3}$; Mg = 15.90 cmol $_c$ .dm $^{-3}$; Al + H = 0.0 cmol $_c$ .dm $^{-3}$; In = 9.18 cmol $_c$ .dm $^{-3}$ and Organic matter = 384.1 g kg^{-1}.

Sowing was done in pots, using four chili cv seeds. They wew all big, distributed and spaced equidistant at a depth of 2 cm. 15 days after sowing (DAS) seedlings were thinned, in order to make them more vigorous. During the experiment, hand weeding was done as the maintenance needs of free culture weed.

Number of fruits per plant, average fruit weight, length and width, number of commercial fruits, weight commercial fruit, pH, total acidity (titration with NaOH 0.1 mol/L) and ascorbic acid were evaluated.

The number of fruits was obtained by counting the fruits. The fruits were weighed in order to obtain the average fruit weight. The length and width of fruits was measured with a digital caliper. The number of commercial fruits was counted by sorting the fruits based on the length and diameter, based on Correia (1984) and Ceagesp (2015), with minor modifications. To get the weight of unmarketable fruits, commercial fruits were weighed on a scale.

The pH analysis, total acidity (titration with NaOH 0.1 mol/L) and ascorbic acid (reduced Tillmans solution) were obtained according to the methods proposed by the Instituto Adolfo Lutz (Zenebon et al., 2008).

The data were analyzed and interpreted from the analysis of variance (F test), by using the statistical program SISVAR, the confrontation of averages Tukey test at 0.05 significance level (5%) and 0.01 (1%) probability according to Ferreira (2011).

RESULTS AND DISCUSSION

There was a significant effect at 1% probability level in the F test for cow urine at all doses in terms of different variables except pH, total acidity (titration with NaOH 0.1 mol/L) and ascorbic acid. They were influenced statistically, except the length of the fruit number of unmarketable fruits, pH, total acidity (titration with NaOH 0.1 mol/L) and ascorbic acid. Organic fertilizers statistically influenced the level of $p < 0.01$ in all variables. No significant response was observed for the interaction doses x organic fertilizers.

The highest number of fruits was found in 100 ml of cow urine. There was an average value of 7.62 fruits as

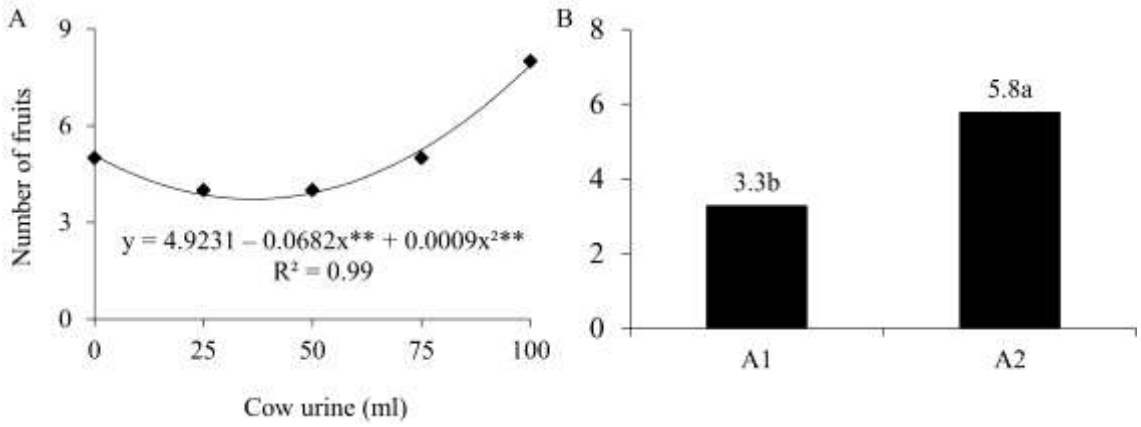

Figure 1. Effect of cow urine doses of (A) under the number of fruits of chili in fertilizer function (B). *,**Significant at the 5 and 1% probability, respectively.

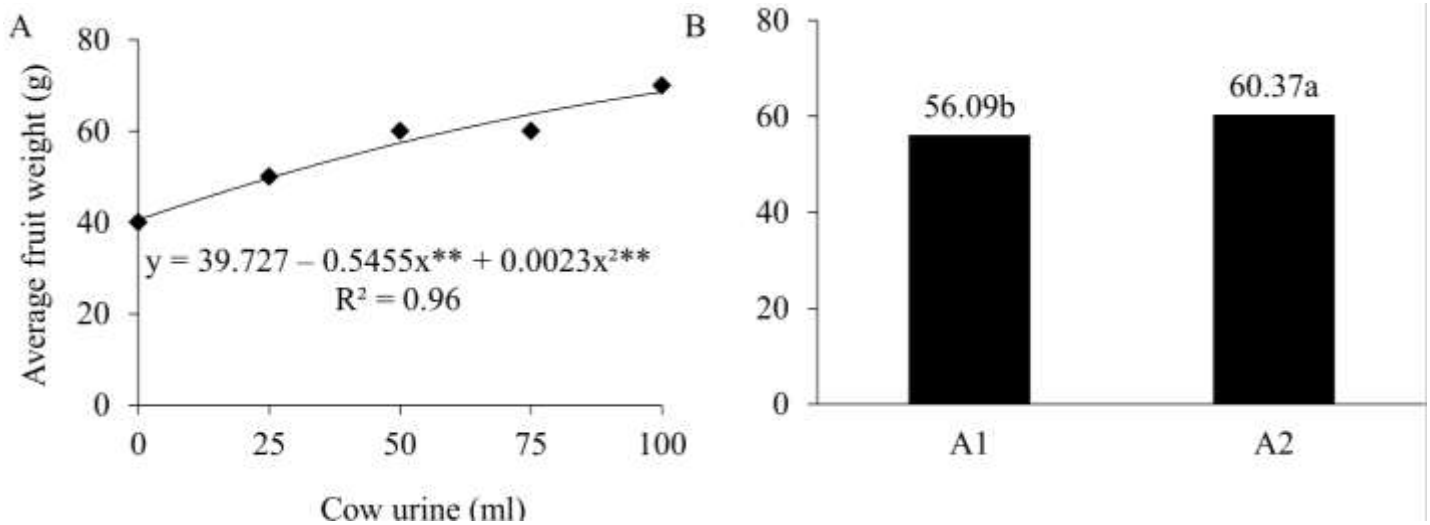

Figure 2. Effect of cow urine doses of (A) under the average fruit weight of chili in fertilizer function (B) *,**Significant at the 5 and 1% probability, respectively.

cow urine increases in dose, with a correlation coefficient of 0.99 (Figure 1A). Perhaps this result is explained by the greater number of nutrients found in cow urine promoting greater fruit production.

For organic fertilizers, it was observed that the best results were obtained with organic fertilizer comprising manure + washed sand in the ratio 2: 1 v / v, with an average of 5.8 fruits (Figure 1B). The use of waste provides several benefits, in terms of environment and expenses: it enables the reduction of spending on mineral fertilizers, and as a result make producers to seek fertilization alternatives to reduce costs and increase productivity (Bonfim Silva et al., 2011).

It was observed for the average weight of fruits quadratic growth, as the cow urine increased there was increase in the average fruit weight, where the maximum dose (100 ml) corresponded to average 72.55 g plant^{-1} (Figure 2A). Regarding organic fertilizers, the combination of cattle manure + sand washed in a 2: 1 v/v excelled to wood dust + manure + sand washed in a 1: 1: 1 v/v (Figure 2B). This explains the greater quantity of manure , the organic fertilizer, as well as the toxic effect of the wood powder.

The use of bovine manure as fertilizer is a viable alternative; in addition, organic feedstock stabilizse the availability of nutrients and increased productivity of crops (Melo et al., 2011; Silva et al., 2011).

The fruit length was adjusted to a quadratic regression model (Figure 3), the maximum length of the fruit (39.15 mm plant^{-1}) was obtained at a dose of 100 ml of cow urine, and the R^2 value = 0.72; the average obtained for the fruit length is outside the standard average (8-13 cm)

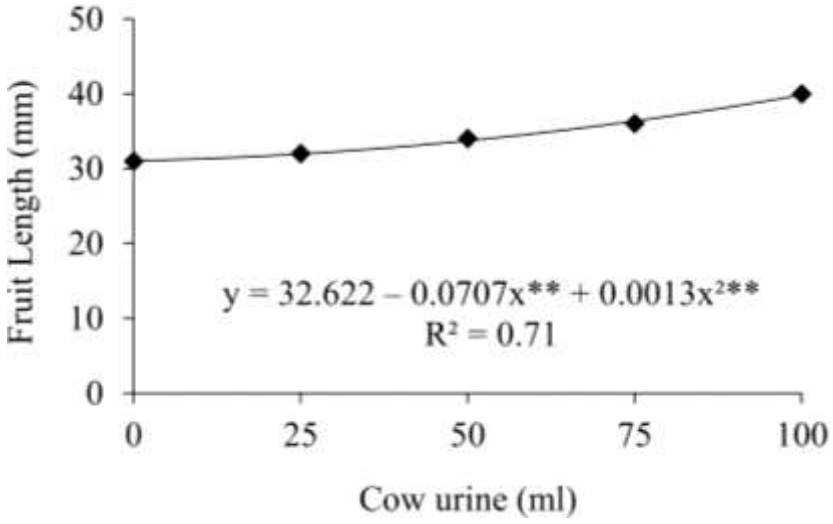

Figure 3. Effect of cow urine doses in the length of chili fruits. *,**Significant at the 5 and 1% probability, respectively.

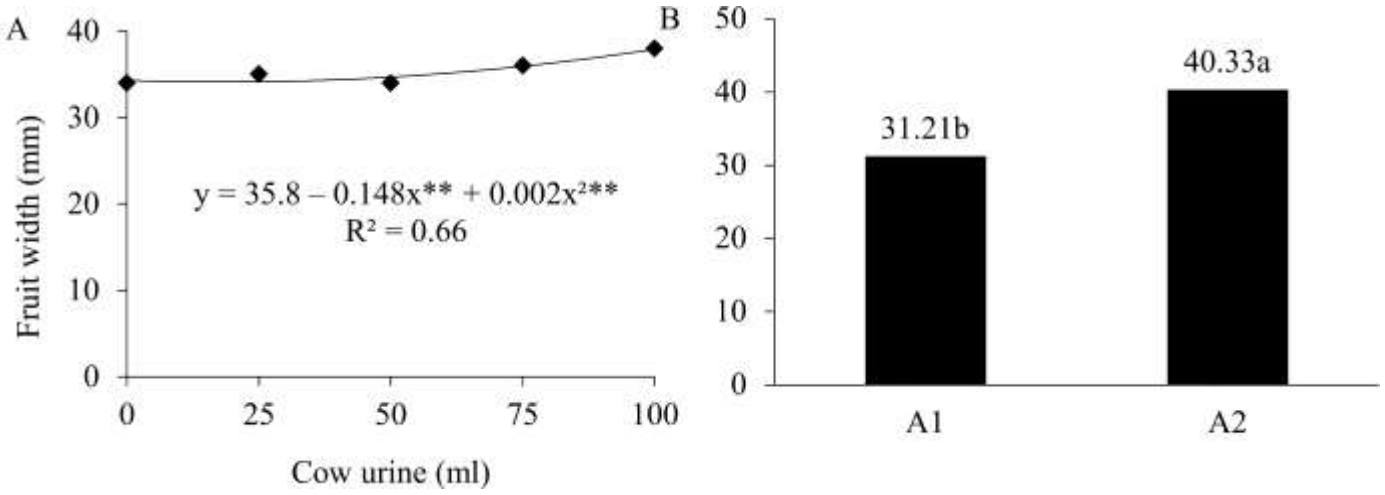

Figure 4. Effect of cow urine doses of (A) in the width of fruits of chili in fertilizer feature (B), *,**Significant at the 5 and 1% probability, respectively.

for this variety. No significant effects were observed for the treatments with organic fertilizers.

It was found that the width of chili fruits obtained a quadratic growth, where best results were obtained at a dose of 100 ml of cow urine (Figure 4A). With regard to the effects of fertilizers on width of chili fruit, it was observed that the optimum fertilizer was composed of manure + sand washed corresponding width of 40.33 mm plant $^{-1.}$ Possibly the toxic effect of this wood powder on the manure (A1) led to a reduction in the width of chili fruit (Figure 4B).

The cattle manure is one of the richest organic fertilizers in nitrogen, and when used for several consecutive years, enables the organic nitrogen accumulation in the soil, and as a result increases the potential mineralization and its availability to plants (Oliveira et al. , 2010).

As the cow urine dose increased, there was increase in the number of marketable fruits (Figure 5). For organic fertilizers, there was no observed significant responses.

In the quadratic growth in the weight of marketable fruits, it was observed that as cow urine dose increased, there was an increase in the weight of marketable fruits (Figure 6A). The organic fertilizer manure + sand washed in a 2: 1 v/v excelled to wood dust + manure + sand washed in a 1: 1: 1 v/v (Figure 6B). Silva et al. (2011) observed that the use of increasing doses of organic compound in chili crops in the rainy season provided a

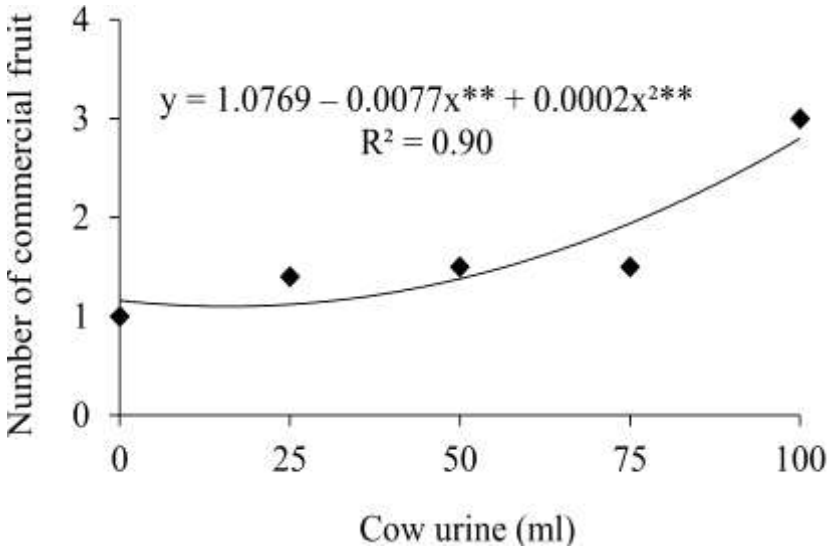

Figure 5. Cow urine doses effect on the number of commercial fruits of chili. *,**Significant at the 5 and 1% probability, respectively.

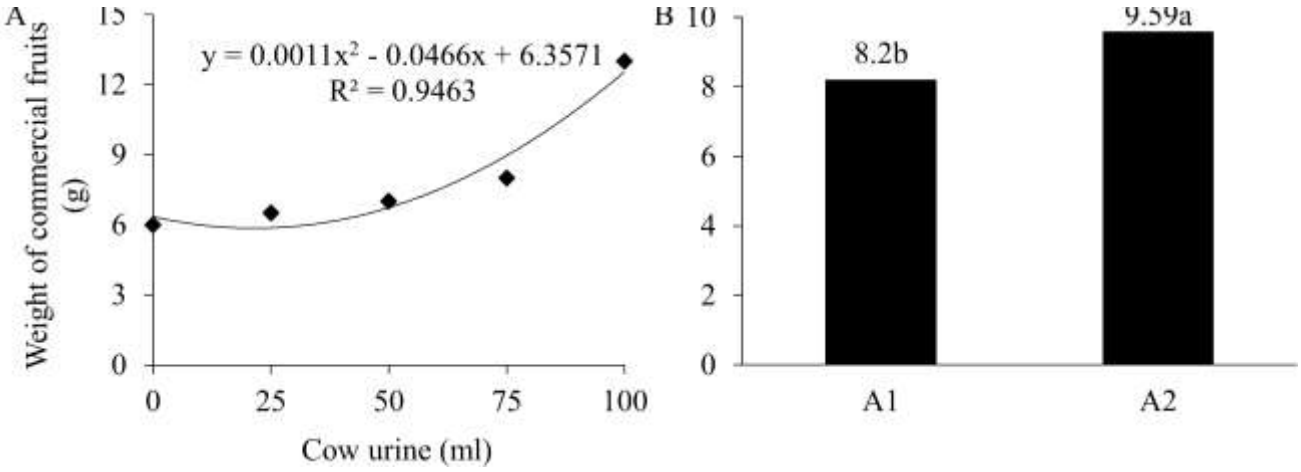

Figure 6. Effect of cow urine doses of (A) in weight commercial chili fruits in fertilizer feature (B), * and ** significant at the 5 and 1% probability, respectively.

linear increase in the average weight of commercial fruits.

Conclusion

The dose of 100 ml of cow urine gave good results in the production of chili. The organic manure fertilizer bovine + washed sand is great for chili. It is not recommended to incorporate wood powder in organic fertilizers.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Root spatial distribution in coffee plants of different ages under conservation management system

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Root system growth and soil structure are interdependent and the threshold of separation between both of them is complex. However, by the evaluation of soil pore space, it is possible to characterize the root system growth environment. The aim of this study was to evaluate the effect of conservation management system over time on pore distribution and on root system development of coffee plantation in Cerrado Oxisol, located in the state of Minas Gerais, Brazil. Two coffee plantation areas were sampled (3 or 6 years old). Trenches were dug lengthwise along the planting row to expose the root system and the vertical profiles were divided into 0.05 × 0.05 m grid cells (0.70 × 1.50 m grid), totaling 420 sample sites. Digital images were taken and using the computer software Safira, it was measured layers along the soil profile, which was spaced 0.10 m apart. Disturbed and undisturbed soil cores the length, the surface area and the volume of the root system were sampled at 0.20 to 0.34, 0.80 to 0.94, and 1.50 to 1.64 m depths layers, in order to determine particle size, total porosity, and pore size distribution. The 3-years coffee stand had the greatest volume of macropores and the largest number of absorbent roots, besides a noticeable root system growth below 1 m depth. The 6-years old coffee stand presented pores reconfiguration due to increase in the intermediate-sized pores and to the uniform root distribution in both horizontal and vertical directions up to 0.9 m depth.

Key words: Gibbsitic Oxisol, pore system, 2D images, geostatistics, root system distribution.

INTRODUCTION

Soil pore space results from mineral particles organization into water-stable aggregates. During soil structural organization, there are alterations in pore distribution and configuration, which interfere with root system distribution and plant growth (Carducci et al., 2013, 2014a, b, 2016; Silva et al., 2014).

Among several reasons, soil structural organization may be related to the use of soil conditioners, such as

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Table 1. Particle size distribution of a very clayey Rhodic Haplustox at different depth under soil conservation management system.

Depth (m)	3-years[(1)]			6-years[(2)]		
	Clay	Silt	Sand	Clay	Silt	Sand
	g kg^{-1}					
0.20-0.34	869	65	66	819	24	157
0.80-0.94	895	59	46	848	25	127
1.50-1.64	904	57	39	886	25	89

[(1)]3 years-old; [(2)] 6 years-old.

agricultural gypsum associated with soil organic matter, which influences the formation of organo-mineral complexes, especially calcium and organic radicals derived from the decomposition of plant residues (Silva et al., 2013, 2014).

Based on this premise, some coffee growers in Minas Gerais have adopted a soil management system which consists of periodically mowed grass cultivation (*Brachiaria decumbens*) between planting rows as a permanent source of organic matter, associated with the building of fertility in deep soil layers, which is possible due to the adoption of a deep, fertilized planting furrow and to the gypsum application on soil surface. This management improves physical and chemical soil conditions, as well as increases root system depth (van Raij, 2008; Serafim et al., 2013a, b, c; Silva, 2012; Carducci et al., 2014b; Silva et al., 2014).

Cerrado Biome, one of the main coffee-producing regions of Brazil, has faced prolonged and more frequent dry spells. The region has the highest proportion of oxidic Latosols (Oxisols). Although, these soils present good physical quality, they have bimodal pore size distribution, that is, this soil has high values of macro and microporosity, but with low proportion of intermediate diameter pores, which hold the readily available soil water to plant roots (Oliveira et al., 2004; Carducci et al., 2013).

Thus, the adoption of water conservation practices might mitigate soil water deficit in Cerrado, which is partly possible due to the increase in intermediate pores in Latosols (Barbosa et al., 2014) and to plant root system development, especially in plants younger than 3-years (Carducci et al., 2016), which improves the use of deep water stored in the soil. This practice is possible and was confirmed by other authors (Silva et al., 2014; Santos et al., 2014; Serafim et al., 2013a, b, c).

The increment of intermediate sized pores and the increase in effective root zone depth in oxidic Latosols contributed to increase coffee production. However, the adoption of new management practices able of altering the pore distribution and promote root growth must be preceded by scientific studies. Thus, the aim of this study was to evaluate the effect of conservation management system over time on pore distribution and on root system development of coffee plantation in Cerrado Oxisol located in the state of Minas Gerais, Brazil.

MATERIALS AND METHODS

Study area description

The study was carried out in two coffee plantations near São Roque de Minas, in the upper São Francisco river basin, Minas Gerais, Brazil. The soil studied is a very clayey Rhodic Haplustox (*Latossolo Vermelho* in the Brazilian System of Soil Classification-Embrapa, 2013; Santos et al., 2013), with ca. 86% clay, from which 55% is gibbsite, and 25% is kaolinite, according to thermal analyses (Carducci et al., 2014a, b) (Table 1). Two plantations were sampled: a young stand (3 years-old, planted in November, 2008) stand, at 20°15'45" S and 46°8'17" W, at 850 m asl, and an old stand (6 years-old, planted November, 2005), at 20°11'35"S and 46°22'07" E, at 841 m asl. Both plantations were planted and conducted according to the soil conservation management system described subsequently.

The fields were managed by using soil conservation practices for coffee cultivation. Planting was carried out in a narrow row, with spacing of 2.5 m between plants and 0.65 m between rows; one plowing and two harrowing were carried out to prepare the land, followed by application of dolomitic limestone (4 mg ha^{-1}) and agricultural gypsum (1.92 mg ha^{-1}) in the total area. Furrows of 0.60 m depth and 0.50 m width were made with a subsoiler coupled to a rotary tiller, which enabled homogeneous distribution of liming and fertilizers to 0.40 m depth.

It was applied 8 mg ha^{-1} (2 kg m^{-1}) of dolomitic limestone and basic fertilizer in the furrow. Afterwards, *B. decumbens* (*Syn*.Urochloa) was planted between rows as cover crop and mowed periodically (Serafim et al., 2013a, b, c; Silva et al., 2013). After the grass had been established, coffee seedlings were planted. Three months later, 7 kg m^{-1} of agricultural gypsum was applied on soil surface of the coffee rows, followed by hilling around the base of the plants (Serafim et al., 2011, 2013a, b, c).

In order to designate the treatment types, terms were chosen according to the age of the coffee plants in each field. According to Nutman (1933a, b, 1934), who published the most comprehensive study about the root system of Arabic coffee, the main morphological and physiological characteristics of the root system complete their development between 5 and 6 years after planting. After this period, the coffee plant is considered adult, and its roots renew constantly; however, it maintains the typical conformation to the end of life cycle.

Soil sampling and physical characterization

Three random trenches were dug lengthwise along the plant row and three replications of disturbed and undisturbed soil cores were sampled from 0.20 to 0.34, 0.80 to 0.94, and 1.50 to 1.64 m depth

layers. The choice of depths was based on the cultural profile method (Tavares Filho et al., 1999), which detects morphological alterations caused by the management system. The disturbed samples were used to determine soil particle size by the pipette method (Embrapa, 2011).

Undisturbed soil cores were collected in volumetric rings (80 cm^3) in the middle of the spacing between plants (0.65 m), using a Uhland-type sampler in order to obtain the water retention curve. Samples were subjected to the following matric potentials of water in the soil: -2, -4, -6, and -10 kPa, using Buchner funnels and -33, -100, -500, and -1500 kPa in the porous plate, with the saturated soil samples placed in pressure chambers. After samples stabilization in each of thematric potential (Ψ_m), they were weighed and kept in incubators at 105 to 110°C for 48 h, to determine the soil bulk density (BD) and the corresponding water content (θ). Macroporosity was calculated by the difference between total porosity (TP = (1 - Ds / Dp)) and microporosity (water retained at Ψ_m -6 kPa) (Embrapa, 2011).

In order to better discriminate the soil pore diameter, information from the water retention curve was used, by using the Bouma equation (1991), which considers cylindrical-shaped pores:

$$D = 4 \sigma \text{ Cos } \theta / \Psi_m$$

where D is the pore diameter (µm); σ is the water surface tension (73.43 kPa uM at 20°C); θ is the contact angle between the meniscus and the capillary tube wall (considered 0), and Ψ_m the matric potential (kPa). The Mesopores (intermediate-sized pores) were classified according to Barbosa et al. (2014).

After reaching equilibrium at the matric potential of -6 kPa (considered as the field capacity for Latosols - Oxisols) (Ferreira and Marcos, 1983; Silva et al., 2014), some samples were weighted and used to determine the soil resistance to penetration (RP), by using a bench top electronic penetrograph (Tormena et al., 1998; Lima et al., 2012).

Assessment of the root system of the coffee plants

For the study of the root system distribution in the 3 and 6-years old stand, three 0.70 × 1.50 × 1.50 m trenches were dug lengthwise the planting row. The vertical trench wall stood in the projection of the coffee canopy at 0.10 m distance from the plant stem. Considering the spacing between plants of 0.65 m, the trench was arranged in order to have a coffee plant in its center. Subsequently, the soil was scarified at 0.03 m to expose the roots, and a grid (0.05 × 0.05 m cells) with the same dimensions as the trench, which consisted of 420 sample units, as detailed by Carducci et al. (2014a, 2015a). The following variables were analyzed: volume (mm^3), surface area (mm^2), length (mm), and root diameter (mm), by using the computer software Safira (Jorge and Silva, 2010), as described in Carducci et al. (2015a).

Statistical analyses

The experiments consisted of complete randomized split-plot design, in both stand age, in which the plot referred to the age of the plantation and the subplot referred to the depth. After the data normality generated by the Shapiro-Wilk method was checked, analysis of variance was carried out, and the means were compared by the Scott-Knott test at 5% of probability, using the computer statistical analysis system Sisvar (Ferreira, 2011).

Data on volume, surface area and root length were subjected to the frequency distribution test and the classes were defined by the Stunges formula:

$$K = 1 + 3.22 \times \log n$$

where K is the number of classes and *n* is the total number of individuals in the population (420 sample units [grid with 0.70 m wide × 1.50 m long, subdivided into 0.05 × 0.05 m cells]).

Roots were classified in three different diameters: 1, fine or absorbent roots (Ø≤1 mm); 2, intermediate or support for the absorbent roots (1<Ø≤3 mm); 3, thick or permanent roots (Ø>3 mm), according to Rena and Guimarães (2000) and Motta et al. (2006).

The surface maps of the root spatial distribution in all soil profiles for both stand ages was carried out by interpolation through the inverse square of the distance method (ISD), using the ArcGIS 9.3 software (ESRI, 2009).

RESULTS AND DISCUSSION

Characterization of the soil physical properties

In both stand ages, it was verified that soils were very clayey in all the profile. Clayey Oxisols generally have low bulk density (BD) and resistance to penetration, as observed in the present study (Table 2).

The soil layer at 1.50 to 1.64 m depth (Bw horizon) is a reference that reflects the intrinsic structural condition of very clayey Oxisols (Ferreira et al., 1999; Severiano et al., 2011a, b). The bulk density (BD) and resistance to penetration (RP) values at other depths confirm the good physical condition of the soil, partly due to the initial effects of the tillage adopted, and to the maintenance of soil organic matter in this management system (Serafim et al., 2013a, b, c; Silva et al., 2012, 2013).

Thus, in order to detect the influence of conservation management practices over time and sampling depth on the pore diameter distribution, factor analysis was carried out with a view towards possible interactions between both factors. The interaction was not observed for all pore diameter classes. However, differences between the conservation management practices over time for some pore diameter classes were detected, as presented in Tables 3 and 4.

The Oxisol under 3-years coffee stand showed higher pore volume in the diameter classes >147 µm (Ψm -2 kPa) and 2.9 to 0.6 6 µm (referring to the Ψm between -100 and -500 kPa), while in the condition of 6 years coffee stand, higher pores volume was observed in the classes of 147 to 9 µm (referring to Ψm between -4 and -33 kPa), and these classes present inter-aggregated pores (macropores and intermediate pores), which suggests a more homogeneous arrangement of the pores with better distribution between intermediate pores (Carducci et al., 2015b), as the management system is consolidated (Table 3)

For the diameter classes < 0.2 µm (Ψ_m -1500 kPa), high volume of micropores was observed (intra-aggregate pores) in both plantations, which means that strong water retention in these gibbsitic Oxisol makes the water unavailable to plants, as previously reported by Carducci et al. (2011).

In both stand ages, the soil at 0.20 to 0.34 m and 0.80 to 0.94 m depth, in comparison with the soil at 1.50 to

Table 2. Rhodic Haplustox physics attributes under 3- and 6-yrs old coffee stand in different depth.

Depth (m)	3-years	
	PR$^{ns(1)}$ (MPa)	Bd*$^{(2)}$ (Mg dm^{-3})
0.20-0.34	0.26^{a} (±0.02)	0.78^{b} (±0.05)
0.80-0.94	0.20^{a} (±0.003)	0.91^{a} (±0.005)
1.50-1.64	0.37^{a} (±0.07)	0.96^{a} (±0.03)
	6-years	
	PR^{ns} (Mpa)	Bd^{ns} (Mg dm^{-3})
0.20-0.34	0.19$^{a(±0.03)}$	0.95$^{a(± 0.05)}$
0.80-0.94	0.14$^{a(±0.04)}$	1.04$^{a(±0.01)}$
1.50-1.64	0.13$^{a(±0.04)}$	1.03$^{a(± 0.03)}$

$^{(1)}$PR: Penetration resistance on -6 kPa potential matric. $^{(2)}$Bd: Bulk density. ns: Non significant, *significant ($p < 0.05$). Means followed by the same letter in the columns do not differ by the Scott Knott test at 5%. Between parenthesis: mean standard error (n=3).

Table 3. Means values of pores diameter distribution in Rhodic Haplustox under 3- and 6-years old coffee stand.

Stand	Pores diameter distribution (µm)						
	> 147**	147 - 73*	49 - 29**	29 - 9*	2.9 - 0.6**	< 0.2^{ns}	TP^{ns}
Age				cm^3 cm^{-3}			
3-years	0.130$^{a(±0.01)}$	0.063$^{b(±0.009)}$	0.031$^{b(±0.004)}$	0.038$^{b(±0.003)}$	0.020$^{a(±0.001)}$	0.259$^{a(±0.02)}$	0.62$^{a(±0.01)}$
6-years	0.087$^{b(±0.006)}$	0.092$^{a(±0.006)}$	0.048$^{a(±0.01)}$	0.050$^{a(±0.002)}$	0.008$^{b(±0.001)}$	0.260$^{a(±0.007)}$	0.61$^{a(±0.01)}$

Means followed by the same letter in the column do not differ by the Scott-Knott test at 5% probability. ns: Non significant; *significant ($p < 0.05$); ** significant ($p< 0.01$). TP: total porosity. Between parenthesis: mean standard error (n = 3).

Table 4. Means values of pores diameter distribution at different depth under soil conservation management system.

Depth (m)	Pore diameter distribution (µm)						
	>147***	147-73**	49-29**	29 -9*	2.9-0.6*	< 0.2^{ns}	TP*
				cm^3 cm^{-3}			
0.20-0.34	0.128$^{a(±0.01)}$	0.087$^{a(±0.006)}$	0.041$^{a(±0.004)}$	0.051$^{a(±0.004)}$	0.015$^{a(±0.003)}$	0.231$^{a(±0.02)}$	0.63$^{a(±0.01)}$
0.80-0.94	0.118$^{a(±0.01)}$	0.093$^{a(±0.006)}$	0.046$^{a(±0.003)}$	0.044$^{a(±0.001)}$	0.016$^{a(±0.002)}$	0.241$^{a(±0.006)}$	0.63$^{a(±0.01)}$
1.50-1.64	0.080$^{b(±0.008)}$	0.052$^{b(±0.01)}$	0.031$^{b(±0.005)}$	0.037$^{b(±0.004)}$	0.010$^{b(±0.002)}$	0.306$^{a(±0.01)}$	0.58$^{b(±0.01)}$

Means followed by the same letter in the column do not differ by the Scott-Knott test at 5% probability. Ns: Non significant; *Significant ($p < 0.05$); **Significant ($p< 0.01$). TP: Total porosity. Between parenthesis: mean standard error (n = 3).

1.64 m depth, showed higher intermediate and inter-aggregate pore volume, especially of the class >147 µm. However, it presented lower pore volume in relation to potential under -1500 kPa (pores < 0.2 µm), which means that there was higher volume of pores with larger diameter at shallow depths (Table 4).

Soil tillage in the row, carried out due to the coffee planting, significantly altered the soil pore distribution, confirming what was observed by Carducci et al. (2013) regarding the pores bimodality of the these Oxisols.

Since intermediate diameter pores are important for roots penetration, aeration and retention of readily available water (Carducci et al., 2015b), a study was carried out with soil pore classes between 73 to 49 µm (referring to Ψ_m from -4 to -6 kPa) and from 9 to 2.9 µm (referring to Ψ_m from -33 to -100 kPa) (Table 5) due to significant interaction between field and sampling depth.

For the diameter classes referring to these intervals, the highest values were found in the 6-years stand age, and were more noticeable at 0.80 to 0.94 m depth. At this

Table 5. Means values of pores diameter (cm^3 cm^{-3}) of intermediate class (73-49 e 9-2,9 µm) at different depth under 3 and 6-yrs old coffee stand with soil conservation management system.

Depth (m)	3-years	6-years
	73 - 49* (µm)	
0.20-0.34	$0.041^{bA(\pm0.007)}$	$0.053^{aB(\pm0.003)}$
0.80-0.94	$0.056^{bA(\pm0.004)}$	$0.104^{aA(\pm0.007)}$
1.50-1.64	$0.044^{aA(\pm0.006)}$	$0.043^{aB(\pm0.006)}$
	9 - 2.9**(µm)	
0.20-0.34	$0.015^{aA(\pm0.0006)}$	$0.019^{aB(\pm0.003)}$
0.80-0.94	$0.018^{bA(\pm0.0009)}$	$0.044^{aA(\pm0.001)}$
1.50-1.64	$0.016^{aA(\pm0.001)}$	$0.013^{aB(\pm0.002)}$

Means followed by the same capital letter in the column and small letter on the line do not differ by the Scott-Knott test at 5% probability; *Significant ($p < 0.05$); **Significant ($p < 0.01$). Between parenthesis: mean standard error ($n = 3$).

depth, the highest water uptake by plants was observed, as verified by Santos et al. (2014), when monitoring the spatial distribution of soil moisture at the same depths and experimental area. Sampling depth was not significant in the 3-years coffee stand, which could be related to the plants at younger stage (3 years age), being insufficient to cause alterations in these pore diameter classes.

It is suggested that subsoil tillage at 0.60 m depth allowed sharp increase in macroporosity along the soil profile (Table 4). Since with the increase in the implantation time, new soil structure reorganization occurred.

This fact was promoted by the joint action of climatic processes and conservation practices carried out in this management system, which caused the inter-aggregate pores go from the largest c diameter pore class to the intermediate class (Tables 3 and 5). This alteration is positive since intermediate diameter pores are responsible for the greater water availability to plants, which may minimize hydric stress during dry spell periods, a typical phenomenon in the Brazilian Cerrado region.

Root classification of the coffee plants

By the frequency distribution tests, roots were clustered into three different classes (Figures 1 and 2). It was verified that the 3-years coffee stand presented greater number of roots with lower surface area (< 77 mm^2) and lower volume (< 35 mm^3), besides being short (< 21 mm) along the whole soil profile.

It is important to highlight that roots growth had excellent performance in 3-years stand age, since they reached depth greater than 1 m, showed typical conformity of a mature plant, and had greater root concentration near the trunk, decreasing the number of roots and the depth gradually in areas close to the periphery of the canopy projection of the coffee plant (Rena and Guimarães, 2000).

Figures 1 and 2 show a great number of the roots with small area and volume (fine roots) in all the soil profile. Since fine roots are the most efficient in water absorption and nutrient uptake (Jesus et al., 2006), this may suggest positive plant response to edaphological conditions caused by management practices.

In both stand age, a great number of fine roots with lower volume (<35 mm^3) was observed along the soil profile, which may be related to the compatibility between the root diameter with some pore diameter classes. This fact is relevant when considering the suggestions of Carducci et al. (2014b) on porosity studies carried out with X-ray CT scan on the 6-years stand age, in which the soil fine macropores (Ø = 1 mm), as well as the large mesopores (Ø = 0.2 mm), favored the development of fine roots (Ø ≤ 1 mm).

From the classification of roots by diameter (Rena and Guimarães, 2000), it was observed that in the 3-years coffee stand a greater number of fine roots (Ø ≤ 1 mm) at the 0.60 to 0.90 m depth layer and intermediate roots (1< Ø ≤ 3 mm) predominated at 0.60 m depth. On the other hand, in the 6-years coffee stand there was a higher concentration of roots with a diameter range of 1 < Ø < 2 mm at 0.90 to 1.20 m depth and the 2 < Ø ≤ 3 mm diameter class at the 0.60 to 0.90 depth layer, and fewer fine roots along the profile (Table 6).

Pore distribution 3D images and coffee root system 2D images were evaluated in the same experimental study area by Carducci et al. (2015a), who also observed the presence of fine roots in layers below 0.80 m depth, especially in younger coffee plants (3 years old), a fact that has not been reported yet in the scientific literature on coffee crop management (Tables 6 and 7).

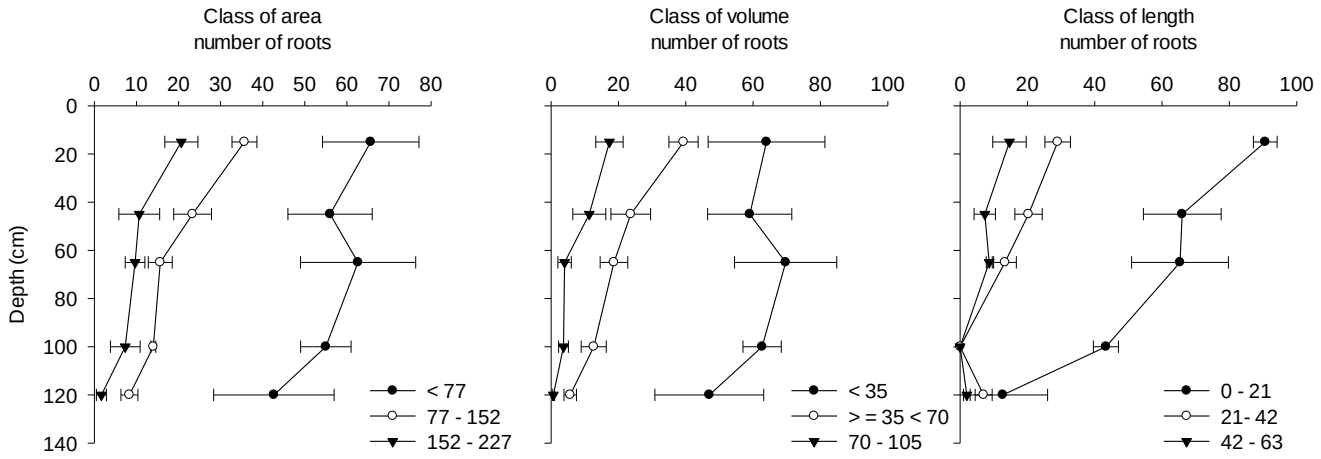

Figure 1. Distribution in classes of the variables: superficial area (mm^2), volume (mm^3) and length (mm) of roots in 3-yrs tillage under conservation system. The Error bars are mean standard error.

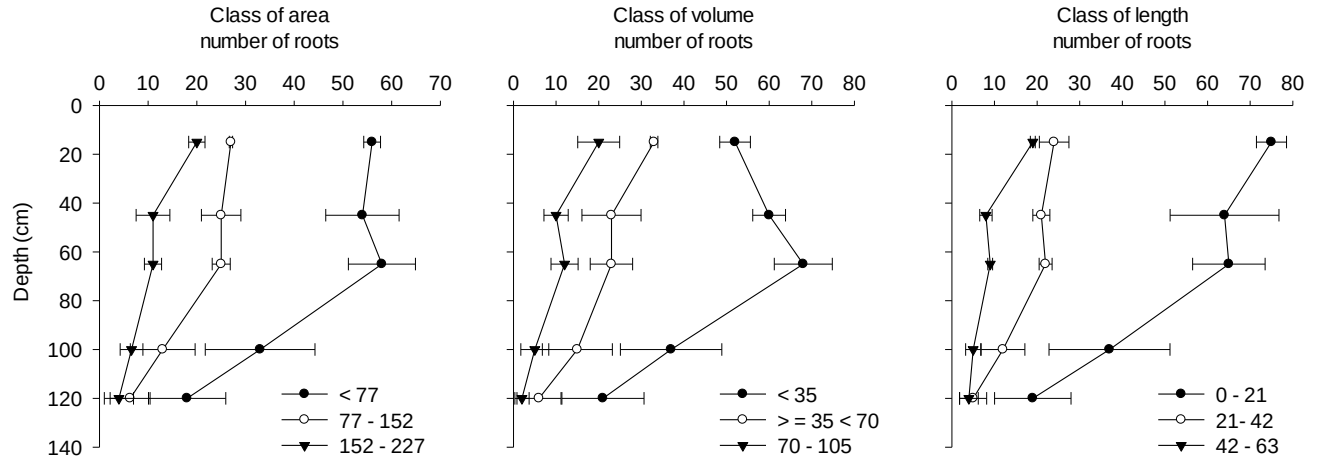

Figure 2. Distribution in classes of the variables: superficial area (mm^2), volume (mm^3) and length (mm) of roots in 6-yrs tillage under conservation system. The Error bars are mean standard error.

Table 6. Mean values of diameter roots class to 3-yrs old coffee stand under soil conservation system.

Depth (m)	Ø ≤ 1	1>Ø < 2	2 > Ø ≤ 3	Ø > 3
0-0.30	$0^{cB(\pm0.3)}$	$74^{aA(\pm3)}$	$55^{aA(\pm6)}$	$34^{bA(\pm10)}$
0.30-0.60	$4^{bB(\pm2)}$	$52^{aA(\pm7)}$	$55^{aA(\pm9)}$	$12^{bB(\pm5)}$
0.60-0.90	$44^{aA(\pm9)}$	$34^{aB(\pm5)}$	$13^{bB(\pm4)}$	$3^{bB(\pm1)}$
0.90-1.20	$20^{aB(\pm11)}$	$40^{aB(\pm2)}$	$14^{aB(\pm3)}$	$3^{aB(\pm0.5)}$
1.20-1.50	$15^{aB(\pm10)}$	$27^{aB(\pm6)}$	$9^{aB(\pm2)}$	$1^{aB(\pm0.3)}$

Means followed by the same capital letter in the column and small letter on the line do not differ by the Scott-Knott test ($p < 0.05$). Ø (mm). Between parenthesis: mean standard error (n=3). Average roots number refers to 0.21 m^2.

In the same study, the authors attributed their results to the higher volume of pores with diameter <2 mm detected by X-ray CT scan. These pores are related to good root system development (Tables 3 and 4). The Pearson's correlation tests showed that the root distribution was associated with the pore distribution of the gibbsitic

Table 7. Mean values of roots diameter class to 6-yrs old coffee stand under soil conservation management system.

Depth (m)	Ø ≤ 1	1> Ø < 2	2 > Ø ≤ 3	Ø > 3
0-0.30	$0^{bA(\pm0.3)}$	$77^{aA(\pm1.5)}$	$52^{aA(\pm5)}$	$26^{bA(\pm5)}$
0.30-0.60	$1^{bA(\pm0.8)}$	$65^{aA(\pm4)}$	$25^{bB(\pm9)}$	$10^{bA(\pm8)}$
0.60-0.90	$19^{bA(\pm18)}$	$54^{aA(\pm11)}$	$28^{bB(\pm10)}$	$8^{bA(\pm5)}$
0.90-1.20	$7^{aA(\pm6)}$	$33^{aB(\pm14)}$	$17^{aB(\pm10)}$	$3^{aA(\pm2)}$
1.20-1.50	$4^{aA(\pm3)}$	$17^{aB(\pm9)}$	$7^{aB(\pm5)}$	$2^{aA(\pm2)}$

Means followed by the same capital letter in the column and small letter on the line do not differ by the Scott-Knott test ($p<0.05$). Ø (mm). Between parenthesis: mean standard error (n=3). Average roots number refers to 0.21 m^2.

Oxisol under the same management system.

The occurrence of dry spells and prolonged drought periods are common in the study area. Thus, the presence of deep fine or absorption roots is positive, since the water content will be almost always available to coffee plants in these layers, as reported by Silva (2012) and Santos et al. (2014), which may minimize the hydric stress and increase yield.

Spatial distribution of coffee root system

In order to simplify the visualization of regions with and without roots, a surface map was made for the root spatial distribution of both stand ages (3- and 6-years old) (Figure 3).

Greater root growth at depth (roots presence detectable at 1.45 m depth) and root concentration in the upper soil layer, with an irregular profile distribution in the profile horizontal direction occurred in the 3-years coffee stand. This result was due to the conservation practices that influenced the increase in organic matter, the fertility building, the erosion control, and boosted the root growth at depth, favored rapid plant establishment during its first years of planting.

The root distribution was relatively uniform in the vertical direction until 0.90 m depth and reached 0.70 m horizontally in the 6-years stand age. Therefore, it had good soil exploration and root growth in both directions, which allows greater water absorption and nutrients uptake. Thus, it is evident that the development of the root system in all the soil profile over the time analyzed for the management practices employed in the area.

It was observed that root system growth stabilization, which was verified by a clear homogeneous occupancy of the area at depth (Figure 4) in the 6-years stand age. The plant evaluated in the 3-years stand age revealed root growth at depth, but lower lateral root branching, differently from the plant in the 6-years stand age, which showed horizontal branching along the profile, up to approximately 0.90 m depth. It should be mentioned that coffee plants are considered physiologically mature at the age of three years; however, their root system completes its development only at the age of 5 to 6 years.

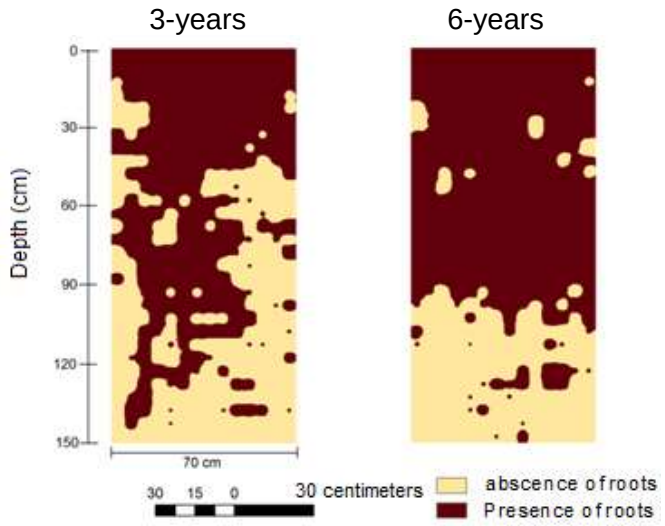

Figure 3. Spatial distribution of roots system in Rhodic Haplustox under 3- and 6-yrs old coffee stand.

In both stand ages, the percentage of the area occupied by roots follows a decreasing trend, according to the soil depth (Figure 4), which is explained by the reduction in the number of intermediate roots 1< Ø < 2 mm; 2 < Ø ≤ 3 mm (Tables 5 and 6), along the soil profile. It was observed that the 3-years coffee stand compared with the 6-years coffee stand presented higher percentage of area occupied by the roots at deeper layers (from 0.90 to 1.20 and 1.20 to 1.50 m), while the 6-years coffee stand presented most of the area occupied by the roots down through the 0.90 m layer.

Ramos et al. (2013), using the same experimental area, verified that even at 0.80 m depth, Ca^{2+} concentration was approximately 2 $mmol_c$ dm^{-3}, which according to Ritchey et al. (1980) is sufficient to normalize root growth and ensure rooting of coffee plants at depth.

Furthermore, the chemical improvement promoted by liming and fertilizers, in addition to the 7 kg m^{-1} of agricultural gypsum applied to the surface associated with the increase of organic residues on the soil surface (Caires et al., 2001) from the grasses grown inter-row

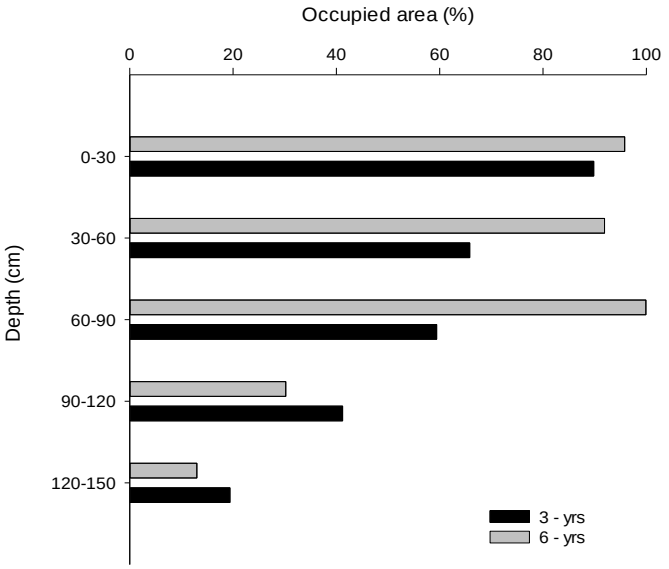

Figure 4. Area occupied by the coffee plant roots along the profile of a Rhodic Haplustox.

(*Brachiaria* species), as well as the coffee itself, should have acted jointly for the construction and maintenance of soil fertility, as well as for the Al^{3+} toxicity reduction, which is primarily responsible for root growth retardation (Carvalho-Pupatto et al., 2003), justifying the uniform growth of the coffee plant roots in the 6-years coffee stand to 0.9 m depth (Figure 4).

Conclusions

Management practices that adopt deep tillage promoted beneficial alteration in the soil pore configuration, which is associated with the use of agricultural inputs, such as gypsum, limestone, together with a balanced fertilization program, and the increase in organic matter, provided the deep root system development of the coffee plants.

During the first years, there was significant amount of inter-aggregate soil pores. However, there was pores reconfiguration with the adoption of management practices over time, which increased the amount of intermediate diameter pores. Coffee plants at the age of three years in the absence of chemical and physical soil limitations had their root system deeper than 1 m. Six years later, there was uniform distribution of roots, both laterally and along the soil profile, reaching up to 0.90 m depth, which evidences the effect of management practices over time on the stabilization of the root network along the gibbsitic Oxisol profile.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Analytical approaches for modeling tree crown volume in black wattle (*Acacia mearnsii* De Wild.) stands

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In this paper, four strategies were proposed for modeling tree crown volume using as independent variable stem variables, crown variables, combination of stem and crown variables, and stem volume. We used a dataset comprised of 170 trees from 12 temporary plots located in forest stands in southern Brazil. Models composed of stem variables presented weaker predictive ability. The best model contained crown variables, which explained 78.95% of observed variability. However, implementation of such model is bounded by its independent variables, which are not often measured in forest inventories. The model composed by diameter at breast height and crown length proved to be an adequate modeling approach. The predictive capability was kept by model $v_c = \beta_0 dbh^{\beta_1} cl^{\beta_2} + \varepsilon_i$, which is composed by most easily measured variable in a forest - diameter at breast height, also by the most easily acquirable crown variable - crown length. In our suggested model, estimates of β_1 and β_2 are coefficients that convert volume of a regular geometric solid – *RGS* is *dbh*² times crown length) - into crown volume, whilst estimate of β_0 is an allometric constant.

Key words: Crown modeling, diameter at breast height, crown length, crown volume.

INTRODUCTION

Tree crowns are responsible for light interception, thus contribute to the regulation of individual growth, and stand yield (Burkhart and Tomé, 2012; Oliver and Larson, 1996; Cluzeau et al., 1995). Tree crown is an important variable to elucidate what occurs in forest stands and its dynamics and, thus, a great effort has been devoted towards quantification of tree crowns (Burkhart and Tomé, 2012), as well as to its modeling (Godin, 2000). Therefore, crown volume turns to be an adequate variable to assess forest dynamics and to improve

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reliability of growth and yield models (Bragg, 2001). Several authors have been exploring three main methods of estimating crown related variables, particularly: 1) approximations to geometric shapes; 2) modern remote sensing techniques aided by computer procedures; and 3) regression methods.

With respect to geometric shapes approximations, Osborn (1962) estimated crown volume using volume of cones while Mawson et al. (1976) compared 15 different Euclidean geometric shapes and obtained crown volumes based on measurements of crown width, length and crown radii. Tucker et al. (1993) estimated crown volume by means of irregular pyramids based on eight measured crown radii. Despite their pioneering approximations of geometric shapes to crown shape and volume, nowadays these techniques are believed to be too restrictive for estimating crown variables because crowns do not necessarily assume geometrical shapes (Crecente-Campo et al., 2009). Due constraints and assumptions of using these shapes, such approximations started to be substituted by more flexible and sophisticated models. Smith (1994) used a distance-dependent individual-tree model to calculate crown shape of Loblolly pine (*Pinus taeda*) in relation to a tree's competitor's crowns. Dubrasich et al. (1997) fitted crown area, taper, width and length models of mixed conifer and mixed conifer-hardwood stands. Hann (1999) proposed adjustable models to predict crown profile for Douglas-fir stand-grown trees by using measurements or predictions of largest crown width. Bragg (2001) developed nonlinear models for crown width estimation of 24 species based on tree diameter at breast height and on local basal area. Bechtold et al. (2002) pointed that regression models were a better alternative for estimating mean crown diameter of hardwoods relatively to field measurements and ocular estimates. Gill et al. (2000) developed individual tree crown radius models for several species to predict canopy cover. Crecente-Campo et al. (2009) used both geometrical shapes and equations to model crown profile. Rupšys (2015) developed stochastic models of crown widths. Power et al. (2012) proposed equations of crown length, profile, shape and surface area of black spruce (*Picea mariana*) and white spruce (*Picea glauca)* to characterize crown characteristics of these species. Sattler and LeMay (2011) proposed a simultaneous system of nonlinear equations to predict crown length and crown radius in structurally complex stands.

Recently, remote sensing methods provide competitive approaches for acquiring not only faster measurements than previous methods, but also accurate estimates of crown (Strîmbu and Strîmbu, 2015; Hu et al., 2014; Eerikäinen, 2009; Næsset, 2002; Hyyppä et al., 2000; Næsset, 1997). However, modern estimation methods based on remotely sensed acquired data may not be affordable in many circumstances due to challenges presented by high computational power, big data, logistics, data acquisition costs (Strîmbu and Strîmbu, 2015; Wulder et al. 2012) and software licenses (Sönmez, 2009; Zhang et al., 2007; Song et al., 2003). Therefore, traditional methods of estimation still show potential. The study of crowns is of considerable importance to assess a forest system. However, major part of studies have been focusing on modeling crown variables other than crown volume. Even though several crown volume studies were carried out, most kept estimating crown volume based on cones volume, cylinders, paraboloids, ellipsoids, hemispheres, or spheres (Villacorta et al., 2015; Fernández-Sarría et al., 2013; Leites et al., 2013; Velázquez-Martí et al., 2012; Pérez-Cruzado and Rodríguez-Soalleiro, 2011; Roberts et al., 2003; Montgomery and Chazdon, 2000; Van Pelt and North, 1996; Jack and Long, 1992; McPherson and Rowntree, 1988; Kuuluvainen, 1988).

Different from approximating volumes, Rautiainen and Stenberg (2005) and Rautiainen et al. (2008) fitted a curve of Lamé curve family to represent crown profile above maximum crown radius, and assumed a cylindrical form for crown below its maximum radius. Dubravac et al (2009) obtained crown volume assuming it to be a cylinder multiplied by a form factor. It is important to note that only one previous study regarding crown modeling of black wattle (*Acacia mearnsii*) was found (Sanquetta et al., 2015). In addition, this species is the third major cultivated species in Brazil, whose bark is the main source of tannin in the world and whose crown biomass has recently gained interest for energy purposes as raw material for production of pellets (Dunlop, 2005).

This paper proposed four approaches for modeling black wattle crown volume by means of acquired stem and crown related variables that could potentially present a biological and mathematical sense to explain crown volume. Our foremost objective was to suggest an operational and reliable crown volume model.

MATERIAL AND METHODS

Field sampling and data

Data was gathered in 2014, during June and July in black wattle stands in the state of Rio Grande do Sul, Brazil. More specifically, this dataset was collected in Cristal, Encruzilhada do Sul and Piratini counties, these are the regions where major part of black wattle commercial stands are found in the country (Figure 1).

In each stand, four 10 m diameter circular plots (covering 78.54 m^2) were randomly located, totaling 12 plots and 170 trees. Sampled stand ages in Cristal, Encruzilhada do Sul and Piratini averaged between 9 and 11 years old - near the end of black wattle's rotation age. In each plot, all trees were felled and the following variables were measured: diameter at breast height - *dbh* (cm and m) measured at 1.30 m above the ground, total height – *ht* (m), crown diameter - *cd* (m), and crown length - *cl* (m), distance attributed to the distance from first branch at the base of the crown to tree tip (Figure 2). All crowns and stems were measured relative to their length so that we could obtain their volume (m^3).

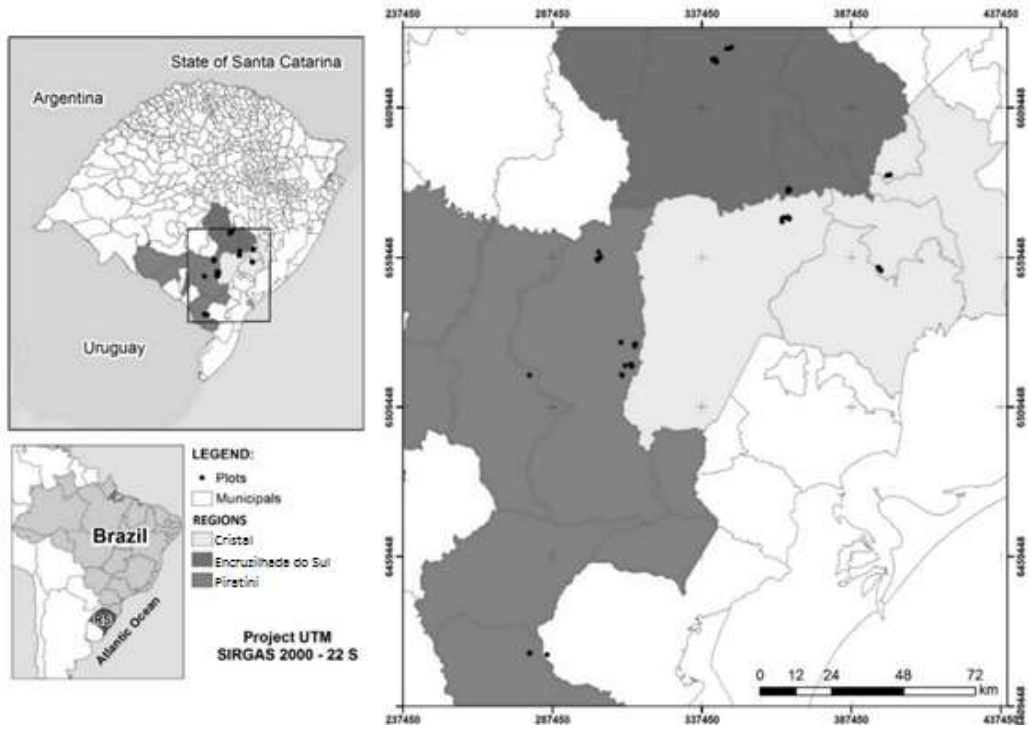

Figure 1. Map of sampled black wattle stands in Cristal, Encruzilhada do Sul and Piratini counties in the state Rio Grande do Sul, Brazil counties.

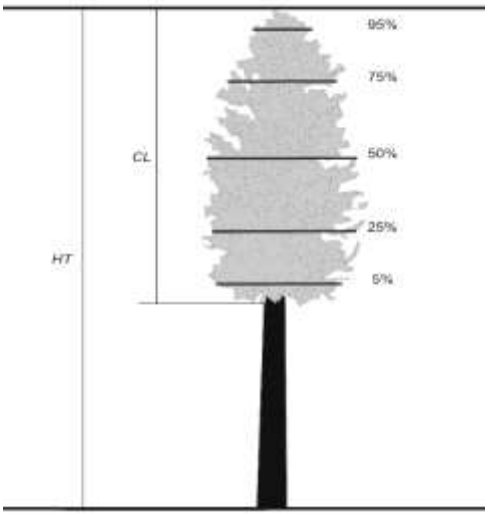

Figure 2. Scheme of how crowns were dissected to obtain volumes of intermediate sections, as well as variables directly measured on the tree, except *dbh*.

In this study, crown volume was considered as a group of components represented by a set of truncated cones and was represented globally, as defined in a review of Godin (2000). Therefore, crown volume was measured based on Huber´s method and it was dissected applying Hohenadl's method. The measurements of crown diameter were taken orthogonally with a tape to obtain an average of measured diameters in a single section. These measurements were taken at positions 5, 25, 50, 75 and 95% of crown length (Figure 2). The measurements were taken after tree felling (Hann, 1999; Biging and Wensel, 1990), since there were no major crown deformations.

The volume of top (95 to 100%) and base (0 to 5%) crown sections were obtained separately and will be further explained. The volume of i intermediate sections (for example, sections 5 to 25%, 25 to 50%, 50 to 75% and 75 to 95% of crown length) were calculated by the following expression:

$$v_{sc} = \sum_{i=1}^{n} \frac{\pi}{4} d_i^2 \, l_i \quad (1)$$

where v_{sc} is the volume of intermediate sections of the crown (m³), d is the average of orthogonal crown diameters (m), and *l* is the length of section *i* (m). Base and top sections volumes of the crown were obtained using the disc method and the volume of a solid generated by rotating the area bounded by the crown axis of symmetry (y-axis), crown length and the function of crown section profile. Volumes of top and base sections were calculated as a concave paraboloid, and were generalized as:

$$v_p = \int_0^l \pi x^2 \, dy \quad (2)$$

where v_p is the volume of the top or base section of the crown (m³) calculated separately, *l* is the section length (m), and *x* is the maximum radii of the section (m). These sections were centered in a Cartesian plane with center on O (0, 0), and symmetry of

parabola is given by the y-axis. The radius measurements were considered half of average diameter of each section and were used as coordinates over the plane. Thus, we could express profile from of top and base sections as a quadratic equation. These coordinates were substituted in the general parabola form function. Crown top and base profile sections were expressed by equation 3:

$$y = a\,x^2 \tag{3}$$

where *y* is the crown profile equation (m), *a* is the coefficient associated with de changes of radius along the section length, and *x* is crown radius along the length of the section (m). Since discs were integrated along the sections' length, radius had to be expressed in terms of section length (*y*):

$$x = \sqrt{\frac{y}{a}} \tag{4}$$

After integrating the found expression for each tree top and base sections profiles, volumes were obtained by the following expression:

$$v_p = \pi \left[\frac{y^2}{2a}\right]_0^h \tag{5}$$

Finally, crown total volume was calculated by:

$$v_c = v_p + v_{sc} + v_p \tag{6}$$

where v_c is tree crown total volume (m³), v_p is volume of crown base or top sections (m³) calculated separately and v_{sc} is volume of a crown intermediate sections (m³). Stem volume (v_s) was obtained using Hohenadl's method and it was measured according to Huber's method. The measurements were taken with a diameter tape along the stem at positions 5%, 15%, 25%, 35%, 45%, 55%, 65%, 75%, 85% and 95% of the total height. The volume of n stem sections was calculated using the following expression:

$$v_s = \sum_{i=1}^{n} \frac{\pi}{4} d_i^2\, l_i \tag{7}$$

where v_s is the volume of sections of stem (m³), d is the mean diameter of section *i* (m), and *l* is the length of section *i* (m). The data set utilized in this study is summarized in Table 1.

Strategic crown volume models

In this paper, we evaluated four different strategies to accurately estimate crown volumes from easily measured variables. These strategies were based on different approaches of crown volume model using stem and crown as independent variables. A Pearson correlation matrix for all independent variables against each other and against crown volume was built to assess relationships. Independent variables were also plotted against tree crown volume aiming to graphically assess explanatory relationships. Our models were adapted to each modeling approach based upon observed relationships.

Strategy 1: stem variables

Initially, we estimated crown volume as a function of diameter at breast height (*dbh*), and total height (*ht*). Four models were proposed and are presented in (8), (9), (10) and (11).

$$v_c = \beta_0 (dbh^2)^{\beta_1} + \varepsilon_i \tag{8}$$

$$v_c = \beta_0 (dbh)^{\beta_1} (ht)^{\beta_2} + \varepsilon_i \tag{9}$$

$$v_c = \beta_0 (dbh^2 ht)^{\beta_1} + \varepsilon_i \tag{10}$$

$$v_c = \beta_0 (1/dbh)^{\beta_1} ht^{\beta_2} + \varepsilon_i \tag{11}$$

Where v_c is crown volume (m³), *dbh* is diameter at breast height (cm), *ht* is tree total height (m), β_i are the coefficients of the models and ε_i is the associated error (m³).

Strategy 2: crown variables

As an alternative strategy, we proposed the same aforementioned models, however, using the largest measured crown diameter (*cd*, m) and crown length (*cl*, m) at this time. Rautiainen et al. (2008) recommended using crown length and maximum crown radius, arguing that these variables were the only ones required to model crown shape. However, in this study, as our objective was to estimate volume, we focused on using crown maximum diameter instead, once it represents a full unidimensional measure of the crown. The models are presented in (12), (13), (14) and (15).

$$v_c = \beta_0 (cd^2)^{\beta_1} + \varepsilon_i \tag{12}$$

$$v_c = \beta_0 (cd)^{\beta_1} (cl)^{\beta_2} + \varepsilon_i \tag{13}$$

$$v_c = \beta_0 (cd^2 cl)^{\beta_1} + \varepsilon_i \tag{14}$$

$$v_c = \beta_0 (1/cd)^{\beta_1} (cl)^{\beta_2} + \varepsilon_i \tag{15}$$

Where v_c is crown volume (m³), cd is crown diameter (m), *cl* is crown length (m), β_i are the coefficients of the models and ε_i is the associated error (m³).

Strategy 3: stem and crown variables

Our third approach uses stem and crown variables (i.g. *dbh* and *cl*) to model crown volume. The fitted models were the same ones as in previous strategies, except for the model which has only diameter as independent variable - model (8) and (12). Models are listed in (16), (17) and (18).

$$v_c = \beta_0 (dbh)^{\beta_1} (cl)^{\beta_2} + \varepsilon_i \tag{16}$$

$$v_c = \beta_0 (dbh^2 cl)^{\beta_1} + \varepsilon_i \tag{17}$$

$$v_c = \beta_0 (1/dbh)^{\beta_1} (cl)^{\beta_2} + \varepsilon_i \tag{18}$$

Where v_c is crown volume (m³), *dbh* is diameter at breast height (m), *cl* is crown length (m), β_i are coefficients of the models and ε_i is the associated error (m³).

Strategy 4: stem volume expansion to crown volume

The last proposed alternative uses of estimates of stem volume ($\hat{v}_s$) for modeling crown volume. For this purpose, Schumacher-Hall model of stem volume (model 9) was fitted and its outputs were

Table 1. Summary of descriptive statistic of the data set used to model crown volume of black wattle stands in Southern Brazil (n=170; 12 sample plots).

Variable	Minimum	Maximum	Mean	Standard deviation	Mean error
dbh (cm)	3.8	23.6	12.9	3.88	0.30
ht (m)	7.7	21.9	16.5	2.92	0.22
cl (m)	0.8	15.6	7.4	2.86	0.22
v_s (m³)	0.0055	0.4773	0.1250	0.08	0.01
cd (m)	0.7	5.6	2.3	0.87	0.07
v_c (m³)	0.2097	150.3095	23.2775	22.22	1.70

Note: *dbh* is diameter at breast height (cm), *ht* is total height (m); *cl* is crown length (m), v_s is stem volume (m³), v_c is crown volume (m³).

used as independent variable in a simple entry crown volume model. The models are presented in (19) and (20).

$$v_s = \beta_0 (dbh)^{\beta_1} (ht)^{\beta_2} + \varepsilon_i \quad (19)$$

$$v_c = \beta_0 + \beta_1 \hat{v}_s + \varepsilon_i \quad (20)$$

Where v_s is stem volume (m³), *dbh* is diameter at breast height (cm), *ht* is tree total height (m), v_c is crown volume (m³), β_i are the coefficients of the models and ε_i is the associated error (m³).

Model fitting

All models were initially fitted using MODEL procedure (SAS Institute Inc., 2002). We have modeled the structural variance of residuals to obtain weights that afterwards were applied to fitted coefficients obtained by *Estimated Nonlinear Generalized Least Squares*, since model outputs resulted in heteroscedastic error terms (Parresol, 2001; Harvey, 1976).

Model comparison and selection

The resulting equations were assessed based on indicators of goodness of fit: adjusted coefficient of determination (R^2_{adj}) and root mean squared error (RSME). Graphical analyses of residuals accounting for model lack of fit was also performed by means of plots of observed versus predicted values, and dispersion and distribution of residuals, following recommendations of Steel et al. (1997). In addition, the White's test accounting for homogeneity of residual variance (White, 1980) and Durbin-Watson's test for independence of residuals (Durbin and Watson, 1950; 1951) were evaluated, as suggested by Greene (2011).

Statistical measures of fit are adequate for original data used, and effectiveness and validation of fitted equations can only be assessed with an independent dataset (Rawlings et al., 1998). Kozak and Kozak (2003) demonstrated that, with seven data sets, cross validation and double cross validation by splitting data rarely granted any additional information on regression models. Thus, due the size of this data set and lack of an additional one, no split data validation analysis was conducted.

In total, thirteen models were fitted: eight models for strategy 1 and 2, three models for strategy 3, and two models for strategy 4 (i.e. one model for estimating stem volume and another for crown volume). To reduce the extensiveness of this study, only the best model for each strategy was discussed. Best overall model was chosen after comparing the best models across proposed strategies.

RESULTS

Strategic crown volume models

The correlation matrix created for all independent variables against each other showed that majority of independent variables were clearly correlated (Table 2). The relationships observed between these variables demonstrated that combinations of inputs should be carefully chosen, once several combinations were highly correlated and that was likely to produce biased regression models due multicollinearity effect. Regarding crown volume, most independent variables presented high capability of explaining the variability of dependent variable, except for *ht*, 1/*dbh*, *cl* and *1/cd*, which presented lowest correlations. The observed relationships indicated that most of the chosen variables for this study presented potential to be effectively utilized for modeling crown volume.

When plotted against crown volume, all independent variables proposed in this paper have presented interesting graphical relationships (Figure 3). Crown variables were the most strongly related variables to crown volume. However, even though stem variables did not present relationships as strong as crown related variables, observed behaviors between stem variables, transformed stem variables and combination of stem and crown variables indicated that reasonable equations were likely to result from models based on such variables.

Strategy 1: stem variables

Models (9) and (11) revealed no statistical significance for both estimates of β_0 and β_2 (Table 3). In model (10) the β_0 estimate was not significant as well. These models presented highest adjusted coefficients of determination and lower RMSE's relatively to model (8). However, when these were refit, no improvements were produced and

Table 2. Pearson correlation matrix for all independent variables and crown volume of black wattle commercial stands in Southern Brazil.

Variable	*dbh*	*ht*	dbh^2	dbh^2ht	*1/dbh*	*cd*	*cl*	cd^2	*1/cd*	cd^2cl	dbh^2cl	v_s	v_c
dbh	1												
ht	0.87	1											
dbh^2	0.98	0.80	1										
dbh^2ht	0.97	0.81	0.99	1									
1/dbh	-0.91	-0.90	-0.82	-0.80	1								
cd	0.77	0.61	0.79	0.79	-0.64	1							
cl	0.29	0.32	0.26	0.26	-0.31	0.28	1						
cd^2	0.72	0.53	0.76	0.77	-0.55	0.97	0.25	1					
1/cd	-0.73	-0.68	-0.69	-0.67	0.70	-0.87	-0.33	-0.73	1				
cd^2cl	0.66	0.50	0.71	0.72	-0.51	0.88	0.51	0.92	-0.64	1			
dbh^2cl	0.83	0.67	0.86	0.86	-0.68	0.72	0.66	0.70	-0.60	0.84	1		
v_s	0.97	0.83	0.99	1.00	-0.81	0.79	0.27	0.76	-0.68	0.72	0.85	1	
v_c	0.71	0.53	0.76	0.77	-0.54	0.87	0.52	0.89	-0.66	0.97	0.88	0.76	1

Note: *dbh* is diameter at breast height (cm), *ht* is total height (m); *cl* is crown length (m), v_s is stem volume (m^3), v_c is crown volume (m^3).

worse fit statistics were observed. Differently from previous fitted models, model (8) generated an equation in which all of its estimators were significant. Additionally, this model did not violate the assumptions of regression adjustments, as indicated by White's and Durbin-Watson's statistics. Model (8) was able to explain 58% of crown volume variability and presented a mean error of the estimate of 14.37 m^3, or 61.73% relatively to mean crown volume. Thus, model (8) was the best one of this strategy.

Strategy 2: crown variables

Models (13), (14) and (15) presented a great capability of explaining crown volume variability, once their adjusted coefficients of determination were, respectively 93, 94 and 93%. Additionally, mean errors for these models were 5.76, 5.27 and 5.76 m^3. However, despite models (13), (14) and (15) presented satisfactory fit statistics, these were dropped from this strategy since modeling of residual variance did not correct problems of hetero-scedasticity of error terms. On the other hand, model (12) presented lower adjusted coefficient of determination for this strategy (79%) and the higher mean error (10.19 m^3, or 43.78%), was the best model for crown volume based on crown related variables. This model was chosen because it presented no problems with any of evaluated assumptions, as previously indicated in Table 3, even though its statistics of fit were not best ones of this strategy.

Strategy 3: stem and crown variables

Model (17) was the first to be dropped because of model singularities, as its large variance calculations and its parameter estimates did not converge. The results obtained for this strategy pointed that remaining models were able to produce satisfactory and reliable estimates, and only slight differences were observed when compared relatively to each other. All estimators were significant, and any models violated assumptions of regression. Model (18) presented somewhat worse statistics of fit when compared with those obtained for model (16). Therefore, model (16) was selected to represent the strategy for modeling crown volume inputting both stem and crown variables. The resulting equation explained 78% of crown volume variability, producing estimates with lower mean error of 10.50 m^3 (45.09 %) for this modeling approach.

Strategy 4: stem volume expansion to crown volume

The model based on stem volume estimates showed no heteroscedasticity problems, neither with correlated error terms as indicated by White's and Durbin-Watson's statistics. All coefficients were significant. This model explained 56% of crown volume variability, having resulted in a mean error of 14.77 m^3, or 63.43% relatively. In this strategy, no other models were fitted and comparisons across strategies will be subsequently discussed.

DISCUSSION

Strategic crown volume models

When comparing the best selected models of each

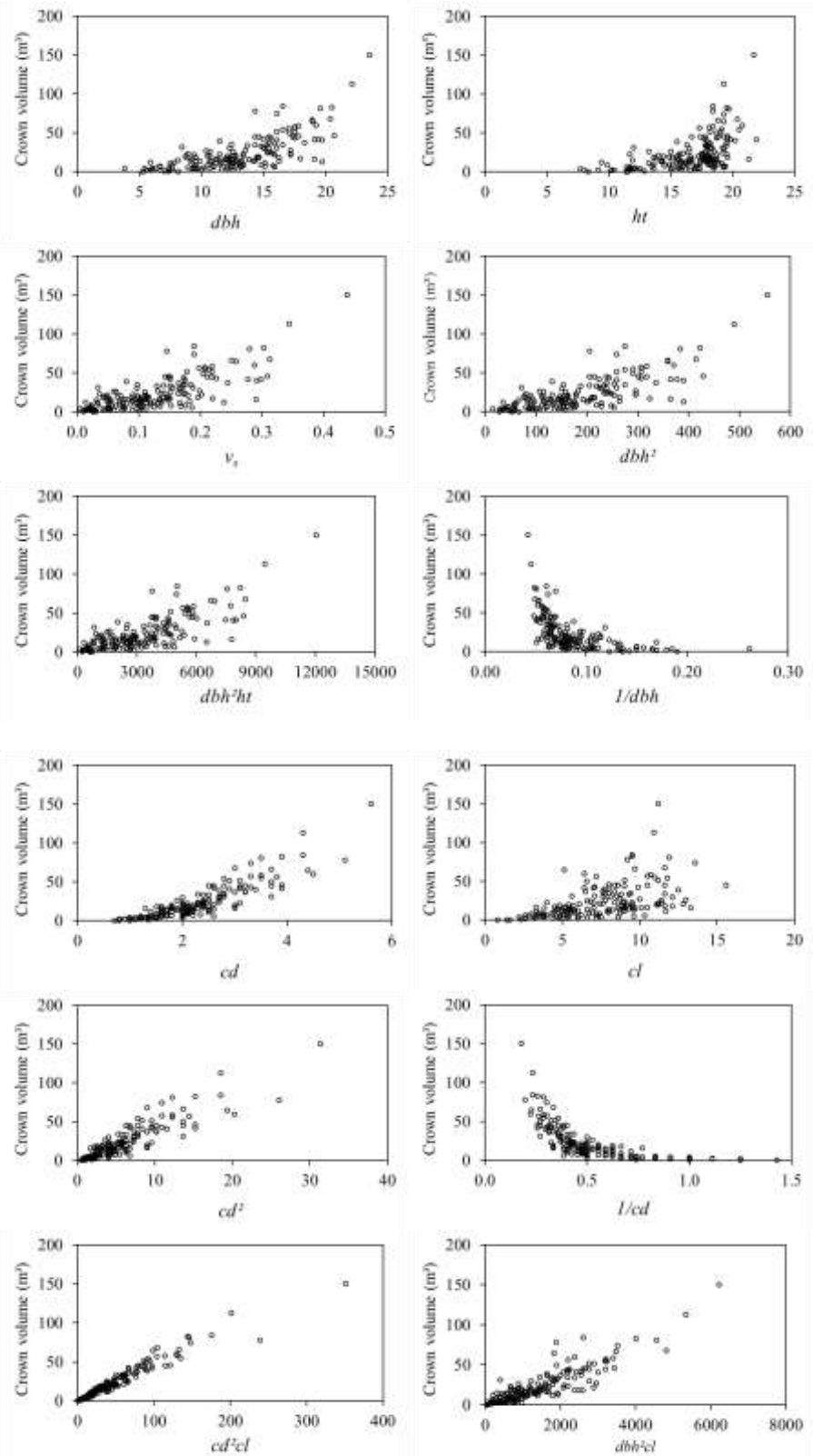

Figure 3. Graphical relationships between independent variables and crown volume in black wattle stands, in estate of Rio Grande do Sul. Where: *dbh* is diameter at breast height (cm), *ht* is total height (m); *cl* is crown length (m), v_s is stem volume (m³), v_c is crown volume (m³).

Table 3. Summary table of crown volume models of black wattle stands in Southern Brazil.

Strategy	Model	Parameter estimates			White's statistic	D-W statistic	R^2_{adj}	RMSE (m^3)	RMSE (%)
		β_0	β_1	β_2					
1	8	0.059233*	1.141983*	-	3.82*	1.7704	0.5816	14.37	61.73
	9	0.2534	2.774462*	-0.96481	4.06*	1.7363	0.5834	14.34	61.60
	10	0.016939	0.895621*	-	6.42*	1.8093	0.5566	14.79	63.55
	11	0.255409	-2.77661*	-0.96957	3.26*	1.7360	0.5836	14.34	61.59
2	12	3.05095*	1.126167*	-	1.27*	2.0353	0.7895	10.19	43.78
	13	0.484012*	1.98941*	1.019193*	53.97	1.8512	0.9328	5.76	24.74
	14	0.67959*	0.926881*	-	94.16	1.9465	0.9437	5.27	22.65
	15	0.483975*	-1.98944*	1.019219*	53.43	1.8511	0.9328	5.76	24.74
3	16	154.2672*	2.070689*	1.089476*	5.42*	1.8704	0.7768	10.50	45.09
	18	163.3499*	-2.09431*	1.085761*	3.18*	1.9347	0.7745	10.55	45.32
4	20	1.657697*	173.0236*	-	6.38*	1.8106	0.5582	14.77	63.43

Note: Asterisk (*) indicates significance at 95% level of confidence ($p < 0.05$).

modeling approach, it was possible to rank them according to their statistics of fit. The best overall strategy for modeling crown volume in terms of goodness of fit was strategy 2: crown related variables. Model (12) was the one with greater capability of explaining tree crown volume, accounting for 79% of observed variability, and produced estimates with lower mean error across strategies, with an error of 10.19 m^3 (43.78%). The subsequent model regarding quality of fit was model (16). This model has produced similar statistics of fit to model (12). It was able to explain 77% of crown volume variability, with a mean error of 10.55 m^3 (45.32%). Differently from these, models (8) and (20) have produced worse fit statistics, substantially smaller than in previously discussed models, as presented in Table 3, thus these were not compared. Residual plots of models (12) and (16) are presented in Figure 4.

Regarding operationality of each fitted model, the most operational ones are model (8) and model (20), once these strategies are based only on the most easily acquirable variables (that is, *dbh* and *ht*), even though model (20) requires an intermediate equation to estimate stem volume. However, as already discussed, these models' statistics were not satisfactory, and were ones with poorest predictive capability, thus they were not recommended despite their operational advantages.

Although model (12) fit statistics were the best ones in this study, this model does not present operational potential. This model's inputs are *cl* and *cd*, and, even though *cl* can be easily obtained when compared to *cd*, measuring this *cd* in all sample plots would greatly enlarge time spent on field. In addition, *cd* was the most difficult variable to be measured in this study, once it was obtained only after felling trees. The use of this model would only be appropriate if a consistent procedure for predicting maximum diameter or crown radius was developed and used with the *cl* variable as explanatory variables in a crown volume model. It is also important to note that a model with multiple estimates as inputs makes it difficult to obtain errors of final estimates, and, for this reason, this approach should be carefully used. Alternatively, model (16) was indicated as the most reliable and operational model for predicting black-wattle crown volume. The resulting equation produced marginal estimates relatively to model (12), even though its statistics of fit were somewhat worse. Model (16) requires *dbh* and *cl* as inputs and these variables make this model operationally advantageous over model (12), since these are easier to measure. Diameter at breast height is the easiest measurable variable on a tree and is the most used variable estimate tree's varialbes, such as crown volume. On the other hand, even though *cl* is not as commonly measured as *dbh* is, it can be easily calculated as the difference of tree *ht* and height to live crown base - commonly named *hblc* (Crecente-Campo, 2008; Rautiainen and Stenberg, 2005). Also, *hblc* can be modeled and estimated for other trees, aiming to reduce the time spent measuring it, in same the fashion as commonly done with hypsometric equations of even-aged single species stands.

Regarding number of variables needed as inputs, model (16) requires only two independent variables - a reasonable number of inputs, what grants model simplicity according to Rawlings et al. (1998), differing from models built by automated procedures of variable selection such as stepwise regression method. Such selection method could yield a "better" model regarding quality of fit, however hardly comprehensible most of

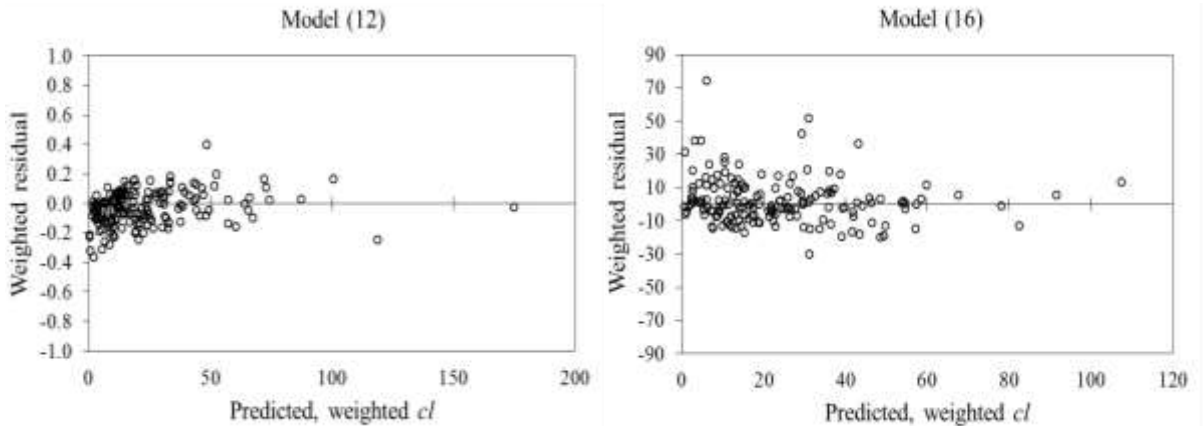

Figure 4. Residual plots of each selected model of different strategies for modeling crown volume of black wattle stands in Southern Brazil.

times due the quantity and different natures of selected input variables (Draper and Smith, 1966). Models that require a greater number of inputs can easily become costly and time demanding due to the number of variables that would have to be measured on field.

In the literature of crown volume modeling, Velázquez-Martí et al. (2012) fitted crown volume models for mandarin (*Citrus reticulata*) that explained between 61 and 77% of crown variability using crown diameter and crown length as inputs. In the study of Rautiainen et al. (2008), estimates of crown volume presented mean error ranging from 14.6 to 21.0 m³ for a Scots pine (*Pinus sylvestris*) and from 22.4 to 48.1 m³ for Norway spruce (*Picea abies*), for different approaches of modeling crown volume that were previously presented. Crown volume models of Pedunculate oak (*Quercus robur*) and Common hornbeam (*Carpinus betulus*) for stands of different age classes proposed by Dubravac et al. (2009) explained 44 to 79% and 23 to 65% of crown volume variability for these species, respectively. Meng et al. (2007) suggested crown volume models for *Pinus contorta* that were build based on uniform stress theory. When using *dbh* to estimate crown volume, this model accounted for 62% of the variability. In his second proposed model, when inputting *dbh*, distance between center of crown and wind speed as independent variables, it has accounted for 70% of crown volume variability. Our proposed model was likely to be significantly advantageous regarding previous studies due its capability of producing consistent estimates of crown volume, and its simplicity of inputting *dbh* and *cl* to do so. Additionally, in relative terms the proposed model was able to explain variability with a similar performance to the models for other species.

Besides being simultaneously the most operational and reliable model of this study, model (16) was biologically and mathematically sound. Model (16) is biologically reasonable because crown volume has implications on *dbh* (Sprinz and Burkhart, 1987), which is also intrinsically related to *cl* (Taiz and Zeiger, 1991). A large crown has potential of intercepting greater amounts of radiation because it comprises greater amounts of active foliage, which are responsible for increasing photosynthesis rates, therefore affecting *dbh* growth (Burkhart and Tomé, 2012; Ottorini et al, 1996; Cluzeau et. al, 1995; Ottorini, 1991). Mathematically, since our data are experimental, b_1 and b_2 are compensatory coefficients, whose product generates the volume of a regular geometric solid – *RGS* ((*dbh*)² times *cl*), whilst the estimate of b_0 may represent a constant of shrinkage from the volume of such solid to the volume of the crown.

The final model (21) with its coefficients and plots of observed crown volume versus predicted values and its residuals distribution plot are presented in Figure 5.

$$v_c = 0.010553\,(dbh)^{2.095694}(cl)^{1.085087} + \varepsilon_i \qquad 21$$

Conclusions

This study was able to suggest a simple, likely unbiased and reasonably accurate crown volume model, relatively to other crown volume models found in literature. Models composed by stem variables did not present a satisfying predictive capability. The model based only on crown related variables was the one with greater predictive ability. However, its independent variables are most onerous to be measured in the field. The predictive capability was kept by model (16), inputting both stem and crown variables. The resulting model strategy using stem and crown variables presented an operational advantage once it only requires *dbh* and *cl* as independent variables. In addition, it is both biologically and mathematically sound, due its inputs and intrinsic

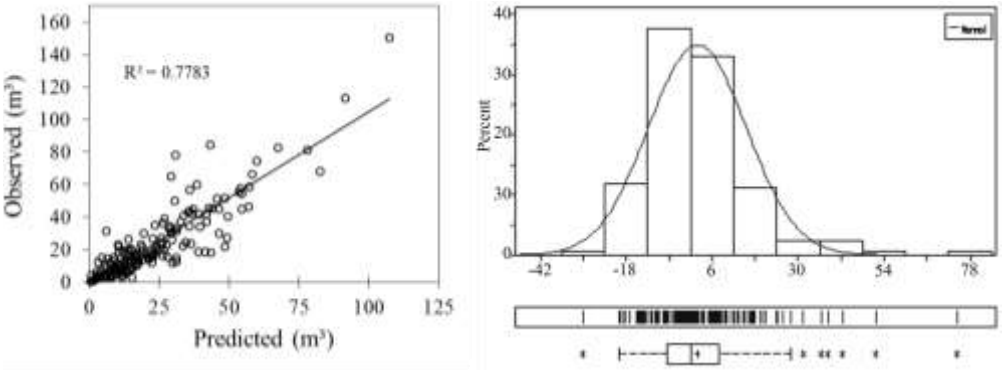

Figure 5. Plot of observed crown volume by predicted values and histogram of residuals of the best overall crown volume model of black wattle stands in Southern Brazil.

mathematical and biological relationships with respect to crown volume.

Conflict of interest

The authors have not declared any conflict of interest.

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Prevalence and variability of the common bean rust in Uganda

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Uganda is the second largest producer of dry beans (*Phaseolus vulgaris* L.) in Africa, but common bean rust caused by *Uromyces appendiculatus* (Pers. Unger), is negatively impacting the production of the crop. There is little information on the occurrence and identity of the rust pathotypes present in the country. Consequently, a field survey was carried out during the 2015 second planting season in fifteen districts, representing the areas of high beans production in Uganda. High common bean rust incidence and severity were observed in the low altitudes and the South-Western Highlands of Uganda. Wakiso and Hoima districts had the highest rust disease incidence 72 and 76% respectively and severity rates of 6 and 5.5, respectively. Rust disease incidence was uniformly high on commercial genotypes and landraces. Similarly, high rust disease incidence and severity were observed in the bean-maize–groundnut cropping system. Twenty-three single rust isolates were collected in Uganda and inoculated on 11 bean rust differentials and Ouro Negro *(Ur-14)* genotypes. Six rust pathotypes were identified and these included 2-0, 4-0, 50-0, 5-1, 4-33 and 63-19. Five of the pathotypes were of Andean origin and only pathotype 4-33 was of Mesoamerican origin. The rust pathotype 63-19 showed similar pathogenic characteristics with the Puerto Rico rust race 19-63. This study provides critical baseline information to integrate breeding and crop protection in the efforts to develop an overall strategy for the management of common beans in Uganda.

Key words: *Phaseolus vulgaris*, *Uromyces appendiculatus*, rust differentials, co-evolution, disease severity.

INTRODUCTION

Uganda is the second largest producer of dry beans (*Phaseolus vulgaris* L.) in Africa (Abate et al., 2012). Common beans are grown in all major agro-ecological regions of Uganda (Ugen et al., 2014) but the production rate is higher in the Western region and lower in the Northern region of the country (Abate et al., 2012). Recently, bean rust has been observed to be an important disease that is devastating farmers' bean fields (Odogwu et al., 2014). Common bean rust is a foliar disease that occurs widely in Africa and has been

observed to be the third major production constraint causing yield loss of about 118.7 tonnes per year in Eastern Africa, after angular leaf spot and anthracnose diseases (Assefa, 1994; Kimani et al., 2001). The disease causes premature leaf chlorosis, senescence and in severe cases complete plant defoliation resulting in yield losses ranging from 18-100% (Souza et al., 2014).

The major mode of dissemination of bean rust is by wind, but other agents of dissemination include migratory birds, insects, water and sometimes through contaminated farm implements and infected plant debris (Liebenberg and Pretorius, 2010). The factors that contribute to the distribution and prevalence of the bean rust disease include altitude, ecological zones and human activities (Helfer, 2014; Lin, 2011). Helfer (2014) and Liebenberg and Pretorius (2010) reported that abiotic factors such as temperature, high humidity and leaf surface moisture; and biotic factor such as rust disease host range contribute to the epidemiology of rust disease. According to Liebenberg and Pretorius (2010), temperatures favoring germination and infection of bean rust range from 17 to 21°C and a sharp decrease in number of pustules per leaf at incubation occurs when temperatures are between 21 to 25°C. Harter et al. (1935) observed high levels of infection when exposed to rust at appropriate temperature ranges, high levels of infection were obtained when plants were exposed to a relative humidity (RH) of 96% or higher, provided free moisture was present on the leaves. However, at lower humidity levels infection levels were reduced, and no infection would take place.

According to Babel and Turyatunga (2014), Uganda has an equatorial type of climate with ample sunshine and heavy rainfall in most parts of the country. Mean annual rainfall near Lake Victoria is about 2,100 mm, while the mountainous regions of the west, southwest, and northeast receive average annual rainfall of about 1,500 mm. The lowest mean annual rainfall (-500 mm) occurs in the extreme northeast (Byrnes, 1990). Mean annual temperatures range from about 16°C in the southwestern highlands to 25°C in the northwest, but in the northeast, temperatures exceed 30°C in the dry season. The maximum temperature ranges between 18 and 35°C and the minimum temperature between 8 and 23°C depending on the part of the country (Byrnes, 1990). Also, Uganda is divided into seven broad agro-ecological zones: the banana-coffee system, the banana–millet–cotton system, the montane system, the Teso system, the Northern system, the West Nile system, and the pastoral system (Mwebaze, 2006). An agro-ecological zone (AEZ), as defined by the Food and Agriculture Organization (FAO 2010), is a land resource mapping unit, defined in terms of climate, landform, soils, and land cover, fairly homogeneous across the AEZ, and having a specific range of potentials and constraints for land use (Babel and Turyatunga, 2014).

Cultural practices were once thought to have only a minimum effect on the prevalence of bean rust (Mmbaga et al., 1996). Lin (2011) suggested that cropping systems such as crop diversification can contribute to the spread or suppression of the disease. Liebenberg and Pretorius (2010) reported that cultural practices such as cultivar mixtures, crop rotation, planting time and age of crop, intercropping and choice of bean varieties could affect the level of infection and spread of the rust disease. Mmbaga et al. (1996) suggested that airborne rust spores produced on volunteer, wild or cultivated beans are sources of infection that could reduce the effectiveness of crop rotation. Ronner et al. (2013) stated that common bean are mostly grown as either as sole crops or as intercrops (on 2/3rd of the total plots planted with beans) with maize, cassava, cotton, bananas and groundnuts in Uganda. The effect of these cultural practices on rust prevalence has not been reported in the country.

According to Steadman et al. (2002), the virulence structure of *U. appendiculatus* and its properties related to the host selection effect on the pathogen population, depends on assessing the pathogen based on the reaction of the pathotypes on a standard differential set of 12 common bean cultivars established during the 3rd Bean rust and 2nd Bean common Bacterial Blight International Workshops in 2002. In this method a binary system based on the position of each cultivar within the series was used to define the virulence level of isolates under study (Pastor-Corrales and Liebenberg, 2010). There is no information on the identity and distribution of bean rust pathotypes in Uganda. Therefore, a survey was conducted to assess rust disease distribution and severity as well as its association with the cropping system and agro-ecological zones in Uganda. Also, other factors contributing to the rust disease epidemiology and uniqueness of the rust pathotypes in the selected bean growing areas were considered.

MATERIALS AND METHODS

Study area and fields

In the second planting season of 2015, 15 districts located in six of the major AEZs of Uganda were surveyed. Bean fields were visited in AEZs and districts are indicated in Table 1. The districts were selected based on their bean production intensity. Fields were selected at random at intervals of 5 to 10 km along the main roads.

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Table 1. Details of agroecological zones and districts surveyed for rust disease assessment in the second planting season of 2015.

Agroecological zone	Districts	Number samples collected
Intensive banana coffee lakeshore system in central region (CR)	Wakiso	10
	Butambala	10
Montane system in the eastern region (ER)	Kapchorwa	10
	Bulambuli	10
	Mbale	10
Montane system in the South-Western highlands of the South-Western Highlands (SWH)	Sheema	10
	Mbarara	10
	Kabale	10
	Masaka	10
Western banana-coffee cattle system of the South-Western region (SWR)	Hoima	10
	Masindi	10
Northern system of Northern Central (NC) region	Amuru	10
	Kole	10
	Lira	10
Annual cropping and cattle West Nile system of the Northern Savannah Grassland (NSG)	Arua	10

When necessary, the sample size (the number of observed fields per region) and the number of sample units (the randomly selected single plants) per field were adjusted to suit the field size and crop distribution. All sampled fields belonged to small-holder farmers and each field was visited once.

Sample units

Following the methodology of Assefa (1994), in each sampled field, the size of the field was estimated and the crop age was determined. Sample units were selected by making a specific number of equally spaced paces following an inverted "V" pattern from the edge of the field. Having made the pre-set number of paces based on the field size, the nearest plant to the right foot was taken as the sample unit. In each sample field, 20 sample plants were selected for disease assessment. A sub-sample of 3 trifoliate leaves per plant was selected, yielding a total of 60 leaves per field. The sub-sample was composed of one leaf from each of the upper, middle and bottom canopy layers of the main stem.

Crop and disease assessment

Data on disease incidence and severity were collected mostly from plants at the V3= first trifoliate leaf stage; V4= third trifoliate leaf stage; R5= pre-flowering; R6= flowering; R7= pod formation; R8= pod filling plant developmental stages (Van Schoonhoven and Pastor-Corrales, 1991). Disease incidence was expressed as the percentage of the number of infected plants over the 20 plants within the sampling point. Rust disease severity was rated using the CIAT 1 to 9 scale following Van Schoonhoven and Pastor-Corrales (1991), where 1-3 = resistant (no visible pustules to few pustules covering 2% of foliar area), 4-6=intermediate (small pustules covering 5% foliar area to large pustules often surrounded by chlorotic halos covering 10% foliar area) and 7-9 = susceptible (large to very large pustules covering 25% foliar area). Also the global positioning (GPS) readings of latitude, longitude and elevation were recorded for each location where data were collected using Garmin eTrex 10x GPS.

Data analysis

Rust disease incidence and severity maps were developed using GPS survey data points obtained from each sampling location and incidence and severity means generated from data analysis (Ddamulira et al., 2014). The altitude was calculated from the elevation data using Spatial Data Download | DIVA-GIS. Maps were exported and visualized in Arc View® GIS3.2 software (Rockware Inc). Analysis of variance and correlation between incidence and severity means were done using GenStat 12th edition (Payne et al. 2011). Multiple mean comparisons for rust disease incidence and severity for all districts surveyed were performed using Tukey's studentised range test, where $\alpha = 0.05$ using the GenStat 14th edition. Bar charts for each factor affecting disease severity and incidence were done using Microsoft Excel 2013.

Fungal isolation and inoculum preparation

Two infected common bean leaves from landraces, commercial and introduced genotypes, with two to three rust pustules, were collected from each sampled point and placed inside a well labeled coin envelope which were left opened while the leaves were left to air dry and taken to the laboratory for isolation. For isolation, the single pustule isolation method currently in use at the pathology laboratory, University of Nebraska, Lincoln, USA was used. Pure isolates were obtained and multiplied by inoculating single pustules' spores on the abaxial surface of the 10-day old leaves of NABE 16, a released cultivar known to be susceptible to rust. From viable pustules, twenty-three isolates were obtained from the diseased bean leaves collected during the survey from the districts of Wakiso, Sheema, Hoima, Masindi and Masaka. The pustules from

Table 2. Analysis of variance of environmental and production on the incidence and severity of in Uganda.

Source of variance	Incidence (%)		Severity
	DF[1]	MS[2]	
Altitude	2	648***	20.36***
Agro-ecological zone	5	128***	41.68***
District	14	648***	20.36***
Crop stage	5	336ns	13.53*
Cropping system	2	988**	33.37**
Intercropping	9	352*	09.73**
Fungicide treatment	2	587*	31.47**
Other diseases	19	211ns	5.05ns
Previous crops	24	232*	8.46ns
Variety	34	299***	8.29**
$LSD_{(0.005)}$		0.058	3

[1]DF: degree of freedom; MS: Mean square, [2]Values with *, ** and *** implies significant at P = .05, P < .01 and P < .001 respectively; [2]ns: not significant.

the other 10 regions were likely not viable since they did not sporulate.

Inoculation and pathotype determination

To determine the rust isolate pathotype, a set of differential cultivars with specific resistance genes where inoculated with each bean rust isolate. The differential set included 11 cultivars from the 2002 set of 12 bean rust differentials suggested by Steadman et al. (2002) which were obtained from the Pathology Lab of the University of Nebraska, USA. They include, 1=Early Gallatin; 2=Redlands Pioneer; 3=Montcalm; 4= Golden Gate Wax; 5= PI260418; 6= Great Northern 1140; 7= Aurora; 8= Mexico 309; 9= Mexico 235; 10=CNC; 11= PI181996 and the 12th genotype was Ouro Negro (*Ur-14*) of the Mesoamerican gene pool that was obtained from Dr. E. Arunga, formerly of the Department of Biotechnology, Eldoret University, Kenya. Six seeds of each cultivar were pre-germinated for two days before planting in 2-L disposable cups containing black soil, manure and sand in a ratio of 3:1:1 and allowed to grow for 10 days. The 10-days old bean plants were inoculated with spores of each isolates and kept in a humid chamber at 18-23°C and 95% relative humidity for 16 h. The plants were air-dried before they were transferred into the screen house. The plants were observed daily for pustule sporulation and size until they are 15 days old. The pustules types were measured using the iGAGING ocular lens (www.iGAGING.com). Rust races were named following the suggestion of Pastor-Corrales and Liebenberg (2010). The virulence reactions of the cultivars were recorded as disease scores based on the rust pustules types (size) using the CIAT scale of 1 to 6 (Van Schoonhoven and Pastor-Corrales, 1991) as follows: 1= no visible symptoms; 2= non-sporulating necrotic spots; 3= sporulating pustules smaller than 300 µm in diameter; 4= sporulating pustules 300-500 µm in diameter frequently surrounded by chlorotic halos; 5= sporulating pustules 500-800 µm in diameter frequently surrounded by chlorotic halos; 6= sporulating pustules larger than 800 µm in diameter frequently surrounded by chlorotic halos. Where several infection grades were present, the predominant or most prevalent was chosen. Cultivars were considered resistant when they were predominantly of grade 3 or lower and susceptible with a predominance of grade 4 and higher. Pathotypes were determined by adding binary values of the differential genotypes that were susceptible to a particular with the respective bean rust isolate based on the predominance of virulence on the Andean [large seeded] or Mesoamerican [small seeded] cultivars (Gepts, 1998). According to Pastor-Corrales and Liebenberg (2010), if a new isolate of the rust pathogen is compatible with Andean bean cultivars Montcalm and Golden Gate Wax, and with Middle American cultivars GN 1140 and Aurora, the new race would be named 20-3. The first digit (20) is obtained from the addition of the binary values of the Andean bean cultivars Montcalm (4) and Golden Gate Wax (16). The second digit (3) is obtained from the addition of the binary values of the Middle American bean cultivars GN 1140 (1) and Aurora (2). Inoculation of the differentials with the different isolates was repeated for authentication of the results. Some isolates were sent to the pathology laboratory, University of Nebraska, Lincoln, USA for authentication.

RESULTS AND DISCUSSION

Rust disease incidence and severity

The results of the rust disease survey showed that bean rust was present in all locations and altitudes sampled confirming findings by Wortmann et al. (1998). The incidence of rust ranged from 10-76% while rust severity scores ranged from 3.0 to 7.2. Rust disease incidence and severity were significantly different (P<0.001) across locations surveyed (Table 2). Significant differences (P<0.001) were observed in incidence and severity across the altitudes, AEZ and districts. Incidence and severity was also significantly (P<0.01) influenced by crop developmental stage and cropping history (previous crop planted). Similar findings were reported by Lin (2011) and Helfer (2014). There was no significant contribution by other disease observed in the field to rust incidence and severity, indicating that the presence of other diseases had not effect on the prevalence of rust disease.

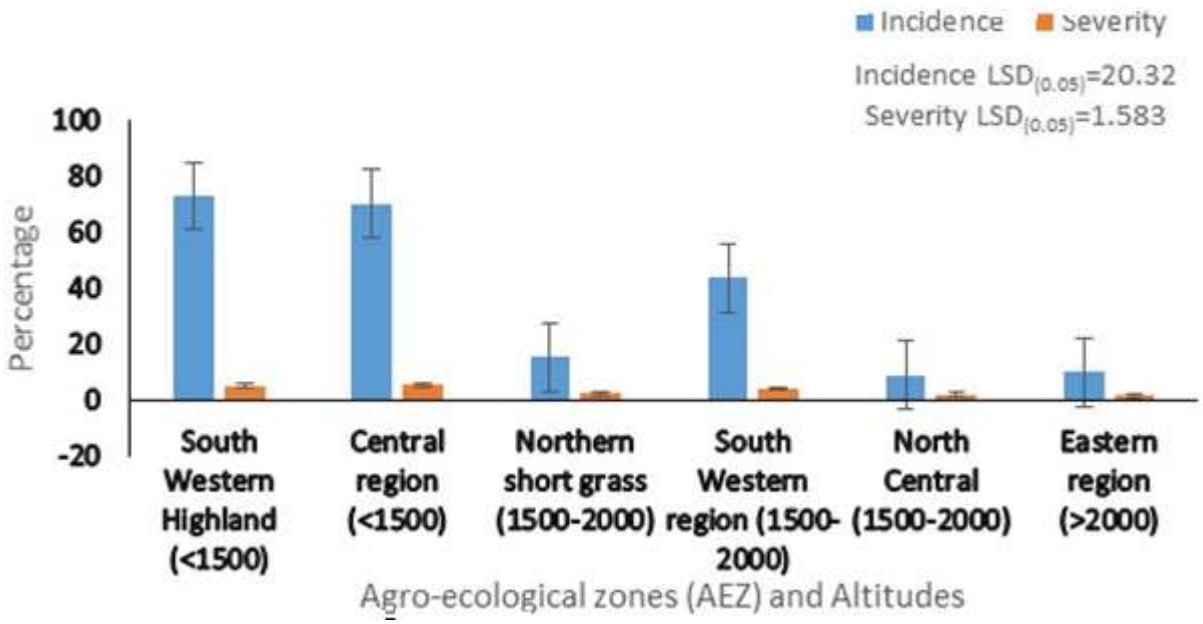

Figure 1. The level of rust incidence and severity based on the AEZ and altitude in Uganda in the 2015 second planting season.

The South-Western Highlands (SWH) had the highest rust incidence of 73% followed by Central region (CR) with 70% and South Western region (SWR) with 44% while the lowest incidence of 9% was observed in North Central (NC) region (Figure 1). On the other hand, CR had the highest rust severity level of 6 followed by SWH (5) and the lowest severity of 2 was observed in the Eastern region (ER). These findings agree with Wortmann et al. (1998) who reported that rust was moderately severe in Western Region (WR), ER, CR and SWH, while it was low in the NC. Both the CR and SWH regions lie at lower altitude (<1500 m) and had the highest rust incidence of 70 and 73%, respectively. While AEZs in altitudes above 2000 m, such as NC and ER had the lowest rust incidence of 9 and 10% respectively, and severity of 2. The recommended temperature threshold of 18°C and relative humidity of 95% are necessary for high rust disease severity to occur (Pastor-Corrales and Liebenberg, 2010). Mwebaze (2006) reported that the SWH had temperatures of 16 to 18°C and the NR had temperatures exceeding 25°C, which may be the reason for the high rust disease prevalence in the SWH and CR AEZs. Similar findings were reported by Wortmann et al. (1998). The highest rust incidence was recorded in Hoima district (76%) followed by Wakiso (72%) and Masindi (71%). Mbale and Kabale recorded the lowest incidence of 10% each (Figure 2). Moderate disease incidence ranging from 67-69% were recorded in Wakiso, Masaka, Butambala and Arua whereas the highest rust severity was recorded in Wakiso district (6) followed by Hoima (5.5). Mbale (2), Kabale (3) and Kapchorwa (3) found in higher altitude recorded the lowest severities.

Paparu et al. (2014) reported moderate rust severity for Mbale and Kabale districts. However, in this study low severity were observed in Mbale, and Kabale. This may be the result of seasonal changes in weather conditions observed in Uganda (Hepworth et al., 2008). The effect of seasonal changes in weather conditions on rust prevalence were reported in Ethiopia by Assefa (1994). In general, rust severity and incidence were found at different levels in all altitudes studied, which suggests that all bean growing areas in Uganda have conditions that favour common bean rust development and spread. It was also observed that the disease severities of Hoima and Wakiso where statistically different with values of 5.55 and 5.75 respectively, while the disease incidence was not statistically different for Masaka, Butambali, Hoima and Wakiso (Table 3).

Effect of cultural systems on rust prevalence

Cropping systems either increased or decreased rust disease prevalence. The effect of cropping system on rust incidence and severity was significant ($P<0.01$) for all the locations surveyed. The mixed cropping system had the highest rust incidence (49%), while sole cropping system was 41%. However, the sole cropping system had the highest rust severity level of 6.2, while mixed cropping system had 3.8. The high severity level of sole cropping system may result from the re-infection of the beans fields with rust pathogens from debris or volunteer crops and weeds. High rust severity in sole cropping system has been reported by Mmbaga et al. (1996). At

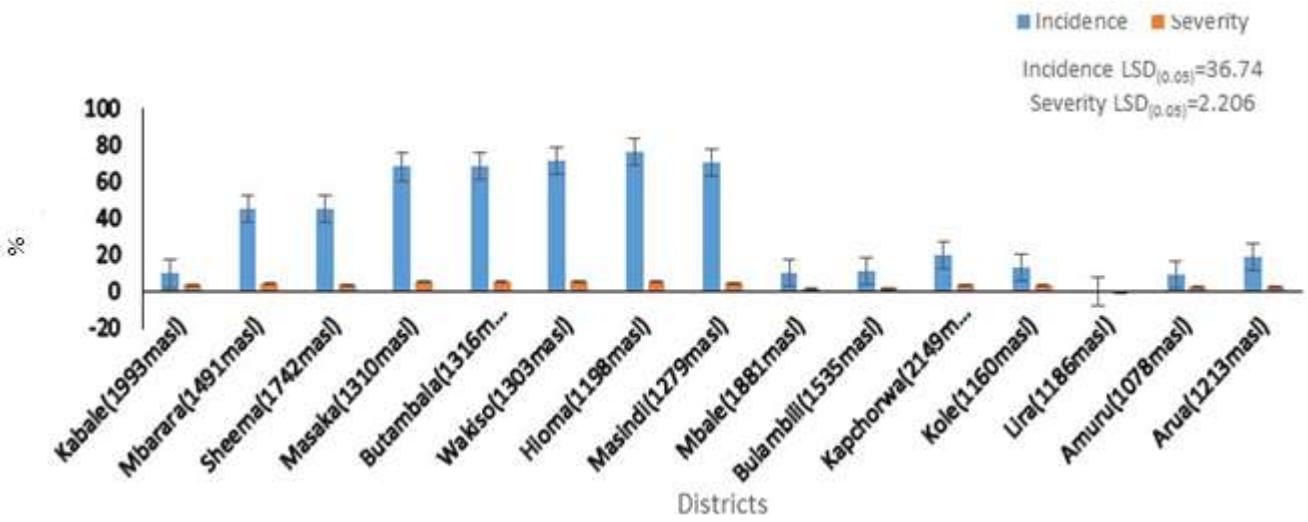

Figure 2. The level of rust incidence and severity on each district in Uganda in the 2015 second planting season.

Table 3. Mean incidence and severity of common beans rust for the districts surveyed in Uganda.

District	Incidence	Severity
Bulambili	0^{a}	0^{a}
Mbale	9^{ab}	2^{ab}
Arua	10^{ab}	2.333^{abc}
Amuru	13^{ab}	2.501^{bc}
Kole	14^{ab}	3.035^{bcd}
Kabale	20^{ab}	3.182^{bcde}
Kapchorwa	27^{abc}	3.429^{bcdef}
Sheema	46^{bcd}	3.515^{bcdef}
Mbarara	50^{bcd}	4.5^{cdef}
Masindi	68^{cd}	4.7^{cdef}
Masaka	69^{d}	5.1^{def}
Butambali	71^{d}	5.167^{def}
Hoima	72^{d}	5.556^{ef}
Wakiso	76^{d}	5.75^{f}

the crop diversity level, the beans-maize-groundnut intercropping system showed the highest disease incidence (100%) while the bean-coffee-banana intercropping system had the lowest score of 2%. However, the beans-coffee and beans-maize-groundnut intercropping system showed the highest disease severity of 6 while the bean-coffee-banana intercropping system had the lowest score of 2. Msuku and Edje (1980) reported that the common bean-maize cropping system had reduced rust severity while Mmbaga et al. (1996) indicated that intercropping beans and maize affected bean rust epidemiology by influencing spore dispersal, spore retention and infection efficiency. However, the high rust severity in the bean-groundnut and maize intercrop might have resulted in the rust disease build-ups in the fields because of the higher relative humidity resulting from the maize-bean-groundnut canopies which favoured uredospore production (Msuku and Edje, 1980).

Rust disease incidence was significantly influenced by cropping history (<0.05), whereas rust severity was not. Fields which were previously grown with bush beans and left to fallow in the 2015A season had high rust incidence levels of 100%, while fields grown with sorghum the previous season had the lowest disease incidence level of 3.33%. The rust disease prevalence in fields previously grown with bush beans and left fallow may be attributed to infected plant debris from previous season (Liebenberg and Pretorius, 2010). Similar findings were reported by Souza et al. (2008).

On the contrary, crop developmental stage had a significant effect on rust disease severity (<0.01) but was not true for rust incidence in all locations surveyed. The plants at the pre-flowering stage (R5) and third trifoliate stage (V4) had more rust disease severity of 4.8 and 4.7 respectively than other stages. While those plants at the pod filling (R8) stage had the lowest rust severity of 2.7. Similar findings have been reported by Assefa (1994). Early planting following the onset of rainfall has been recommended as a strategy in managing rust disease which enables the bean plants to escape the onset of the rust disease which is high and prevalent during the mid-season (Steadman et al., 2002; Ronner, 2013), that is October and November for the second cropping season in Uganda.

Production practices significantly influenced rust incidence and severity in all locations surveyed (Figure 3). Fields without fungicide treatment showed high rust incidence of 53% while those with fungicide application had low disease incidence (45%). The fungicide commonly used by farmers was the Indofil-M45

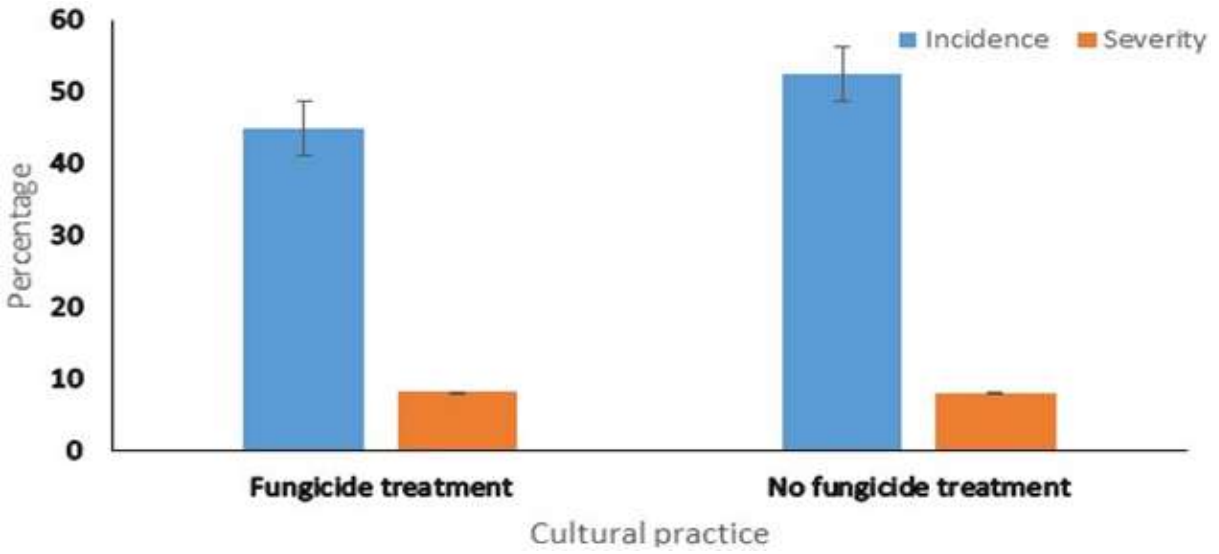

Figure 3. The level of rust incidence and severity based on the cultural practice in Uganda in the 2015 second planting season.

(Mancozeb 80%W. P). Similarly, unsprayed fungicide fields showed high rust severity (6.6) while those sprayed with fungicide treatment had lower rust severity (4.1). These findings confirm the importance of a disease management in small-holder farms. Farmers could also reduce rust disease on their farms by planting resistant varieties (Mmbaga et al., 1996). In this study, rust disease incidence and severity were significantly influenced by the type of bean variety grown (Figure 4). The commercial varieties NABE 1, NABE 5 and NABE 16 had the highest rust disease incidence levels of 100% while fields cultivated with the introduced genotypes, Mac 44 and Nutri-beans, screened by the Ugandan legume programmes had no rust disease. On the other hand, the landraces Kamenyameggo, Kanyebwa and Masindi yellow had the highest rust severity levels of 7, 6.5 and 6.1 respectively. In general, high disease severity was observed in fields cultivated with farmers' preferred landraces and commercial cultivars. These findings provide information that will guide the rust resistance breeding programme in Uganda. Also, the genotypes Mac 44 and Nutri-beans which were rust free needs to be further investigated to confirm and determine the resistance genes they may possess.

Rust pathotypes virulence

The results on the pathogenic reaction of 23 *U. appendiculatus* isolates on 12 bean cultivars with specific resistance genes are presented in Table 4. The reaction of the isolates on the different cultivars revealed the existence of pathogenic variability among rust isolates. Most isolates were pathogenic to both Andean and Mesoamerica differentials but one isolate, Masindi-4, was more pathogenic on Andean than Mesoamerican cultivars. Based on the isolates' pathogenic reactions on the rust resistance cultivars, 22 isolates were classified as Andean group because they were virulent on most of Andean cultivars, while one isolate, Masindi-4, was virulent on most Mesoamerican cultivars and is grouped as Mesoamerican. All the 23 isolates were grouped in six pathotypes 2-0, 4-0, 50-0, 5-1, 4-33, and 63-19. Five of the pathotypes, 2-0, 4-0, 50-0, 5-1, and 63-19, were most virulent on the Andean differentials, indicating the co-evolution of host and pathogen as the large-seeded beans are most preferred in Uganda. Similar findings were reported in South Africa and Kenya by Liebenberg and Pretorius (2011) and Arunga et al. (2012) respectively.

The pathotype 63-19 was identified based on the complete differential set at the University of Nebraska, USA. This pathotype had resistant reactions on the Mesoamerican differentials 'Mexico 309' with the resistant gene *Ur-5*, 'Mexico 235' (*Ur-3*+) and 'PI 181996' (*Ur-11*). Susceptible reactions were observed on the Andean differentials 'Early Gallatin' (*Ur-4*), 'Redlands Pioneer' (*Ur-13*), 'Montcalm' and 'PC-50' (*Ur-9*, *Ur-12*), 'Golden Gate Wax' (*Ur-6*), and 'PI 260418' and Mesoamerican differentials 'GN 1140' (*Ur-7*), 'Aurora' (*Ur-3*) (Figure 4), and 'Compuesto Negro Chimaltenango' (CNC). The Andean differentials were all susceptible to the 63-19 pathotype but half of the Mesoamerican differentials were resistant. Based on the reaction of the differentials with the pathotype 63-19 we can conclude that pathotype 63-19 had similar pathogenicity with the highly virulent race 19-63 identified in Puerto Rico (Vega, et al., 2009).

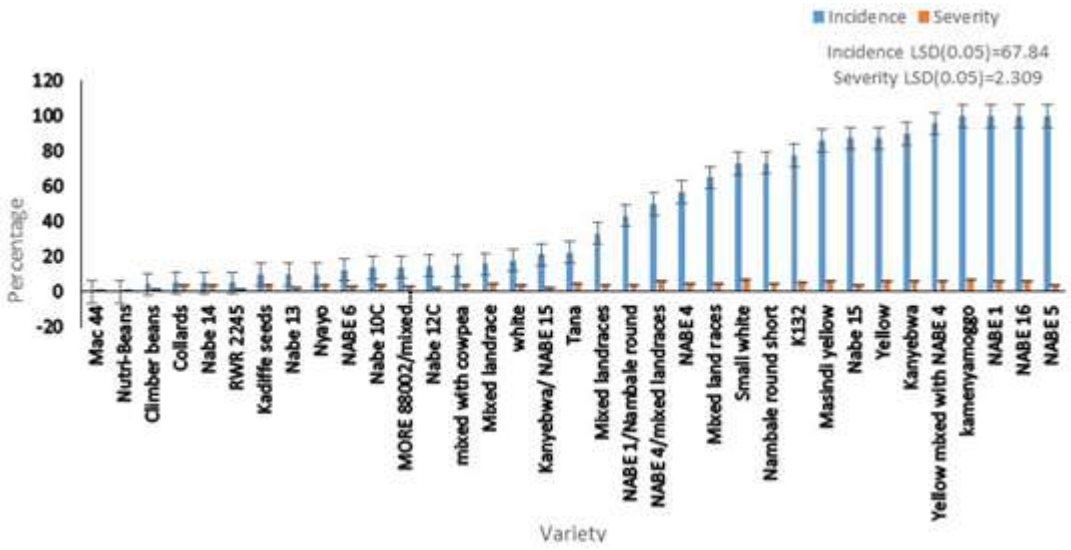

Figure 4. The effect of the variety type on the level of rust incidence and severity in the 2015 second planting season.

Table 4. Pathogenicity of 23 *U. appendiculatus* isolates on 12 common bean rust differential genotypes possessing specific resistance.

Genotypes*[1]	Andean origin					Mesoamerican origin							Rust pathotypes
	1	2	3	4	5	6	7	8	9	10	11	12	
Rust genes (*Ur*)[2]	4	13	UNK	6	UNK	7	3	5	3+	CNC	11	14	
Linkage map(PV)[3]	06	08	UNK	11	UNK	11	11	04	UNK	UNK	11	04	
Binary number	1	2	4	8	32	1	2	4	8	16	32	UNK	
WAKISO-1	1	1	5.6	1	1	1	1	1	1	1	1	1	4-0
WAKISO-2	5.4	5.3	5.3	5.2	4.3	6.2+	5.2+	3.2+	2	4.1	1	0	63-19†
WAKISO-3	1	1	5.6	1	1	1	1	1	1	1	1	1	4-0
SHEEMA-1	1	1	5	1	1	1	1	1	1	1	1	1	4-0
HIOMA-1	1	5.6	1	1	3	1	1	1	1	1	1	1	2-0
HIOMA-2	1	5.6	1	1	3	1	1	1	1	1	1	1	2-0
HIOMA-3	1	5.6	1	1	3	1	1	1	1	1	1	1	2-0
HIOMA-4	1	5.6	1	1	3	1	1	1	1	1	1	1	2-0
MASINDI-1	6	1	6	1	1	6	1	1	1	1	3	1	5-1
MASINDI-2	1	6	1	4	3.4	1	1	1	1	1	1	2.3	50-0
MASINDI-3	1	6	1	4	3.4	1	1	1	1	1	1	2.3	50-0
MASINDI-4	1	1	6	1	1	6	1	1	1	1	4	5.4	4-33
MASINDI-5	1	6	1	4	3.4	1	1	1	1	1	1	2.3	50-0
MASAKA-1	1	5.6	1	1	3	1	1	1	1	1	1	1	2-0
MASAKA-2	1	5.6	1	1	3	1	1	1	1	1	1	1	2-0
MASAKA-3	1	5.6	1	1	3	1	1	1	1	1	1	1	2-0
MASAKA-4	1	5.6	1	1	3	1	1	1	1	1	1	1	2-0
MASAKA-5	1	5.6	1	1	3	1	1	1	1	1	1	1	2-0
MASAKA-6	1	5.6	1	1	3	1	1	1	1	1	1	1	2-0
MASAKA-7	1	5.6	1	1	3	1	1	1	1	1	1	1	2-0
MASAKA-8	1	5.6	1	1	3	1	1	1	1	1	1	1	2-0
MASAKA-9	1	5.6	1	1	3	1	1	1	1	1	1	1	2-0
MASAKA-10	1	5.6	1	1	3	1	1	1	1	1	1	1	2-0

† Pathotype 63-19 was validated at the Plant Pathology Laboratory at University of Nebraska, Lincoln, USA; [1]1=Early Gallatin; 2=Redlands pioneer; 3=Montcalm; 4= Golden Gate Wax; 5= PI260418; 6= Great Northern 1140; 7= Aurora; 8= Mexico 309; 9= Mexico 235; 10=CNC; 11= PI181996; 12= Ouro Negro; *Ur*= rust resistance genes; Pv= chromosomes. [2]UNK=unknown.

The pathotypes 2-0 and 4-0 were the most predominant. The occurrence of several rust pathotypes in a district was an indication that there was high variability of the rust pathogens within those locations. Masaka district had only one pathotype, 2-0 which indicates low variability of the rust pathogens in that location. The diversity of the isolates collected in Uganda has implications on the release of common bean cultivars, for instance, the three pathotypes 5-1, 50-0 and 4-33 were found in Masindi district whereas two races, 63-19 and 4-0 were found in Wakiso district. These two districts represent the major common bean growing regions (WR and CR) where farmers grow different types of common bean cultivars which are susceptible to diseases including rust as reported by Abate et al. (2012). Deploying cultivars with single resistance genes will not be appropriate in such areas because of the different rust pathotypes, and therefore requires the use of a combinations of resistance genes as suggested by Arunga et al. (2012).

The cultivars with the Mesoamerican background, Mexico 309 (*Ur-5*) and Mexico 235 (*Ur-3*$^{+}$) were resistant to all rust pathotypes, followed by Aurora (*Ur-3*), CNC (*UNK*) which were resistant to all rust pathotypes except pathotypes 63-19 and Ouro Negro (*Ur-14*) which was susceptible to only pathotype 4-33. On the other hand, the genotype Montcalm (UNK) of the Andean background were susceptible to five rust pathotypes 4-0, 63-19, 5-1, 4-33 and 2-0. The pathotype 4-33 was observed to be pathogenic to Mesoamerican cultivars, GN1140 (*Ur-7*) and PI181996 (*Ur-11*) and Ouro Negro (*Ur-14*). The Mesoamerican cultivars Mexico 309, Mexico 235, Aurora, CNC and Ouro Negro could be good sources of resistance to bean rust in Uganda. Similar Mesoamerican sources of resistance have been recommended in South Africa, Kenya and Brazil (Arunga et al., 2012; Liebenberg and Pretorius, 2011; Souza et al., 2011).

Conclusion

High severity of common bean rust was observed in the low altitudes and in the South-Western Highlands of Uganda. In addition, higher rust disease severity was observed for the bean-maize–groundnut cropping systems, and fields cultivated with either commercial genotypes or landraces. Six unique Ugandan rust pathotypes where identified. Most of the pathotypes had Andean background. The rust pathotype 63-19 showed similar pathogenic characteristics with the Puerto Rico rust race 19-63. Also, the similarity in these two races will help in identification of new rust resistance sources to manage bean rust disease in Uganda.

Conflict of Interests

The authors have not declared any conflict of interests.

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Authors' contributions

This work was carried out in collaboration between all authors. Author BAO designed the study, carried out the experiment and wrote the first draft of the manuscript. Authors STN, CM, PP, PR, JK and JS reviewed the experimental design and all drafts of the manuscript. Authors BAO, STN and PR performed the statistical analysis and managed the literature searches. All authors read and approved the final manuscript.

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Full Length Research Paper

Chemical and microbiological changes in a sandy soil with pig liquid waste application in Southern Brazil

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Liquid residue from pig farming contains nutrients that can be used for the fertilization of cultivated soils. The aim of this study was to evaluate chemical and microbiological changes in a sandy soil under pasture with Bermuda Grass (*Cynodon* spp) that received doses of pig liquid waste (PLW). The experiment was conducted in Cianorte-PR, Brazil, in a Typic Hapludox soil with sandy texture. The treatments consisted of 30, 60 and 90 m^3 ha^{-1} yr^{-1} of PLW or chemical fertilizer (CF) applied for two years in a randomized block design, with three replications. Soil samples were taken at 0-10 cm, 10-20 cm and 20-40 cm layers, after three months of the second consecutive application of PLW in the second year, before grazing. PLW increased the concentrations of P, C and K at 10-20 and 20-40 cm soil depth, in addition to increasing the microbial biomass carbon and nitrogen and the population of rhizobia at 0-10 cm, in the treatment with 90 m^3 ha^{-1} yr^{-1}. PLW improved the chemical fertility at deeper soil layers and the biological fertility at 0-10 cm of a sandy soil under pasture.

Key words: Microbial biomass, organic fertilizer, phosphorus, potassium, rhizobia.

INTRODUCTION

Brazil is the fourth largest global pork producer and exporter, and Santa Catarina, Paraná and Rio Grande do Sul are the main producing states (MAPA, 2016), resulting in the production of pig liquid waste (PLW), which use as a source of nutrients can reduce the costs of agricultural production. However, we have to be aware of the possibility of negative effects of PLW on the soil and water quality due to nitrates, phosphates, salts, trace elements such as copper and zinc, xenobiotic compounds such as antibiotics, as well as potentially pathogenic organisms (Plaza et al., 2004; Scherer et al., 2010; Guardini et al., 2012).

Organic matter is a key component of soil fertility, affecting physical, chemical and biological properties. It includes microorganisms that act in the biogeochemical cycles of C, N, P, among others (Paul and Clark, 1996). Microorganisms are widely recognized to perform important processes in biogeochemical cycles and affect the functioning of natural ecosystems. In addition, the microbial community regulates the plant productivity by direct mechanisms, like symbiosis, and indirect effects on plant diversity through their influence on the availability of

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Table 1. Chemical analysis of pig liquid waste (PLW) and soil at 0-20 cm depth before the installation of the experiment.

	DM	N	P	K	Ca	Mg	Al	Al+H	pH	C
PLW [1]	-- g L^{-1} --		---------- g kg^{-1} ----------							
	1.7	31.3	31.6	71.2	39.8	17.1	-	-	-	-
Soil [2]			mg dm^{-3}		---------- $cmol_c$ dm^{-3} ----------					g dm^{-3}
	-	-	9.4	0.10	0.76	0.23	0.13	3.42	4.3	7.48

[1]DM, dry matter determined in the *in natura* residue; average of three repetitions. The nutrients in the PLW represent the total concentration determined in the DM. N determined by Kjeldahl method; P, K, Ca and Mg determined in nitric-perchloric extracts. [2]Nutrients and Al represent the available concentrations. K and P extracted by Melich-1; pH determined in $CaCl_2$ 0.01 mol L^{-1}; Ca and Mg extracted in KCl 1 mol L^{-1}, Al determined in SMP buffer; C: Walkley-Black method.

nutrients (Van Der Heijden et al., 2008). Even in agricultural soils, the microbial community plays a pivotal role (Paul and Clark, 1996).

Soil is usually the final recipient of wastes from human activity, where the biodegradable organic fraction is used as source of carbon and nutrients for microorganisms, which simultaneously carry out mineralization and immobilization processes. Most of the carbon added to the soil through waste returns to the atmosphere as CO_2, while the mineral fraction is immediately made available to plants or is partially immobilized in microbial biomass, which acts as a nutrient reserve (Paul and Clark, 1996). The microbial immobilization is important when the supply of nutrients is greater than the absorption and immobilization performed by plants, as it helps to prevent nutrient losses, as nitrogen by leaching of nitrate and phosphorus by fixation of phosphate, as they are temporarily protected in the microbial cells.

In general, agricultural studies on waste application focuses on agronomic and environmental changes mainly in clayey soil, due to its higher buffering capacity, whereas less frequent studies are conducted on sandy soils. In many cases, however, logistics involved in the transport of residues to be applied in clayey soil is unfeasible, which requires applying it in sandy soils close to the generating source. However, the environmental risks are higher and, therefore, careful monitoring is essential to reduce the risks of environmental degradation.

This work arose from the need to define safe doses of pig liquid waste in sandy soils of agricultural areas near the pig farmers in the state of Paraná, Brazil. At first, the main concern was about the effects of PLW on soil chemical and physical characteristics. Later, micro-biological and biochemical characteristics were included as they are pivotal components of the soil quality. This study aimed to evaluate the effects of PLW application on chemical and microbiological attributes of a sandy soil under pasture, compared with the use of chemical fertilizer.

MATERIALS AND METHODS

The study was developed in the second year of consecutive PLW application, in a field trial conducted in Cianorte, state of Paraná (PR), Brazil (23º 39' 28" S, 52º 42' 47" W), 490 m above sea level, humid subtropical climate (C*fa*) according to Köppen (Figure 1). The soil granulometric fraction is formed by 870 g kg^{-1} of sand, 90 g kg^{-1} of clay and 40 g kg^{-1} of silt, classified as Typic Haplustox (Soil Survey Staff, 2014; Santos, 2013).

The soil acidity was corrected with dolomitic limestone (20% of CaO and 20% of Mg) at 2.2 t ha^{-1} before the installation of the experiment. The four treatments consisted of three applications of PLW at doses of 30, 60 and 90 m^3 ha^{-1} yr^{-1}, and a control with only chemical fertilizer applied on the soil surface under pasture with Bermuda Grass (*Cynodon spp*). The experiment followed a randomized complete block design with three replications and plots of 10 m × 5 m, spaced 2 m apart. Doses of PLW were based on models to estimate the amount for each soil type, taking into account the texture, chemical properties and topography (Castro Filho et al., 2001). PLW was first applied in 2002 twice a year: half in early summer and half in early winter. The compositions of PLW and soil chemical properties at 0-20 cm are shown in Table 1. The control treatment with chemical fertilizer (CF) received 60 kg ha^{-1} of P_2O_5 (Triple Superphosphate), 60 kg ha^{-1} of K_2O (KCl) and the N recommended for group II pastures (eg., Bermuda grass), 80 kg ha^{-1} (urea). The fertilizers were divided in two applications a year, at the same time of PLW application, in the respective plots.

In October 2004, before grazing and three months after the application, 10 sub-samples were taken in each plot at 0-10 cm, 10-20 cm and 20-40 cm of soil depth using a Dutch type auger, and pooled to form a composite sample. For chemical analysis, samples were dried at 60°C and sieved (2 mm). Total organic C (TOC) was obtained by oxidation with $K_2Cr_2O_7$ in acid medium and conversion to the equivalent of organic matter (OM) by a factor of 1.724 (Van Bemmelen factor), pH ($CaCl_2$), H+Al (SMP - Shoemaker, McLean and Pratt - buffer), Ca and Mg (KCl), P and K (Mehlich) (Pavan et al., 1992). Microbiological analyzes were performed in field moist samples collected at 0-10 cm layer. Populations of ammonifying microorganisms (Amo) and rhizobia (Rhiz) nodulating common bean (*Phaseolus vulgaris L*.) were estimated by the most probable number (MPN) technique (Hungria and Araújo, 1994). Carbon (C-MB) and nitrogen (N-MB) in the soil microbial biomass were estimated by the fumigation-extraction method (Brookes et al., 1985; Vance et al., 1987). The C-MB/TOC ratio was also calculated.

Statistical analysis

The analysis of variance (F-test, $P < 0.05$) was performed using the software SISVAR v. 4.6 (Ferreira, 2011), with MPN data for ammonifyers and rhizobia transformed to Log_{10}. Once the effects of the treatments were observed, the averages were compared by Student's *t*-test ($P<0.05$). A principal component analysis (PCA) was performed using the results of chemical and microbiological

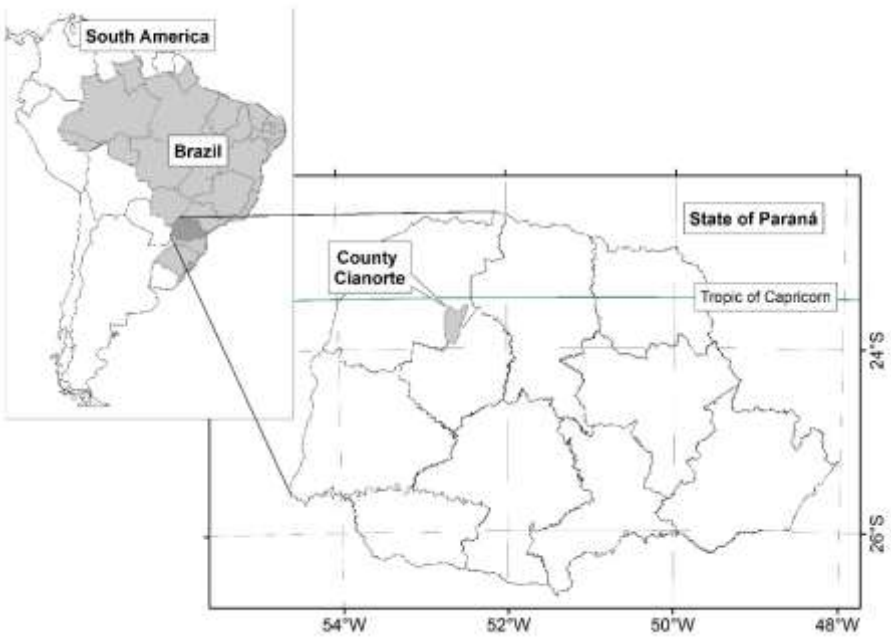

Figure 1. Location of the sampling site in Cianorte, State of Paraná, Southern Brazil.

soil properties at the 0-10 cm layer (Addinsolft, 2009).

RESULTS AND DISCUSSION

Chemical Properties

The highest dose of PLW and the chemical fertilization resulted in higher P concentration in the topsoil, which decreased with depth. At the 0-10 cm layer, applications of PLW in doses from 30 to 90 m^3 ha^{-1} yr^{-1} resulted in lower P concentrations in the soil in relation to the chemical fertilizer. At the 10-20 cm layer, the addition of 90 m^3 ha^{-1} yr^{-1} significantly increased the P levels in relation to the other treatments. At 20-40 cm layer, there was a P increase with the addition of 60 or 90 m^3 ha^{-1} yr^{-1} of PLW compared with chemical fertilizer and 30 m^3 ha^{-1} yr^{-1} of PLW (Figure 2).

Increments of available P in the soil surface by the addition of PLW were observed by Scherer et al. (2010), with successive applications for 20 years from 30 to 60 m^3 ha^{-1} of PLW in Latossolo (Oxisol), Cambissolo (Inceptisols) and Neossolo (Entisols), containing 42, 42 and 36% of clay, respectively. Da Veiga et al. (2012) found smilar results in a Latossolo Vermelho Distroférrico (Hapludox) that received 50, 100 or 200 m^3 ha^{-1} yr^{-1} of PLW for nine years, as well as reported by other authors (Ceretta et al., 2010; Guardini et al., 2012). This result was expected because of the high content of P in the PLW, that is, 31.6 g kg^{-1} (Table 1).

The distribution of P along the soil layers have different mobility depending on the source, that is, CF or PLW. Greater mobility of P was observed in the soil profile in treatments with PLW. Taking the highest dose of PLW as example, the P concentration at 10-20 cm was 44% of the value found at the surface layer, whereas at 20-40 cm it was 37%. In turn, CF provided lower concentrations, both in absolute and relative figures, that is, only 18 and 8%, respectively, at the same layers (Figure 2).

P levels at 0-10 cm in treatments that received the highest dose of PLW or CF are above 42 mg dm^{-3}, considered high according to the Commission of Chemistry and Soil Fertility of the States of Rio Grande do Sul and Santa Catarina (CQFSESCRS, 2004), for a clay content of 9%. According to Ceretta et al. (2010), the soil P added via PLW is predominantly inorganic, especially $H_2PO_4^-$ and HPO_4^{2-}, and considerable amounts of P precipited with Mg^{2+}. This fact favors P losses by runoff and increases the risk of eutrophication of water resources (Scherer et al., 2010; Guardini et al., 2012). This requires special attention to conservation practices on soils intended to receive PLW. Moreover, the doses must be adjusted to the plants' nutrient requirement in order to avoid contamination of deep waters due to leaching.

The content of C and OM at 0-10 cm increased with doses of PLW and did not differ in relation to the CF treatment for doses 60 and 90 m^3 ha^{-1} yr^{-1} (Table 2). At 10-20 cm, the dose 90 m^3 ha^{-1} yr^{-1} significantly increased the C and OM contents in relation to the CF, while at 20-40 cm, the doses 60 and 90 m^3 ha^{-1} yr^{-1} resulted in higher levels. Conversely, the application of pig manure up to 200 m^3 ha^{-1} yr^{-1} for 9 years on an Oxisol, and 150 m^3 ha^{-1} yr^{-1} of pig slurry for 4 years on a Calcic Luvisol did not

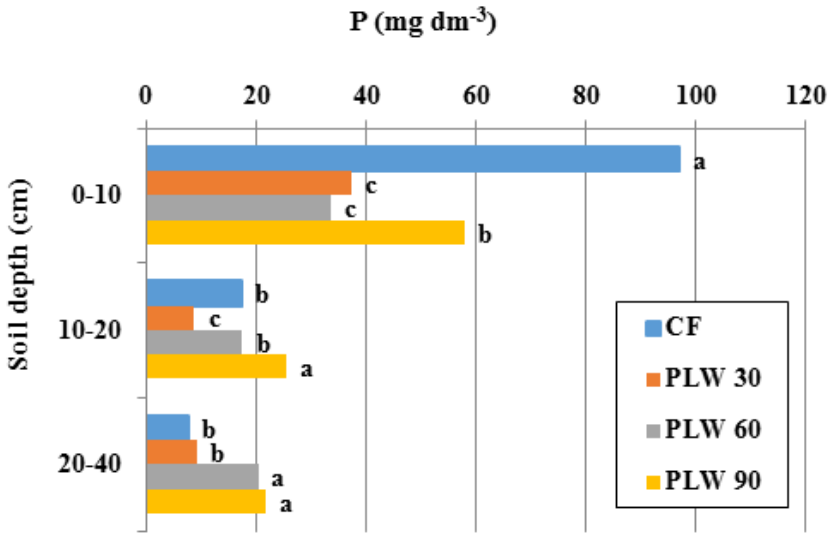

Figure 2. P levels at three depths of a Typic Haplustox soil under pasture, submitted to successive applications of doses of pig liquid waste (PLW) (30, 60 or 90 m^3 ha^{-1} yr^{-1} split in two applications) or chemical fertilizer (CF) (60 kg ha^{-1} of P_2O_5 as Triple Superphosphate, 60 kg ha^{-1} of K_2O as KCl, and 80 kg ha^{-1} of N as urea, split in two applications) in Cianorte-PR, Brazil. Coefficient of variation CV (%): 11.4% (0-10 cm), 17.4 (10-20 cm) and 28.9 (20-40 cm). Same letters in the column do not differ by Student's *t*-test (P <0.05).

Table 2. Chemical characteristics at three soil layers of a Typic Haplustox under pasture, submitted to successive applications of doses of pig liquid waste (PLW) or chemical fertilizer (CF) in Cianorte-PR, Brazil.

Treatments	C	OM	pH	H+Al	Ca	Mg	K
	g kg^{-1}	g kg^{-1}	$CaCl_2$	----------cmolc dm^{-3} ----------			
				Depth: 0-10 cm			
CF[1]	18.3^{ab}	31.5^{ab}	5.5^{a}	2.8^{a}	2.8^{a}	1.6^{ab}	0.17^{c}
30 PLW[2]	15.0^{c}	25.8^{c}	5.3^{a}	2.9^{a}	1.6^{b}	1.0^{c}	0.30^{b}
60 PLW	17.2^{ab}	29.6^{ab}	5.2^{a}	2.9^{a}	1.8^{b}	1.2^{bc}	0.37^{ab}
90 PLW	24.5^{a}	42.1^{a}	5.6^{a}	2.7^{a}	2.5^{a}	1.8^{a}	0.44^{a}
CV (%)	22.7	22.7	5.8	8.2	16.1	15.8	21.0
				Depth: 10-20 cm			
CF	8.1^{b}	14.0^{b}	5.0^{a}	2.9^{a}	1.3^{a}	1.0^{ab}	0.11^{c}
30 PLW	8.1^{b}	13.9^{b}	4.9^{a}	2.9^{a}	1.0^{a}	0.8^{b}	0.17^{bc}
60 PLW	11.7^{ab}	20.2^{ab}	4.9^{a}	3.0^{a}	1.4^{a}	0.9^{ab}	0.19^{ab}
90 PLW	12.6^{a}	21.7^{a}	5.4^{a}	2.7^{a}	1.4^{a}	1.3^{a}	0.25^{a}
CV (%)	20.8	20.8	8.5	12.0	17.0	17.0	22.0
				Depth: 20-40 cm			
CF	6.8^{b}	11.7^{b}	5.0^{a}	2.9^{a}	1.0^{a}	0.6^{a}	0.07^{b}
30 PLW	8.4^{ab}	14.5^{ab}	5.0^{a}	2.9^{a}	1.1^{a}	0.7^{a}	0.11^{ab}
60 PLW	11.2^{a}	19.3^{a}	5.0^{a}	2.9^{a}	1.3^{a}	0.8^{a}	0.15^{a}
90 PLW	10.3^{a}	17.6^{a}	5.1^{a}	2.8^{a}	0.9^{a}	0.8^{a}	0.17^{a}
CV (%)	17.4	17.4	6.8	13.7	23.5	16.3	29.5

Average of three replications. K extracted by Melich-1; pH determined in $CaCl_2$ 0.01 mol L^{-1}; Ca and Mg extracted in KCl 1 mol L^{-1}, Al determined by SMP buffer; C: Walkley-Black and OM: organic matter (carbon × 1.724). Same letters in the column do not differ by Student's *t*-test (P <0.05). [1]Chemical fertilizer (60 kg ha^{-1} of P_2O_5 as Triple Superphosphate, 60 kg ha^{-1} of K_2O as KCl, and 80 kg ha^{-1} of N as urea, split in two applications). [2]Pig liquid waste (30, 60 or 90 m^3 ha^{-1} yr^{-1} split in two applications). CV: coefficient of variation.

Table 3. Microbiological characteristics at 0-10 cm of soil depth in a Typic Haplustox under pasture, submitted to successive applications of doses of pig liquid waste (PLW) and chemical fertilizer (CF) in Cianorte-PR, Brazil.

Treatments	C-MB[1]	N-MB[2]	C-MB/TOC[3]	Amo[4]	Rhiz[5]
	---------- µg g^{-1} of soil ----------		%	------ Log_{10} MPN g^{-1} -----	
CF[6]	260[b]	52[b]	1.51[a]	7.7[a]	3.5[b]
30 PLW[7]	213[b]	68[b]	1.43[a]	7.8[a]	3.9[b]
60 PLW	262[b]	58[b]	1.51[a]	7.7[a]	2.8[b]
90 PLW	351[a]	87[a]	1.47[a]	7.7[a]	4.7[a]
CV (%)	15.2	12.6	23.0	5.6	16.8

Average of three replications. Same letters in the column do not differ by Student's *t*-test ($P < 0.05$). [1]Carbon microbial biomass; [2]Nitrogen microbial biomass; [3]Ratio between microbial biomass carbon and total soil organic carbon; [4]Population of ammonifying microorganisms; [5]Population of rhizobia nodulating common bean. [6]Chemical fertilizer (60 kg ha^{-1} of P_2O_5 as Triple Superphosphate, 60 kg ha^{-1} of K_2O as KCl, and 80 kg ha^{-1} of N as urea, split in two applications). [7]Pig liquid waste (30, 60 or 90 m^3 ha^{-1} yr^{-1} split in two applications). CV: coefficient of variation.

change the soil OM (Da Veiga et al., 2012; Plaza et al., 2004). Differences in climate, soil type, management and crops may reflect different accumulations of OM in the soil profile due to PLW applications.

No effect of the application of PLW was observed in relation to soil pH and potential acidity at any layer (Table 2). However, Ca increased at the higher dose at 0-10 cm, compared with 30 and 60 mg ha^{-1} yr^{-1}, without differ from CF. Similar behavior was observed for Mg at 0-10 and 10-20 cm. The use of PLW at doses of 60 and 90 m^3 ha^{-1} yr^{-1} increased K concentration in relation to CF. Although the levels decreased with soil depth, this effect was observed up to 20-40 cm. These results are consistent with several studies that demonstrated higher levels of K at the topsoil with texture ranging from 15 to 42% of clay, and receiving up to 200 m^3 ha^{-1} yr^{-1} of PLW (Scherer et al., 2010; Guardini et al., 2012).

Microbiological characteristics

The application 90 m^3 ha^{-1} yr^{-1} of PLW resulted in significant increases in C-MB, N-MB and the population of rhizobia (Rhiz) nodulating of common bean. However, there was no effect on the C-MB/TOC ratio and in the population of ammonifying microorganisms (Amo) (Table 3).

In the present study, only two years of implementation of the trial were enough to observe increases by 35 and 67% in the C-MB and N-MB in soil, which reached 351 µg C g^{-1} and 87 µg N g^{-1}, respectively. This increase was probably due to the increased content of organic matter caused by the application of PLW, as the C-MB/TOC ratio remained unchanged, ranging from 1.43 to 1.51%, with no significant differences between treatments. Increases in C-MB and N-MB were observed in Neossolo with 19% clay after four years of applications (Plaza et al., 2004). Moreover, the application of PLW also contains microbial biomass that can be incorporated into the soil (Plaza et al., 2007; Zornoza et al., 2013).

These results for microbial biomass are in agreement with works on applications of PLW in soil, where values for C-MB are between ~37 and ~570 µg g^{-1} (Deng et al., 2006; Zornoza et al., 2013) and N-MB ranges between ~30 to ~120 µg g^{-1} (Deng et al., 2006). Differences in values in soil microbial biomass depend on the PLW and on soil and environmental characteristics, such as the contents of C and N in manure and in soil, temperature and moisture regimes, etc.

In general, the levels of soil organic matter resulting from application of PLW did not differ significantly between treatments with and without PLW (Hernandez et al., 2007; Plaza et al., 2007). Slight variations in soil organic matter in sandy soil and in clay soil receiving 90-300 m^3 ha^{-1} yr^{-1} was enough to stimulate the soil microbial carbon by 36 to 68%, between 340 and 572 µg C-MB g^{-1}, in periods ranging from 14 days to 5 years (Hernandez et al., 2007; Plaza et al., 2007). It is possible that effects of PLW application are more noticeable in soils with low initial levels of organic matter as in the present study (12.9 g dm^{-3} before the applications). Studies of Zornoza et al. (2013) showed that soils receiving the same dose of PLW but with different contents of organic matter (4.1 or 21.1 g kg^{-1}) resulted in similar levels of C-MB. However, in soils with higher content of organic matter (57.0 g kg^{-1}), Morales et al. (2016) found no effect even after the application of 209 kg N as PLW ha^{-1} yr^{-1} for fourteen years. Thus, in soils with low initial content of organic matter, organic carbon added via pig manure provides a source of energy and organic and inorganic nutrients to the soil microorganisms and boosts the microbial biomass.

In this study, microbial biomass was a sensitive biological indicator with significant responses to organic matter changes in the soil, as a result of C and N inputs with the application of PLW in a short period. However, these effects may be temporary due to the presence of readily degradable compounds, associated to a lower C/N ratio and low stability of the organic matter added (Guerrero et al., 2007; Hernandez et al., 2007). In this

regard, relatively high doses are required to keep the MB values significantly higher than the control soil (Guerrero et al., 2007) and depend on the continuous C input over time.

Compared with the treatment with chemical fertilizer, the application of 90 m^3 ha^{-1} of PLW stimulated almost 16 times the population of rhizobia that nodulates common bean living saprophytically in the soil (Table 3). Although growing in a soil without a legume host, such microorganisms also act in the biogeochemical cycles, besides being plant growth promoters. Thus, its increase in soil is considered beneficial, although not directly benefiting the pasture with *Cynodon* sp.

Similar populations of rhizobia in soil that received wastes as organic fertilizers have been found (Zengeni et al., 2006; Kimiti and Odee, 2010). In general, the cowpea cultivation in sandy soil that received animal manure, application of P and a combination of both resulted in a population of rhizobia ranging from 2.7 to 4.3 Log_{10} MPN g^{-1} (Kimiti and Odee, 2010). Increases of 100 times in the rhizobia population were observed soils with 20 to 30% clay, from 10^1 CFU g^{-1} in the control to 10^3 CFU g^{-1} in the treatment that received 10 t ha^{-1} of manure and cultivated with soybean for two years (Zengeni et al., 2006). The application of PLW provided a favorable environment for the population of rhizobia due to increases in soil organic matter (Plaza et al., 2007), in addition to the supply of nutrients and improvement of soil fertility (Zengeni et al., 2006; Kimiti and Odee, 2010).

Although moderate doses of PLW may favor the soil chemical and biological fertility, excessive doses of PLW for long periods may accumulate high concentrations of heavy metals in the soil, such as copper, zinc and other elements. Therefore, there is a great environmental concern about the disposal of PLW in soil that might lead to accumulation of metals and other elements, and could have negative impacts on soil microorganisms. Excess of metals may impair the activity, the survival, growth and N fixation capacity of rhizobia, and change populations in relation to metal tolerance (Chaudri et al., 1992; Tindwa et al., 2014).

Regarding metal tolerance, strains of *Rhizobium leguminosarum* biovar *trifolii* isolated from root nodules of clover grown in sandy soil contaminated with heavy metals (Zn, Cu, Ni and Cd) were more tolerant to those metals, thus allowing their survival, but lost their ability to fix nitrogen with *Trifolium repens*. On the other hand, strains originating from non-contaminated soil can fix nitrogen, but due to intolerance to metals, they are not able to survive in contaminated soil (Chaudri et al., 1992).

The absence of adverse effects of a higher dose of PLW on the population of rhizobia in the soil of the present work probably indicates low input of toxic elements because of the short period of two years and four applications of PLW on soil under pasture.

Furthermore, it should be considered that the presence of vegetation provides greater protection to rhizobia, reducing the availability of metals and consequently its toxicity (Renella et al., 2007).

In theory, the physiology of microbial groups related to the nitrogen cycle such as ammonifyers and nitrobacteria are responsible for controlling ammonification and nitrification processes, respectively (Jiang et al., 2015). The ammonifying microorganisms are represented by various prokaryotes, algae (Cyanophyceae) and fungi using nitrogenous organic compounds as source of carbon and N, releasing ammonia, which in part volatilizes to the atmosphere. In the soil solution, ammonia is converted into ammonium ion in the presence of H^+, which may be adsorbed on negative charges of soil or converted into amino acids by plants and microorganisms. It may be microbiologically oxidized to nitrate in the nitrification process and thereafter follow denitrification (Paul and Clark, 1996). In the present work, the density of ammonifyers was not affected by the treatments, averaging 7.7 Log_{10} MPN g^{-1} (Table 3). The lack of effect of treatments on the population of ammonifyers is probably related to the low C/N ratio of PLW. As it is a source of easily degradable carbon, we must consider the time between the PLW application and the soil sampling. The elapsing time might have been enough to recover any eventually transient effect on the ammonifying population.

Ammonifying microorganisms play important role in the soil biological fertility and its capacity to supply nitrogen to the soil microbiota and plants (Acea and Carballas, 1996). Therefore, management practices that maintain the population of ammonifying microorganisms is important to keep the soil health and its role in the nitrogen cycling (Aparna et al., 2014). As seen in the present work, the application of PLW was not harmful to the soil ammonifying microorganisms.

Principal component analysis

Principal component analysis (PCA) correlates the set of chemical and microbiological soil attributes to treatments (Figure 3), where factors 1 and 2 accounted for 86% of the total variability.

The factor 1 was more related to the variables C, pH, H+Al, Ca, Mg, N-MB and the rhizobial population, explaining 55% of the variability. The factor 2 was more associated to the variables P, K, C-MB, C-MB/ TOC and the population of ammonifyers, explaining 31% (Figure 3). The factor 1 was associated with the doses of PLW 30 (24%), 60 (16%) and 90 (56%) m^3 ha^{-1} yr^{-1}, whereas the factor 2 was associated with the CF (54%).

The CF treatment influenced the values of P, Ca, Mg and the C-MB/TOC ratio. The dose of 90 m^3 ha^{-1} yr^{-1} was more related to the variables C, pH, K, C-MB, N-MB and the rhizobia population. The dose of 30 m^3 h^{-1} yr^{-1} showed effects on the population of ammonifying microorganisms, whereas the dose 60 m^3 h^{-1} yr^{-1} only

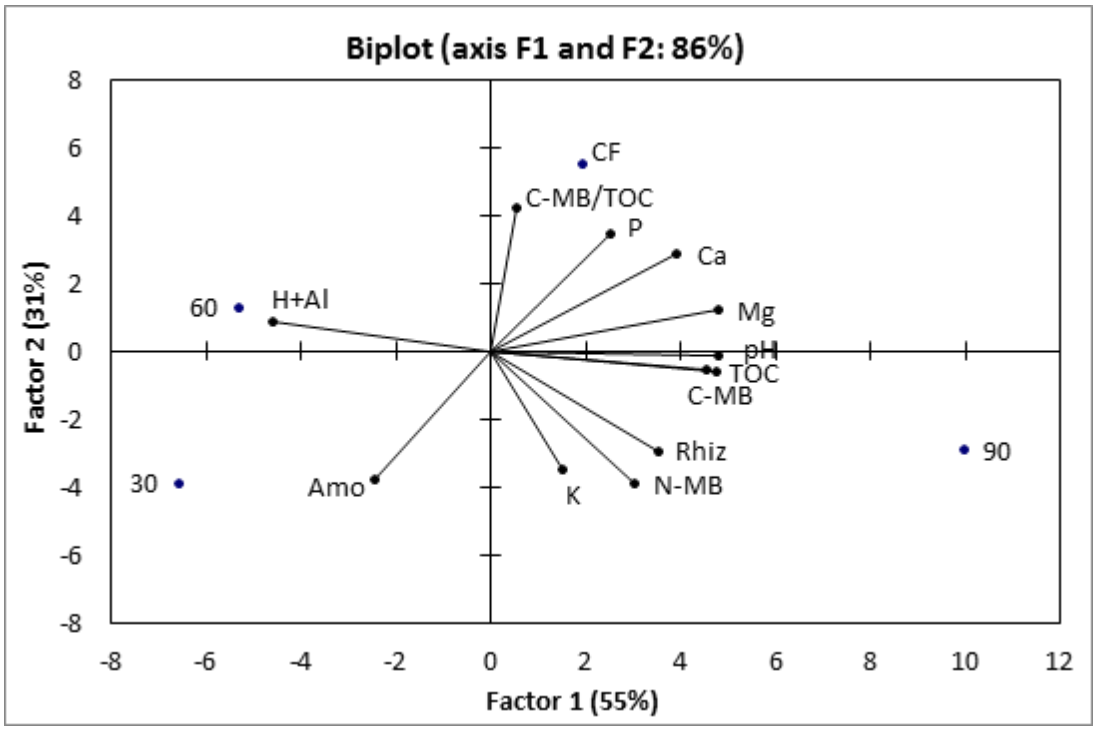

Figure 3. Principal component analysis based on eight chemical and five microbiological attributes at 0-10 cm of depth in a Typic Haplustox soil under pasture, submitted to application of three doses of pig liquid waste (PLW) and chemical fertilizer (CF) in Cianorte-PR, Brazil. CF: Chemical fertilizer (60 kg ha^{-1} of P_2O_5 as Triple Superphosphate, 60 kg ha^{-1} of K_2O as KCl, and 80 kg ha^{-1} of N as urea, split in two applications); PLW: Pig liquid waste (30, 60 or 90 m^3 ha^{-1} yr^{-1} split in two applications); C-MB: microbial biomass carbon; N-MB nitrogen microbial biomass; TOC: total organic carbon; C-MB/TOC (%): carbon microbial biomass in relation to the total organic carbon; Amo: ammonifying microorganisms; Rhiz: *Rhizobium* sp.

presented a relation with the potential acidity. The dose 90 m^3 ha^{-1} yr^{-1} was associated with microbiological variables, K, total carbon and pH, which are related to the availability of nutrients in the soil. Soil pH is directly correlated with the availability of nutrients and also influences the microbial activity.

Conclusions

Applications of pig liquid waste in soil increase C, P, and K levels, especially at deeper soil layers compared with chemical fertilization.

Applications of pig liquid waste for two years change nutrients and soil microbiota, contributing to improve the chemical and microbiological properties of a sandy Typic Hapludox soil under pasture.

Overall, the absence of negative effects of pig liquid waste on the soil populations of rhizobia and ammonifying microorganisms suggests low concentration of available harmful elements in the residue.

Finally, the application of pig liquid waste to pastures on sandy soils with original low levels of soil organic matter seems promising as final destination of residues and improvement of soil chemical and biological fertility. However, the doses must be criteriously established and the soil monitoring must be done to minimize risks of soil and water degradation.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effects of the application of treated domestic sewage via surface and subsurface drip irrigation on the solution and chemical properties of the soil in an orange plantation

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The use of treated domestic sewage (TDS) in agriculture by drip irrigation in the soil subsurface can serve as an extra treatment and also provide higher availability of water and nutrients to plant roots. It represents safer and healthier cultivation for crops, producers and consumers, and allows the direction of better quality water for domestic consumption. This study aimed to analyze the possible effects of TDS irrigation on chemical and biological properties of the soil in an orange plantation. The experiment was conducted on Lagoa Bonita Farm, at the Adventist University Center of São Paulo - UNASP - Engenheiro Coelho Campus, SP. A block design with three replications was used in the following treatments: soil irrigated with TDS on the surface, soil irrigated with TDS in the subsurface, and soil with no irrigation. Soil samples were collected for chemical and biological analyzes, as well as samples of the treated domestic sewage used. The TDS characteristics were within the parameters allowed by law. Considering the chemical parameters and the types of irrigation analyzed, the highest results observed were: K: 11 mmolc.dm^3, Ca: 30 mmolc.dm^3, Mg: 7 mmolc.dm^3, and pH: 5.5.

Key words: Reuse, citrus, soil chemistry, soil solution.

INTRODUCTION

The rational use of soil and water is currently seen as vital to the existence and livelihood of future generations around the world. Brazil has the largest availability of arable land and water in the planet, however, new ways of producing food and utilizing sustainable alternatives must be studied due to the rapid reduction and scarcity of land and water throughout the planet (Scolari, 2005).

Treated domestic sewage (TDS) collected from

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stabilization ponds for use in agricultural irrigation is an alternative water source, as it provides essential nutrients to the crops and may partially or fully replace commercial chemical fertilizers. The application of TDS in the soil subsurface, despite having higher costs and risks of clogging, represents economic benefits due to the better use of water and nutrients by plant roots, less evaporation and improved plant health by avoiding surface runoff (Souza et al., 2012).

Subsurface drip irrigation with wastewater may maximize land use, allowing the reduction of production costs and the intensive use of machines with expertise and implementation of manpower, besides reducing seasonality, providing more favorable prices for producers and consumers, incorporating new areas, facilitating the establishment of agricultural industries, improving the quality and standardization of products, allowing the opening of new markets, producing noble crops, minimizing climate risks, among others (Andrade Neto, 1997).

The application of treated domestic sewage via subsurface drip irrigation tends to be a relevant form of irrigation due to the characteristics of the system, which disposes water directly in the root system and thus presents a high uniformity of application, avoiding wetness of the soil surface and plant shoots. It also eliminates drift issues, increasing the efficiency of fertilizer application. Even though several studies have been conducted on the use of treated domestic sewage via subsurface irrigation in agriculture, the application of this technique by farmers is still limited.

The reduction or minimization of environmental impacts in agriculture is essential for the adoption of models that measure the quality of the environment considering the various factors that promote environmental sustainability, such as product quality and production indicators, consumption and pollution of water resources, soil quality indicators and flow of gases with a global warming potential.

The advantage of using treated domestic sewage is to allow better conservation of water bodies and contribute with considerable amounts of nutrients to the soil, resulting in improved fertility and reduced costs with mineral fertilizer (Souza et al., 2012). With the expansion of sewerage networks, public awareness, and the monitoring and inspection by environmental agencies, the disposal of treated domestic sewage by soil subsurface irrigation in agriculture will certainly be adopted (Oliveira et al., 2013).

Throughout the last years, several studies have shown concern about the application of wastewater to the soil. The operation and maintenance of the systems must be constant in irrigation with post-treated sewage, as the water used has characteristics that may modify and destroy soil fertility if no proper precautions are taken (Lima et al., 2006). However, the soil is a natural filter. It is a combination of physical and chemical treatments and biological processes occurring in the soil-plant-atmosphere system, but the clearance rate of said system is slow. Despite providing nutrients to the plants, wastewater is also a source of contaminants to the soil, in addition to causing nitrate leaching into groundwater and emissions of greenhouse gases (Tzanakakis et al., 2007).

This study aimed to assess chemical behavior parameters in soil irrigated with treated domestic sewage applied via subsurface drip irrigation in an orange plantation in the city of Engenheiro Coelho, São Paulo.

MATERIALS AND METHODS

The experiment was installed in 2015, in the city of Engenheiro Coelho - SP (22° 29' 18" S, 47° 12' 54" W), on Lagoa Bonita Farm, in an orange plantation owned by the Brazilian Central Union of the Seventh-day Adventist Church, located at UNASP, next to a sewage treatment plant. The plots measured 6 m x 50 m, totaling an area of 300 m^2 per experimental plot. The study was conducted between August 2015 and January 2016.

According to the Köppen classification, the local climate is humid subtropical, with temperatures above 22°C in the hottest month of the year and below 18°C in the coldest month. Annual rainfall in the region is 1,328 mm and the predominant soil is classified as typical eutrophic Red Yellow Argisol.

The wastewater used was collected from a secondary facultative pond at UNASP's sewage treatment plant (STP) by a pressurized sand filter system to remove suspended solids. The STP consists of Australian-type stabilization ponds, with a receptive anaerobic pond and two facultative ponds (one primary and the other secondary).

The application of treated domestic sewage was performed by a surface irrigation system and by a subsurface irrigation system at 0.2 m deep from the soil surface. The drippers used (Super Typhoon, by Netafim) were spaced every 0.5 m, with a flow rate of 1.75 L h^{-1} and a wall thickness of 0.38 mm.

Irrigation was performed every day from Monday to Friday during the months of August to December, 2015, based on the following treatments: Drip Irrigation (DI), Subsurface Drip Irrigation (SDI) and no irrigation (NI). Data concerning to evapotranspiration were estimated by the Hargreaves-Samani method, based on temperature data from a weather station installed next to the experimental area.

Treated domestic sewage samples were collected for the verification of the following parameters: pH, Fe, EC (in dS m^{-1}), Calcium, Iron, Total Phosphorus, Nitrate, oils and grease, COD and Sulfide. The pH was determined by the hydrogen- ion activity of the soil-water solution by means of potentiometry. The exchangeable acidity (Al^{+3}) was extracted with potassium chloride (KCl) 1 mol L^{-1} and quantified by titration with sodium hydroxide (NaOH) at 0.025 mol L^{-1} (Embrapa, 1999). Phosphorus (P) was extracted with Melich solution and determined by calorimetry and flame photometry. The calcium ion (Ca^{2+}) was extracted with KCl at 1 mol L^{-1} and quantified by inductively coupled plasma optical emission spectrometry (ICP-OES).

Transversely to the crop row, a trench was opened (0.2 m wide and 0.4 m deep), using as a reference the center of the planting furrow for collecting soil samples for physical and chemical characterization. Three simple soil samples were collected per depth (0.20 m and 0.20-0.40 m) in the direction parallel to the surface. The following soil parameters were assessed: Organic Matter (OM), pH, P, K, Ca, Mg, $H^{+}Al$, Al, SB, CEC, S, Cu, Fe, Mn, Zn and B.

Samples of soil solution were collected by extractors installed in

Table 1. Mean values of the main physicochemical parameters of the TDS.

Parameter	Legal standards (Conama-430/2011)	Mean values	SD
pH	5 to 9	7.22	0.05
EC ($dS.m^{-1}$)	1.0 to 3.1	0.83	38.11
Ca (mgL^{-1})	20 to 100	23.3	12.26
Iron ($mg\ L^{-1)}$	15.0	0.34	0.21
Total Phosphorus ($mg\ L^{-1}$ P)	0.025	8.40	3.15
Nitrate ($mg\ L^{-1}$)	10.0	0.14	0.09
Oils and grease ($mg\ L^{-1}$)	30	45	17.44
COD ($mg\ L^{-1}$)	360	35	14.10
Sulfide ($mg\ L^{-1}$)	0.002	0.71	0.27

Source: Biosciences laboratory – UNESP – Botucatu/SP.

the treatment units for the analysis of the following parameters: N, P, K, Ca, Mg, S, B, Cu, Fe, Zn, Mn, pH and EC.

The experiment consisted of a randomized block design with three replications and five treatments, totaling 20 experimental units. The treatments were based on the employment of treated domestic sewage, which was applied through different blades for total irrigation of the subsurface and surface according to the atmospheric demand, and on the Hargreaves-Samani method, which adapts to dry periods.

Data were subjected to analysis of variance and mean values were compared by Tukey's test at 5% probability using the computer system SISVAR version 5.1 Build 72 (Ferreira, 2011).

RESULTS AND DISCUSSION

Table 1 shows the results of the TDS samples collected from UNASP's Sewage Treatment Plant during October, November and December as well as the legal standards regarding effluent disposal established by Resolution 430, from May 13, 2011, by the National Environmental Council – CONAMA.

Most mean values of the parameters of the TDS employed in the irrigation during the experiment are in accordance with the Brazilian legal standards. The pH was slightly basic. Ayres and Westcot (1991) recommend pH between 6.5 and 8.4 for water used in irrigation.

The concentration of H+ and OH- in irrigation water may exert influence on the availabilty and absorption of nutrients by plants, on soil structure and properties, and also on irrigation systems. Some parameters showed values above the standard set by the Brazilian regulation for the use of effluents in irrigation, such as phosphorus, with a value of 8.40 mg L^{-1}, whereas 0.025 mg L^{-1} is permitted; sulfide, with a value of 0.27 mg L^{-1}, whereas 0.002 mg L is permitted; and oils and grease, with a value of 45 mg L^{-1}, whereas 30 mg L^{-1} is permitted.

The use of treated domestic sewage in irrigation depends on the correct management of the irrigation system and on the monitoring of the charecteristics of the soil and crop, since the effects of salinity, sodicity and alkalinity hinder the continuous usage of wastewater in the irrigation of agricultural crops in general.

Total coliform content was 460 per ml of water and thermotolerant coliform content was 43 per ml of water. According to CONAMA Resolution 20/86, the limit is up to 5,000 total coliforms per 100 ml (CONAMA, 1986).

Rainfall during the period of the experiment was 832.9 mm. Figure 1 shows climatological and evapotranspiration data.

Table 2 shows the results of the soil analysis before irrigation with TDS and of the analysis of variance of the different treatments applied.

Based on the results of the analysis of the organic matter found in the soil after the application of TDS, the subsurface drip irrigation caused a significant positive effect. As shown in Table 2, the organic matter content in the soil after the application of treated domestic sewage via SDI was higher than the content in the initial, DI and NI treatments. Most likely, the higher nitrogen and carbon concentration in the other treatments favored the quick mineralization and decrease in the organic matter content of the soil. Feigin et al. (1991) state that the typical C:N ratio of secondary domestic sewage is near five or lower, which causes these elements to easily decompose in the soil and be assimilated by plants.

The pH was also higher in the application of treated domestic sewage via SDI. Higher pH is related to effluent alkalinity, addition of exchangeable cations and anions to soil, and changes in the N cycling production of OH- ions due to enhanced denitrification or nitrate reduction. Fonseca (2005), in a study on irrigation with treated sewage effluent, obtained low soil alkalinization during the experiment regardless of the irrigation system employed.

The values of H + Al of the applications presented variation when compared to the values of the initial chemical analysis. Application via DI presented the highest value, 52 $mmolc.dm^3$. Moreover, the soil that received TDS via SDI presented the lowest values of potential acidity, contrasting with its high organic matter value - that should have contributed for an increase in the potential acidity - and serves as evidence of the inversely proportional relationship between organic matter and pH.

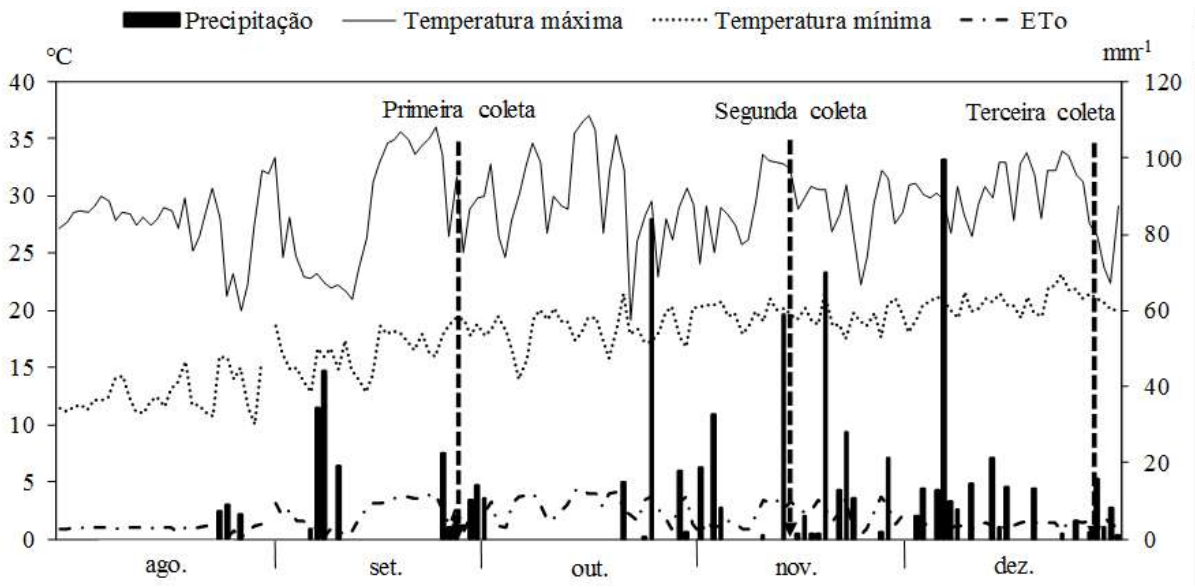

Figure 1. Climatological data during the experiment.

With respect to K, the mean values found were lower in both DI and SDI in comparison with the initial treatment and non-irrigated areas. Silva and Borges (2008) found similar results in a study of an orange crop under successive irrigation with treated domestic sewage. Irrigation with waste water allows an increase in sodium concentration, which consequently benefits the displacement of K in the soil exchange complex. In some cases, there might be a decrease in K due to its leaching and plant absorption.

The highest value of P was found in the application of treated domestic sewage via DI. In relation to Ca, the lowest values were found in the superficial layers of the soil, which might be associated to the migration of Ca^{+} from the soil to the solution in the exchange complex, as there is more nutrient absorption by plants, mainly citrus. Carbonate accumulation, especially calcium carbonate, caused by prolongated irrigation can promote soil cementation, thus hindering the penetration of irrigation water and roots (Egreja Filho et al., 1999; Rego Filho, 2014).

A directly proportional relationship between the decrease of pH and Mg values was observed in the treatments, suggesting a correlation between Mg and soil acidity.

Medeiros et al. (2005) studied a coffee crop under irrigation with TDS and reported that the factors that represent the main negative impacts for the soil in this type of management were the increase in the exchangeable sodium concentration and in the electrical conductivity (EC), the sodium adsorption ratio (SAR) and the exchangeable sodium percentage (ESP).

Table 3 depicts the mean values of the chemical properties of the soil solution after irrigation with treated domestic sewage.

The pH values of the soil solution were higher than the pH values of the soil. Interestingly, under SDI, the pH of the soil solution was the lowest, whereas the pH of the soil was the highest. The pH is an extraordinary indicator of the soil chemical conditions as it has the capacity to interfere directly or indirectly in the way other chemical elements are disposed and interact in the soil.

Electrical conductivity was higher in the application of the TDS via SDI. The precipitation that occurred during the experiment might have influenced the dissolution of effluent salts, causing the decrease in the EC of the soil solution. As the dispersion in the subsurface layers takes more time, the highest electrical conductivity values were found in this type of application.

The highest concentration of K was found in the treatment via SDI, possibly due to its absorption by plants and leaching. Authors such as Silva and Borges (2008) and Leal (2007) state that K concentration in the soil is variable and might be associated to the dynamics of K in the soil-plant-effluent system.

Similarly, the highest concentration of Ca was found in the same type of application, what evidences that the dynamics of this element was influenced by its absorption by plants and leaching.

Magnesium dynamics were similar to the dynamics of Ca. Both presented higher concentration in the application via SDI.

In general, treated domestic sewage applied via SDI presented the highest concentrations in most of the parameters analyzed.

Azevedo and Oliveira (2005), in a study on a cucumber crop (*Cucumis sativus* L.) under irrigation with treated sewage effluent, did not report significant difference between phosphorus concentrations in the soil water regarding different types of applications. Statistically

Table 2. Analysis of variance of the soil chemical parameters.

Soil samples	OM (g.dm^3)	pH	P (mg.dm^3)	K	Ca	Mg	H + Al	S.B.	CEC (mmolc.d^3)	V%	S	Cu	Fe	Mn	Zn	B
				----------mmolc.dm^3----------							----------mg.dm^3----------					
Initial	19	4.2	38	1.6	12	5	41	18	59	30	-	13.5	56	2.0	4.2	0.39
DI	23	4.8	61	0.9	17	6	52	24	76	31	11	20.7	46	8.6	5.7	0.48
SDI	30	5.3	48	1.1	30	7	36	39	74	52	16	22.6	44	2.8	10.4	0.56
NI	18	4.7	34	1.7	15	4	43	21	64	33	29	12.9	57	1.8	5.5	0.57

DI- Drip irrigation; SDI – subsurface drip irrigation; NI – no irrigation.

Table 3. Mean values of the chemical properties of the soil solution under different treatments.

Treatment	N	P	K	Ca	Mg	S	B	Cu	Fe	Mn	Zn	pH	E.C (mS)
	----------mg.L^{-1}----------												
DI	55	0.9	18	23	10	10	0.07	0.04	0.06	0.14	2.6	6.54	0.607
SDI	62	1.5	29	43	11	13	0.1	0.35	0.08	0.41	2.1	6.21	0.769
NI	52	0.8	20	24	10	11	0.1	0.02	0.09	0.15	2.4	6.45	0.602

Source: Soil Fertility Laboratory– FCA UNESP – Botucatu/SP.

higher values of potassium, calcium and magnesium content were observed in the soil irrigated with TDS, as a possible result of the accumulation of these nutrients in the retained soil water.

Conclusion

The application of treated domestic sewage via subsurface drip irrigation provided an increase in the organic matter concentration of the soil in the orange plantation as well as in its nutrients, such as potassium, calcium and magnesium, leading to better plant development and productivity. However, research for a longer period of time in order to thoroughly assess the behavior of the nutrients applied to the soil and their relationship with orange productivity is recommended.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Induction of coffee wilt disease infection using different types of contaminant in field conditions in Democratic Republic of Congo

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A study was conducted on the induction of coffee wilt disease (CWD) infection with different types of contaminants using a randomized complete block design with four replications. Coffee seedlings were inoculated with infected bark, sub-bark tissues, wood necrotic and an artificial inoculum. Statistical analysis did not show significant differences (P>0.05) between treatments. Results obtained show that all contaminants used induced main symptoms of CWD. Chronologically, wilting appeared an average 55 DAI, followed by leaf browning (60 DAI), defoliation (69 DAI), leaf drying (82 DAI) and seedlings mortality (86 DAI). The lowest rate of wilting and leaf browning (11.1%) was recorded on seedlings inoculated with infected bark, and the highest rate (33.3%) was observed on seedlings inoculated with wood necrotic. Seedlings inoculated with sub-bark tissue expressed 11.1% of leaf drying, and those inoculated with an artificial inoculum presented 22.2% of mortality. Seedlings inoculated with sub-bark tissue expressed 22.1% of defoliation, while those inoculated with artificial inoculum expressed 39.4% of defoliation. The presence of *Fusarium xylarioides* was confirmed in dead woods of seedlings inoculated with sub-bark tissue, and those inoculated with artificial inoculum. Results obtained confirm the potential danger of wood debris from infected coffee trees, which can act as a source of infection and promote the spread of CWD when dragged through plantations.

Key words: *Coffea canephora*, coffee wilt disease, *Fusarium xylarioides*, field infection, types of contaminants, Democratic Republic of Congo.

INTRODUCTION

Coffee represents one of the most important agricultural commodities, ranking second in international trade after crude oil (Mishra and Slater, 2012). In Democratic Republic of Congo (DRC), coffee represents both an industrial and export product, which promotes the jobs creation and allows for currency inflows to the treasury

(Anonymous, 1998). However, its production is experiencing a remarkable decline due to aging plantations, the degeneration of planting materials, use of uncertified plant materials and attacks of pests and diseases (Tshilenge-Djim et al., 2004). Among these, coffee wilt disease (CWD) is one of the most important diseases dramatically limiting coffee production (Coste, 1989; Tshilenge-Djim et al., 2004; Girma et al., 2005; Sihen et al., 2012, 2013). This fungal disease caused by *Fusarium xylarioides* Steyaert (teleomorph: *Gibberella xylarioides* Heim & Saccas) had devastated plantations in West Africa and also in DRC (Tshilenge-Djim et al., 2004).

In general, coffee plant infected by CWD shows no sign outside to notice the presence of the pathogen. This disease is manifested by a sudden stop of vegetation, the terminal buds of young shoots turn black; young leaves present along the main veins, chlorotic bands which reach quickly the entire limb. All leaves of terminal shoots turn yellow become flaccid, dangling towards the floor, turn brown, then darkening ensues with curling. The young shoots that carry them blacken also (Steyeart, 1948; Tshilenge-Djim et al., 2004). The disease spreads a few days after appearance of the first symptoms. In most cases, the first symptoms appear unilaterally on a single branch, the ends of the leaves wither, and soon after shoots, twigs and the entire plant, but the rest of the shrub seems intact. With older plants, this kind of phenomenon can last for several weeks, even several months (Saccas, 1951; Tshilenge-Djim, 2007).

In young plants, the appearance of external signs is occurs in a short time; this can be explained by the incubation period of the disease ranging from six to ten days as opposed to the older coffee trees. This means that this parameter is based on the volume of tissue of infected plants (Saccas, 1951; Tshilenge-Djim, 2007). Inside, the bark becomes dry and adheres to wood, deeper it turns brown then necrosis. Woody tissues appear gray in places and the central cylinder presented pink lines purplish or blackish blue mainly near the cambium. Drivers vessels are congested, many mycelial hyphae are colorless and tylose clogging. The vessel occlusion to the periphery and at the level of cortical crevices becomes complete (Saccas, 1951; Tshilenge-Djim, 2007). Having disappeared after the strict implementation of control strategies, CWD resurgence has been reported in the DRC where it continues to cause damage in the coffee growing areas of the Eastern and North Kivu provinces (Katenga, 1987; Mfwidi-Nitu, 1994) and affects up to 90% of plantations (Flood, 1996). In agronomic practice, solving phytosanitary problems lies not only in-depth knowledge of the host plant, its environment and the conditions of its culture, but also and especially on the pathogens and conditions of pathogenesis (Semal, 1989).

The presence of a primary inoculum in a field constitutes one of the most important factors in the spread of an epidemic disease. In the case of CWD, Kalonji and Onyembe (1996, unpublished data) mentioned the possibility of infection and propagation by different woods of infected coffee tree. These authors showed that control methods are not strictly respected by farmers. The farmers usually leave tracts of sick coffee on the ground or carry them through the plantations to their homes for domestic use: hedges of plots or firewood. It is in this context that the present study aimed to determine the role played by this parasitic wood in the infection of CWD. This study was initiated through different types of inoculum resulting from the fragments of the wood harvested on *Coffea canephora* tree infected with CWD.

MATERIALS AND METHODS

Description of experimental site

The study was conducted in the Experimental Garden of the Department of Biology, Faculty of Sciences, University of Kinshasa, DRC. The geographic coordinates recorded with the GPS (extrex Summit Garmin) indicated 4°19'S latitude, 15°8'E longitude, and 330 m of altitude. The experimental site falls within the Aw4 climate type according to Köppen classification characterization with 4 months of dry season (from second mid-may to first mid-September) coupled with 8 months of rainy season (from second mid-September to first mid-may) sometimes interrupted by a short dry season in January/February. Daily temperature averages 24.5°C and accuses small variations, and the annual rainfall is close to 1500 mm. The July and February are respectively the coldest and warmest month. The relative humidity is highest in April and May, and is minimum in September and October (Makoko and Mananga, 1986; Makoko et al., 1992). Data related to climatic conditions prevailing during the field trial are reported in Table 1.

The rainy season (October – November) corresponding to the period of intense flow of raw sap was favorable to the growth of the fungus, and promotes the transport of the fungus in vessels of plant.

Coffee seedlings used in the study

The plant materials used in the present study were 6 months old, obtained from seeds of *C. canephora* var *robusta* harvested in Research Station of Kiyaka (Institut National pour l'Etude et la Recherche Agronomiques/INERA) in DRC. The coffee genotype used in this study was identified as I1010203/OG. After germination, coffee seedlings were transplanted into polyethylene bags of 15 x 35 x 0.05 cm filled with forest soil from the valley of the Monastery Prieuré Notre Dame de l'Assomption. The soil used was characterized by dark brown coloration according to Munsell scale

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Table 1. Temperature, relative humidity and rainfall conditions prevailing during the experimental period.

Month	Temperature (°C)	Relative humidity (%)	Rainfall (mm)
August (2004)	27.8	76.0	17.6
September (2004)	28.2	76.0	20.1
October (2004)	28.7	80.6	183.4
November (2004)	28.6	81.3	248.7
December (2004)	27.7	89.6	266.6
January (2005)	30.5	98.9	24.0
February (2005)	26.7	81.4	27.5

Source: Department of Physics and Soil Hydrology (Regional Center of Nuclear Studies of Kinshasa: CREN-K).

Table 2. Interval of time (days) between coffee seedlings inoculation and the expression of CWD symptoms.

Contaminant	Mean time (day) of CWD symptoms expression (Mean±SD)				
	Wilt	Browning of leaves	Drying of leaves	Defoliation	Mortality of seedlings
Bark	17±2.8	22±1.2	N.O	50±2.4	N.O
Sub-bark tissue	51±1.7	56±3.4	61±2.5	58±2.3	66±2.4
Wood necrotic	60±2.4	65±3.5	N.O	66±2.5	N.O
Artificial inoculum	93±3.5	98±3.7	103±2.5	100±2.5	105±2.7
Control	N.O	N.O	N.O	45±1.2	N.O
LSD (0.05)	N.S	N.S	N.S	N.S	N.S

N.O: Not observed; N.S: not significant; SD: standard deviation.

(Anonymous, 2000), high porosity and a pH of 5.1. The polyethylene bags containing coffee seedlings were placed under a natural shade of *Acacia* spp. and *Eucalyptus* sp. Seedlings were watered every two days during dry periods.

Types of inoculum used

In the present study, natural and artificial inoculum were used. The natural inoculum was obtained from the infected coffee tree harvested in Yeboka region (province of Equateur, DRC), and consisted to wood shards of 4 x 3 x 0.5 mm taken in bark (colonized by perithecia), sub-bark tissue and wood necrotic. An artificial inoculum was isolated from a sample of the same infected material and purified in the Unit of Phytopathology laboratory (Faculty of Agronomy, University of Kinshasa, DRC), where it is stored as parent-strain in test tubes containing the synthetic nutrient agar (SNA) medium under paraffin. The SNA medium used constituted of: KH_2P0_4: 1 g; KNO_3: 1 g; $MgSO_4.7H_2O$: 0.5 g; KCl: 0.5 g; glucose: 0.2 g; sucrose: 0.2 g; agar Merck®: 20 g; and distilled H_2O: 1000 ml) (Tshilenge-Djim et al., 2004, 2011). Artificial inoculum used consisted of a pellet of 5 mm in diameter cut with a sterile scalpel blade outskirts of the mycelium of strain-girl previously obtained on SNA. This inoculum was taken where high concentration of conidia was previously observed upside of the Petri dishes under microscope (Olympus BX 40).

Technique of inoculation

The inoculation was done by technique of incision as described by Tshilenge-Djim et al. (2011), and consisted of inserting the inoculum into a notch made at the base of the stem of plant. The shards of infected tree representing different types of natural inoculum, and the stem of seedlings were first superficially disinfected with 70% ethanol which was allowed to evaporate for 10 min. The incision was made using a sterile scalpel blade at 1 cm below the insertion point of cotyledonary leaves in the plane of the first pair of true leaves; then, inoculum was inserted into incision and maintained in place by a ligature made with parafilm.

Experimental design, data recorded and statistical analysis

The study was performed using a randomized complete block design (RCBD) replicated four times using five treatments and nine seedlings per plot. Each treatment represented a type of contaminant, and non-inoculated seedlings were used as control. Seedlings inoculated were observed every 7 days during 4 months. Data collected were focused on the following variables: the time (days) between inoculation and the appearance of symptoms of CWD. The rate of each symptom was also recorded. The height and collar diameter of seedlings were measured, respectively with a ruban meter and a caliper. At the end of the trial, the re-isolation of the pathogen was made to check Koch's postulate. Data collected were submitted to analysis of variance (ANOVA) using R (R-2.12.0) software. Means comparison was performed by LSD test at 5% of probability level.

RESULTS

Data related to the interval of time (days) between inoculation and expression of symptoms of CWD are reported in Table 2. Results related to the rate of

Table 3. Rate (%) of different symptoms of CWD after inoculation with different contaminants.

Contaminant	Rate (%) of symptoms of CWD (Mean±SD)				
	Wilt	Browning of leaves	Drying of leaves	Defoliation	Mortality of seedlings
Bark	11.1±0.5	11.1±1.2	0	24.0±2.1	0
Sub-bark tissue	11.1±0.6	11.1±0.5	11.1±1.5	22.1±1.5	11.1±2.5
Wood necrotic	33.3±1.4	33.3±2.5	0	31.7±2.3	0
Pellet of SNA	22.2±1.2	22.2±2.4	22.2±2.0	39.4±2.1	22.2±1.6
Control	0	0	0	38.8±1.5	0
LSD (0.05)	N.S	N.S	N.S	N.S	N.S

N.S: Not significant; SD: standard deviation.

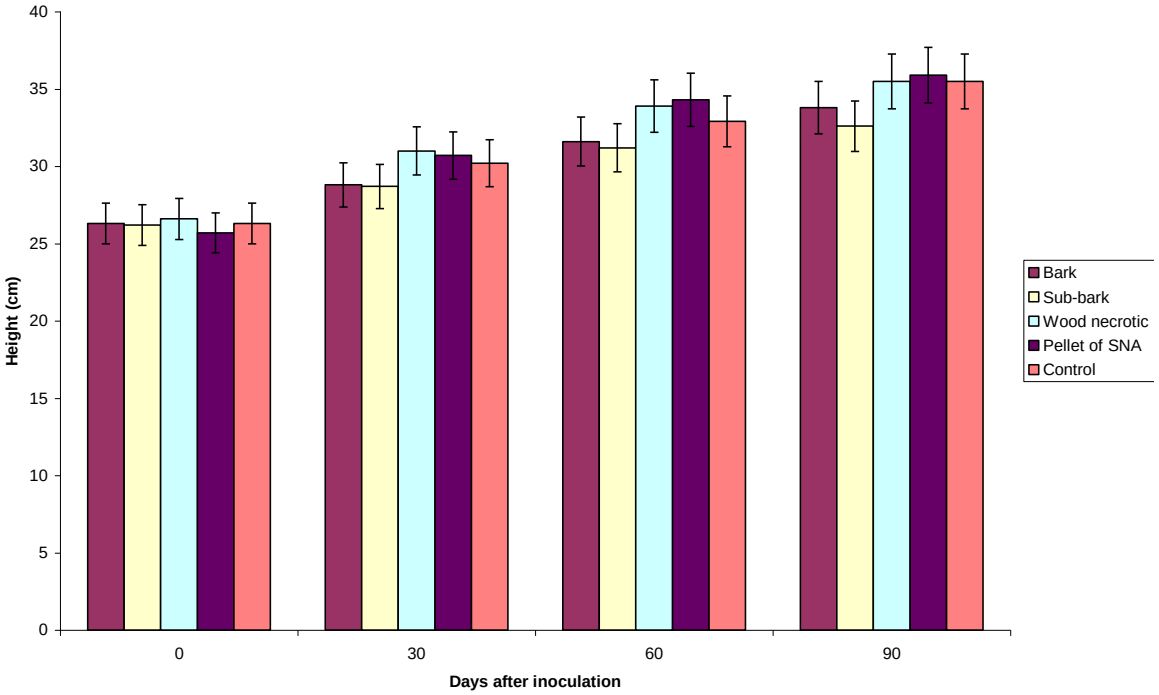

Figure 1. Height of coffee seedlings inoculated with different contaminants of *F. xylarioides*

Table 4. Presence/absence of *F. xylarioides* in dead woods.

Contaminant	Presence (+) or absence (-) of *Fusarium xylarioides* in dead woods
Bark	-
Sub-bark tissue	+
Wood necrotic	-
Pellet of SNA	+
Control	-

symptoms of CWD are presented in Table 3. Figures 1 and 2 illustrate the vegetative development of coffee seedlings. Results of the re-isolation of *F. xylarioides* are reported in Table 4.

Assessment of the pathogenicity of different contaminants used

Data reported in Table 2 show that wilting, leaf browning

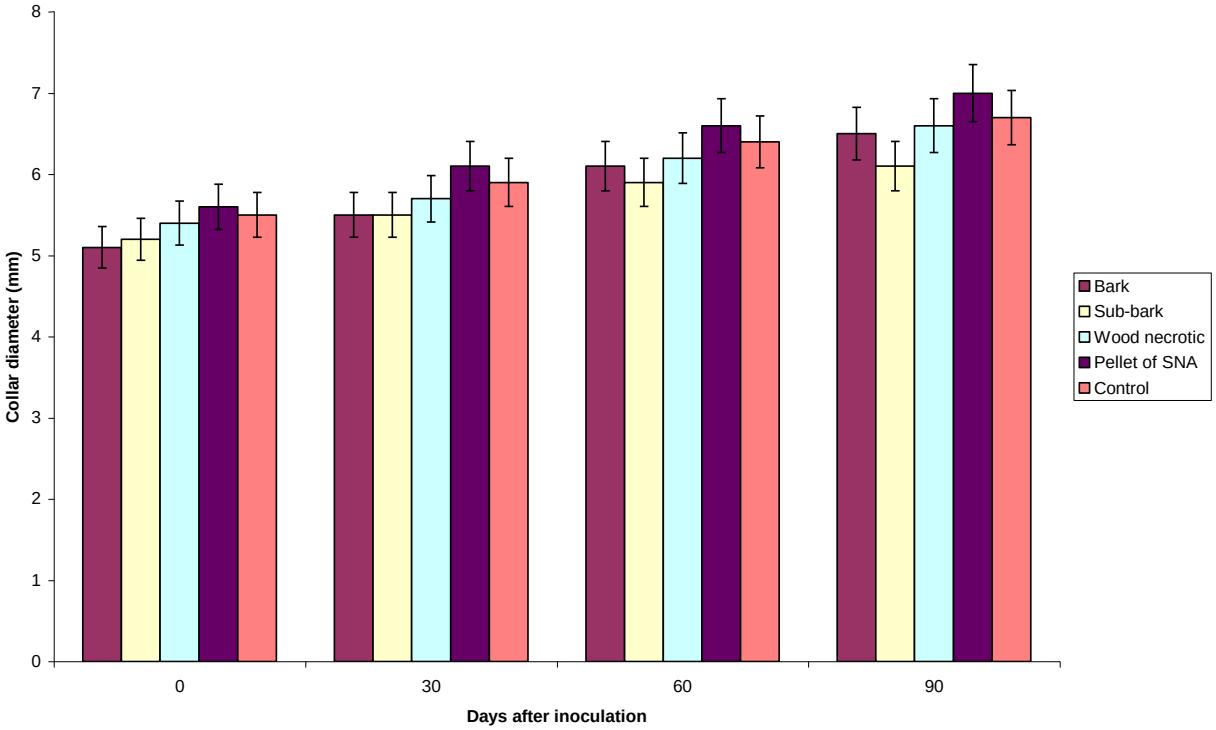

Figure 2. Collar diameter of coffee seedlings inoculated with different contaminants of *F. xylarioides.*

and drying, defoliation and seedlings mortality were main symptoms of CWD observed in inoculated seedlings. Seedlings of the control plot expressed only symptom of the defoliation. Statistical analysis did not show significant differences (P>0.05) between treatments. The wilting was recorded at an average 17 days after inoculation (DAI) on seedlings inoculated with infected bark, followed by leaf browning and defoliation at 22 and 50 DAI, respectively. The same symptoms were recorded an average at 93, 98 and 100 DAI, respectively, on seedlings inoculated with artificial inoculum. The leaf drying and seedlings mortality were recorded at an average of 61 and 66 DAI, respectively, on seedlings inoculated with sub-bark tissue, and an average of 103 and 105 DAI on seedlings inoculated with artificial inoculum.

Rate (%) of different symptoms of CWD

Results in Table 3 show that the percentage of inoculated seedlings expressing CWD symptoms did not vary significantly (*P>0.05*) between treatments. The lowest rate of wilting and leaf browning (11.1%) was recorded on seedlings inoculated with infected bark, and the highest rate (33.3%) was observed in seedlings inoculated with wood necrotic. Seedlings inoculated with sub-bark tissue expressed 11.1% of leaf drying, and those inoculated with an artificial inoculum presented 22.2% of mortality. The lowest rate of defoliation (22.1%) was recorded in seedlings inoculated with sub-bark tissue, while the highest rate (39.4%) was noted on seedlings inoculated with artificial inoculum.

Assessment of vegetative development of inoculated seedlings

Analysis of variance related to data presented in Figures 1 and 2 did not show significant differences (P>0.05) between inoculated seedlings. In Figure 1, thirty DAI, the highest height (31 cm) was recorded on seedlings inoculated with wood necrotic, while the lowest height (28 cm) was recorded on seedlings inoculated with infected bark, and sub-bark tissue. Sixty and ninety DAI, seedlings inoculated with artificial inoculum expressed the highest height (34.3 and 35.9 cm, respectively), while seedlings inoculated with sub-bark tissue expressed the lowest height (31.2 and 32.6 cm, respectively), compared to other seedlings. According to Figure 2, thirty DAI, seedlings inoculated with artificial inoculum expressed the highest collar diameter (6.1 mm), while seedlings inoculated with infected bark, and sub-bark tissue expressed the lowest collar diameter (5.5 mm). Sixty and ninety DAI, the highest collar diameter (6.6 and 7.0 mm, respectively), was recorded on seedlings inoculated with artificial inoculum, while the lowest value (5.9 and 6.1 mm, respectively), was noted on seedlings inoculated

with sub-bark tissue.

Re-isolation of *F. xylarioides* on dead woods

According to results presented in Table 4, the presence of *F. xylarioides* was confirmed in dead woods of seedlings inoculated with sub-bark tissue, and those inoculated with artificial inoculum.

DISCUSSION

Coffee wilt disease (CWD) is one of the main factors constraining coffee production in DRC. This fungal disease attacks all commercial *Coffea* spp. at any growth stage (Sihen et al., 2012). Results obtained in the present study show that all contaminants used are capable of inducing the following symptoms: wilting, leaf browning and drying, defoliation and seedlings mortality. Those symptoms were earlier described by Coste (1989) and Tshilenge-Djim et al. (1998, 2004) such as main symptoms of CWD.

Chronologically, wilting appeared at an average of 55 DAI, followed by leaf browning (60 DAI), defoliation (69 DAI), leaf drying (82 DAI) and seedlings mortality (86 DAI) (Table 2). The same trend was earlier reported by Tshilenge et al. (2010) and Tshilenge-Djim et al. (2011) who mentioned that CWD symptoms varied in their nature and their chronological sequence from the time of their appearance. The time of onset of CWD symptoms varies from one contaminant to another. The initiation of disease begins with wilting that is early on seedlings inoculated with infected bark, while it is late on seedlings inoculated with pellet of SNA. On inoculated seedlings, leaves turned brown at an average of 5 days after wilting, and the defoliation appeared at an average of 1-2 days after leaf browning on seedlings inoculated with wood necrotic, sub-bark tissue and a pellet of SNA. In general, when different contaminants induced the same symptom, it appears early on seedlings inoculated with infected bark, followed by sub-bark tissue, wood necrotic and pellet of SNA (Table 2).

According to various authors such as Sihen et al. (2012) and Muengula-Manyi et al. (2016), coffee plants infected with CWD always end up dying. In the present study, the mortality of seedlings was observed at an average of 12-15 days after wilting. Results reported by Tshilenge-Djim et al. (2011) showed that the wilting appeared at 16-17 days after inoculation, and the seedlings mortality appeared at 4-6 days after wilting. This difference can be due to the age of seedlings and pathogenicity of *F. xylarioides* strains used in those different studies. It is clear that mortality early appears when seedlings are youngest or when pathogen strain is more aggressive. In addition, according to Saccas (1951) cited by Tshilenge-Djim (2007), in young plants, the appearance of external signs of CWD is observed in a short time as compared to older coffee trees, and can be explained by the incubation of the disease which ranges from six to ten days. This means that this parameter is based on the volume of tissue of infected plant. By analyzing results recorded on the rate of symptom onset, it is possible to understand that there is no relationship between the types of contaminant and the degree of development of different symptoms.

The leaf drying and mortality were observed on seedlings inoculated with sub-bark tissue, and with pellet of SNA. The defoliation observed on control seedlings can be due to the senescence of leaves or other physiological cause. The absence of mortality on seedlings inoculated with infected bark bearing perithecia and with wood necrotic would be due in part to the identity of these perithecia which may be other than of *G. xylarioides*, capable of causing common symptoms of CWD except mortality. Indeed, Tshilenge-Djim et al. (2004) indicated that *F. solani*, *F. stilboides* and *F. falciforme* species are able to induce main symptoms of CWD without causing death of inoculated plant. In Ethiopia where CWD occurs, Girma (2004) isolated six fungal species belonging to the genus *Fusarium* from infected coffee samples. In Kenya, Baker (1972) indicated that *F. solani* and *F. oxysporum* induced varying types of wilting on coffee. In view of results reported by these many authors, it is possible that bark and wood necrotic tissues used in the present study were colonized by *Fusarium* species than *F. xylarioides*, which would explain the absence of mortality on seedlings inoculated with these contaminants. Results illustrated by Figures 1 and 2 revealed that during the trial, different contaminants did not influence the vegetative development of inoculated seedlings as compared to the control. Our observation corroborates findings reported by Muengula-Manyi et al. (2016), which revealed that inoculation of *Gibberella xylarioides* did not significantly influence the vegetative development of coffee seedlings.

Conclusion

The present study demonstrated that the different contaminants used induced characteristic symptoms of CWD at varying moment and degrees. Results obtained confirm the potential danger of wood debris from infected coffee trees, which can act as a source of infection and promote the spread of CWD when dragged through plantations. The use of shards of infected tissue taken at depth may constitute an alternative to culture on agar medium for inoculation under conditions where microbiological manipulations are not possible. The role of *F. xylarioides* in the pathogenesis of CWD was confirmed in the case of inoculation with sub-bark tissue and with pellet of SNA, whereas in case of seedlings that did not die, the potential role of other species of *Fusarium*

was suggested. The inoculation has not influenced the vegetative development of inoculated seedlings.

Conflict of Interests

The authors declared that there is no conflict of interests.

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Full Length Research Paper

Plant defense elicitors' purification in soybean and bean from pathogenic nematode

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Plant resistance induction against pathogens is an alternative disease control method, which involves the activation of the plant defense mechanisms, such as the phytoalexins induction. The eliciting molecules have the capacity of inducing and activating such responses and thus, techniques have searched to isolate and characterize fractions with eliciting aspect. This study aimed to purify, through ion exchange chromatography and gel filtration chromatography, eliciting molecules from phytopathogenic nematodes, and test them in the induction of phaseolin in bean hypocotyls and gliceolin in soybeans cotyledons. A Tris HCl 0.05 M (pH 6.8) buffer was used as control treatment and acibenzolar-S-methly (50 mg i.a. L^{-1}) and *Saccharomyces cerevisiae* (20 mg m L^{-1}) was used as induction standard treatment. Ion exchange chromatography (IEC) and gel filtration chromatography (GFC) were applied to separate power eliciting fractions from five hundred female root knot nematodes (*Meloidogyne javanica*). The purification of elicitors, through IEC, resulted in sixty glicidic fractions and six glycoprotein ones. They were classified according to their nature, being twenty-six glicidic fractions and thirty-seven glycoprotein ones, with molecular masses ranging from 29.19 to 2,989.25 kDa. From the purified fractions, eight of them presented phaseolin inducing effect potential, whereas fifteen fractions presented gliceolin inducing effect. Chromatography proved to be efficient in purifying the eliciting compounds. Compounds having gliceolin and phaseolin suppressing characteristics were verified in the bioassays. For those fractions obtained through IEC and posteriorly submitted to GFC that did not induce phytoalexin, it is suggested that the molecules need to act jointly so there is eliciting effect and thus induce a defense response in the plant.

Key words: gel filtration chromatography, ion exchange chromatography, *Meloidogyne javanica*, phytoalexins, resistance induction.

INTRODUCTION

In phytopathology, resistance is the rule and susceptibility to pathogenic agents is the exception. The flourishing and development of an illness is the result of the interaction between susceptible host, virulent pathogenic agent and favorable environment. The incompatibility of these three components results in the non-manifestation of the disease. In this sense, one of the primary events to occur is the recognition of the pathogenic agent by the hosting plant and the activation of its defense mechanisms (Pascholati, 2011). Such mechanisms are responsible for the plant resistance, so that it can be induced by the expression of a set of defense genes, which aim to restrain the pathogen growth and/or activity, as well as diminish the incidence of diseases (Métraux, 2001) of biotic origin in plants. Thus, the resistance induction in plants against illnesses has become a fundamental tool for research tuned at studies over phytosanitary issues. One of the main focus of agro-ecological agriculture is the alternative control of diseases by the induction of plant defense mechanisms. Bonaldo et al. (2005) suggested that the pathogenic agent control such as viruses, bacteria, fungi and nematodes could be established by resistance mechanism induction and that, according to Stangarlin et al. (1999), are activated by eliciting molecules, which they produce against phytopathogens.

The elicitors are molecules released by the pathogen or by the plant itself, by the pathogen action, and that are recognized by present receptors in the plant cell membrane (Durrant and Dong, 2004; Jalali et al., 2006). The pathogen elicitors belong to a signaling molecule class that participates in the signal swapping between plant and pathogenic agent (Kamoun, 2006) able to induce the phytoalexin synthesis (Hahn, 1996), which to Barros et al. (2010) and Mazaro et al. (2013) are biocide compounds that act on the plant biochemical defense against pathogens. The soybean isolated gliceolin (Paxton, 1995) and phaseolin in beans (Müller, 1958), are examples of phytoalexins being studied currently and that perform an important role as a plant-pathogen interaction defense response (Franzener et al., 2000). Currently, there are resistance inductors available in the market, whose purified and tested molecules presented an elicitor aspect able to activate and/or induce defense responses in plants, such as Messenger™, a formulation obtained from the harpin protein from the bacteria *Erwinia amylovora* (Sobrinho et al., 2005). The elicitor characterization present in nematode females has as its objective to optimize the advance in studies of elicitor molecule products and develop viable sustainable alternatives for disease control. The elicitor molecule capacity originating from nematode females in the phytoalexin induction is still unknown.

This study seeks to purify, through ion exchange chromatography and gel-filtration chromatography, protein and glicidic elicitor molecules, from the root knot nematode (*Meloidogyne javanica*) and characterize them as being molecules with phaseolin phytoalexin inductor potential in bean hypocotyl and gliceolin in soybean cotyledons.

MATERIALS AND METHODS

The *Meloidogyne javanica* population used was obtained from "Santa Clara" tomato cv. plants and identified based on the perineal configuration (Hartman and Sasser, 1985). With the aid of a needle, 500 females were detached from their galls and were macerated in a 200 µl Tris HCl buffer at 0.5 M, pH 6.8 in a microcentrifuge tube with the aid of a glass rod. The sample volume was completed to 1 ml using the same buffer and subsequently filtered in syringe filters (Millipore) with 0.45 µl in diameter, being the sample final volume adjusted to 2 ml with the same buffer.

Firstly, an ion exchange chromatography (IEC) was conducted. A volume of 1.5 ml from the prepared sample was applied and eluted with a buffer (Tris-HCl 0.025 M (pH7.5)), in a 1.5 ml min^{-1} flux being collected in 6 ml fractions. After the non-absorbed material removal, the material retained was displaced by NaCl linear gradient on the buffer in sub sequential concentrations from 0 to 100%, and was determined by electrical conductivity. The fractions were eluted in NaCl and only those on the Tris-HCl buffer were dialyzed in 12 to 16 KDa molecule exclusion limit membranes against polyethylene glycol (PEG) 20,000, according to Franzener (2011). The aliquot of fractions obtained were adjusted to a final volume of 3 ml with a Tris-HCl 0.05 M (pH 6.8). From this elution pattern, 12 fractions were obtained, which represent the treatments.

Next, purification was performed by the gel-filtration chromatography (GFC). From the fractions compiled through IEC, 1.5 ml of sample was applied over the column bed, eluting the sample with the buffer in a flux of 0.5 ml min^{-1}, with 1 ml of the GFC elution pattern fraction collected which resulted in 63 fractions, and this represent the treatments. The protein relative molecular masses of each fraction, obtained by GFC, were calculated by the equation:

$$y = 8867.4e^{-3.231x}$$

The standard curve for the dosage of carbohydrate reducers was performed through the Lever method (1972):

$$y = 0.0414x - 0.0297$$

The total content quantification of proteins was calculated by a standard curve, the Bradford method (1976):

$$y = 0.0434x + 0.0431$$

The IEC and GFC protein and glicidic fractions were collected according to their distribution patterns and monitored via

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spectrophotometer at 280 and 410 nm, respectively. They were then tested for their capacity of inducing gliceolin phytoalexins in soybean cotyledons and bean hypocotyls.

Bioassay for the production of phytoalexins in soybean cotyledons

Soybean seeds (*Glycine max* L.), Cultivar VMAX, were sanitized in sodium hypochlorite 1% (5 min), washed with distilled water, and sowed in plastic trays containing autoclaved sand (three times for 1 h at 121°C and 1 atm). After 7 days, the recently-opened cotyledons were detached from the seedlings for the bioassay execution. The cotyledons were placed on a petri dish (five cotyledons/dish) containing two sheets of paper filter sterilized and moistened with 1 ml of sterilized distilled water. A “wedge” was made on each cotyledon, on the adaxial surface, with the aid of a stiletto and, on each one of them was added an aliquot of 20 µl of the treatments (fractions). The dishes were incubated in a biochemical oxygen demand (BOD) incubator at 25°C, in the dark, for 20 h. After this period, the cotyledons were transferred to vials containing 15 ml of sterilized distilled water and left for shaking in an orbital shaker/150 rpm for 1 h, for gliceolin extraction.

Subsequently, the phytoalexins (gliceolin) were measured by supernatant reading in a spectrophotometer at 285 nm. The cotyledons were washed with distilled water, dried up and weighed on an analytical balance. The gliceolin content in the sample was obtained by the absorption value divided by the cotyledons’ mass (ABS/gmf^{-1}). As treatment control, a solution buffer Tris-HCl 0.05 M was used, and as induction treatment pattern, the *Saccharomyces cerevisiae* cell suspension was used (20 mg ml^{-1} of commercial product Fleischmann Fresh Biological Yeast) (Meinerz et al., 2008).

Bioassay for the production of phytoalexins in bean hypocotyls

Bean seeds (*Phaseolus vulgaris* L.) Cultivar IPR-Colibri were sanitized in sodium hypochlorite 1% for 5 min and washed with distilled water, and sowed in plastic trays containing autoclaved sand (three times for 1 h at 121°C and 1 atm). After 7 days, etiolated hypocotyls segment with 0.5 cm were detached from the seedlings, washed with distilled water and dried up over a sterile paper filter. Four (4) segments of hypocotyls were placed in a microcentrifuge tube and immerged in 500 µl of the purified fractions. The tubes were kept at 25°C in the dark for 48 h. The treated hypocotyls were transferred to vials containing 4 ml of ethanol 80%, kept at 4°C, for 48 h, and shaken (orbital shaker/150 rpm) for 1 h for phaseolin extraction. Next, the phytoalexins (phaseolin) were measured by supernatant reading in a spectrophotometer at 280 nm. Afterwards, the hypocotyls were washed in distilled water, dried up and weighed on an analytical balance. The content of phaseolin in the sample was obtained by the cotyledons’ mass (ABS/gmf^{-1}). As treatment control, a solution buffer Tris-HCl 0.05 M was used, and as induction treatment pattern the plant defense inductor acibenzolar-s-methyl (50 mg $i.a.L^{-1}$) (Bion ™) was used (Bailey and Burden, 1983).

Statistical analysis

The phytoalexins assays were conducted by completely randomized design, with 12 treatments (fractions) for IEC and sixty-three fractions for GFC, with four repetitions. The data were submitted to analysis of variance and Scoot-Knott test ($P \leq 0.05$) was executed. The homogeneity of the variances was determined by the Liliefors test. Data transformation was used when made necessary. The software used for statistical analysis was Genes (Cruz, 2006).

RESULTS

The majority of peaks for purified carbohydrates presented little magnitude, however, the presence of a peak of glicidic nature of higher absorbency was registered (0.132 nm), not coinciding with the protein peak, which corresponds to the material retained in the resin. As an anionic exchange, resin was used, the carbohydrates which remained attached to it presented negative liquid charge; thus they needed to be eluted in NaCl conditions. For the other protein peaks which coincided with the glicidic ones, they were considered to be glycoproteins (Figure 1). The molecule purification of female nematodes *M. javanica* by IEC that presented fractions with similar spectrophotometric aspect were collected in three fractions of glycoprotein nature (4, 5, 6) and three of glicidic nature (fractions: 1, 2, 3) to those not attached to the resin (eluted against a NaCl concentration gradient and subsequently, dialyzed with distilled water) three fractions of glycoprotein nature were registered (10, 11, 12) and three of glicidic nature (7, 8, 9,) (Figure 1, Table 1).

Bioassay of phytoalexins in bean hypocotyls

The fractions purified by IEC from females of *M. javanica* and tested in the production of phaseolin phytoalexin in bean hypocotyls are presented on Table 1. For the Scoot-Knott test, fractions 1, 6 and 5 (not attached to the resin) were grouped in the 1st batch, which presented elicitor aspect, with activating potential of phaseolin phytoalexin, of which, the total protein concentrations detected were 0.000 μl^{-1}, 0.412 µg ml^{-1}, and 0.343 µg ml^{-1}, respectively. For carbohydrate contents, 1.249, 2.529, and 2.214 µg ml^{-1} were obtained, respectively. Also, for fraction 3, which responded similarly to acibenzolar-s-methyl (ASM), the total protein and carbohydrates were 0.000 and 0.838 µg ml^{-1}, respectively. It should be pointed out that, for these fractions, as well as for the ones grouped in other batches, the carbohydrate concentrations presented themselves to be always higher when compared with the ones of total protein.

The sample quantity used to accomplish the total protein analysis was 50 µl, due to the amount available for running the tests. This amount, in turn, may exercise an influence on the reading and not represent the real quantity of protein existing in the sample. Fractions 1, 6 and 5 inducted the phaseolin synthesis in 35.17, 29.05 and 28.44%, respectively, values superior to the ASM induction standard treatment. In relation to fraction 3, it delivered the same phaseolin induction level as the standard treatment. Regarding fractions 7, 10, 4 and 2, no influence was verified on the phytoalexin induction, being their values similar to the Tris HCl 0.05 M control treatment. As to fractions 11, 8, 12 and 9, they presented suppression effect on the phaseolin phytoalexin induction,

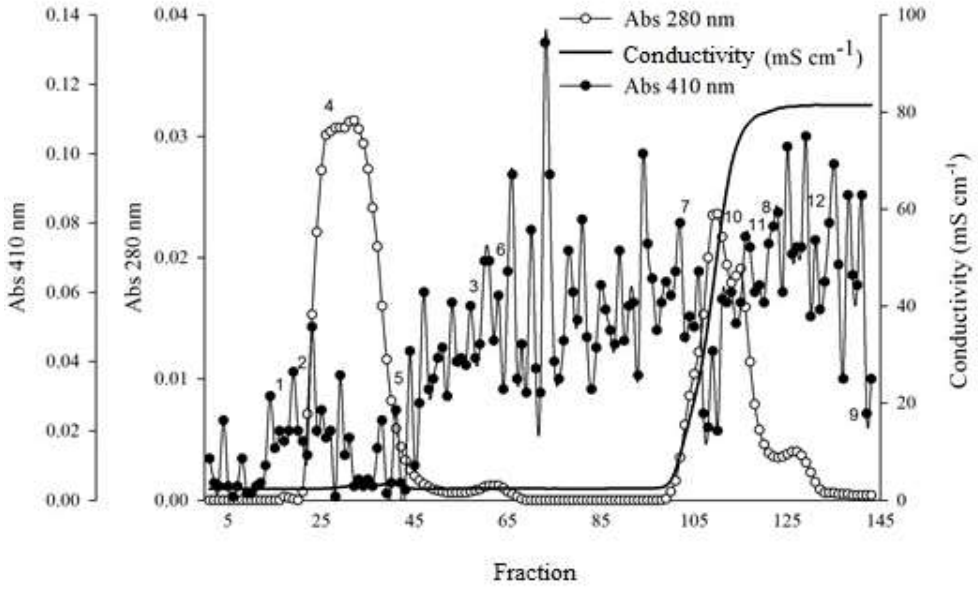


Figure 1. Ion exchange chromatography from samples of females of the nematode Meloidogyne javanica. A sample of 1.5 ml was applied over a glass column (5 cm × 20 cm) fulfilled with anion-exchange resin (UNOsphere™ Q Strong Anion Exchange) balanced and eluted with Tris-HCl 0.025 (pH 7.5) buffer, being collected fractions of 6 ml, in a 1.5 ml min^{-1} flux. The absorbed material was eluted with a NaCl linear gradient (0 to 100%) on the same buffer. Protein (○) was determined in 280 nm and carbohydrates (●) by the Lever method (1972). Concentrations of NaCl (-) were determined indirectly by conductivity.

Table 1. Fractions, nature, total protein content (µg mL^{-1}), carbohydrate content (µg mL^{-1}) and the phaseolin accumulation in bean hypocotyls (nm $g.m.f^{-1}$), treated with the glycoprotein and glicidics fractions from ion exchange chromatography (IEC), from *Meloidogyne javanica.*

Fractions of IEC	Nature	Total protein content (µg protein mL^{-1})	Carbohydrate content (µg glucose mL^{-1})	Phaseolin (280 nm $g.m.f.^{-1}$)
1	Glicidic	0.000	1.249	17.68^{A}
6	Glycoprotein	0.412	2.529	16.88^{A}
5	Glycoprotein	0.343	2.214	16.80^{A}
3	Glicidic	0.000	0.838	15.01^{B}
7	Glicidic	0.000	1.683	11.83^{C}
10	Glycoprotein	0.786	1.852	11.32^{C}
4	Glycoprotein	0.878	1.877	10.90^{C}
2	Glicidic	0.000	1.128	10.73^{C}
11	Glycoprotein	0.117	1.732	7.64^{D}
8	Glicidic	0.000	0.789	6.69^{D}
12	Glycoprotein	0.343	2.456	6.30^{D}
9	Glicidic	0.000	1.828	5.74^{D}
ASM *	--	--	--	13.08^{B}
Tris HCl **	--	--	--	10.62^{C}
CV %				15

*Acibenzolar-S-methyl (50mg i.a. L^{-1}), used as inducting standard treatment; ** Buffer solution used as mobile phase in the chromatography (control treatment); Scoot-Knott test at 0.05%.

with a reduction of 28.06, 37.00, 40.68 and 45.95%, respectively, in relation to the control treatment.

In the attempt of purifying new fractions of females of *M. javanica* with phytoalexin potential inductor, those obtained by IEC were subjected to GFC and it was possible to obtain different peaks. It is observed that the GFC patterns are represented on Table 2, whose total collected fractions, according to the elution pattern, resulting in 63 fractions. The molecule batch purified by the technic presented glycoprotein and glicidic nature with known molecular masses. The majority of purified fractions presented in their composition a higher concentration of carbohydrates than of proteins, except fractions 7 (10.873 and 3.326 µg ml^{-1}), 33 (7.102 and 3.085 µg ml^{-1}), 20 (4.848 and 3.930 µg ml^{-1}), 26 (6.833 and 3.374 µg ml^{-1}), 36 (4.614 and 3.423 µg ml^{-1}) and 5 (2.793 and 2.674 µg ml^{-1}) in which the protein concentration was superior to the carbohydrate, all of them being from glycoprotein nature.

The utilization of GFC proved to be efficient to the purification of compounds coming from IEC, with potential for activity over bean hypocotyls, although not all fractions presented the same property (Table 3). Through the Soot-Knott test, the fractions 6, 15, 17, 2 and 22 were grouped in the batch I, and indicated a greater potential of phaseolin inducting activity, characterized as being elicitors of glycoprotein natures, with average values of 124.59, 98.68, 94.06, 86.14 and 77.56, respectively, greater than the ASM standard treatment. These fractions correspond to those obtained at IEC and not attached to the resin (positive charge).

IEC fractions 1 and 5, which presented phaseolin phytoalexin inducting activity, when submitted to GFC resulted in the fractions 15, 17 and 2, which inducted defense responses in bean hypocotyls, with molecular masses around 329.31, 78.89 and 107.62 kDa, respectively. As to fraction 2 (positive liquid charge molecules) and 7 (negative liquid charge molecules) purified by IEC they did not deliver phaseolin inducting effect, however when submitted to GFC it resulted in fractions 6 and 22 of elicitor aspect, with molecular masses of 114.51 and 188.25 kDa, respectively.

Regarding fractions 7 (glycoprotein nature and molecular mass of 69.66 kDa), 56 (glicidic nature) and 3 (glycoprotein nature and molecular mass 61.52 kDa) obtained via GFC, these also presented phaseolin inducting potential, with 45.05, 44.39 and 38.94%, respectively, compared to ASM treatment. Fraction 7, which demonstrated inducting potential, corresponds to fraction 2 from IEC, which did not deliver phaseolin inducting effect, whereas, fraction 56, corresponding to fraction 9 (negative liquid charge molecules) from IEC, presented phytoalexin suppressing aspect. Yet, fraction 3 obtained at GFC, corresponding to fraction 1 (positive liquid charge molecules) from IEC, inducted phytoalexin synthesis.

Fractions 33, 20, 27, 34, 16, 26, 29, 36, 55, 1, 54, 45, 24, 42, 40, 41, 21, 38 and 25 presented the same level of phaseolin induction as ASM standard treatment, so that, for those of glycoprotein nature, molecular masses are known. The other fractions presented did not induct phytoalexins, being the values similar to Tris HCl 0.05 M standard control.

Bioassay of phytoalexin on soybean cotyledons

The fractions purified by ion exchange chromatography from females of *M. javanica* and tested in the production of gliceolin phytoalexin on soybean cotyledons are presented on Table 4. Based on the Scoot-Knott test, fraction 12 (attached to the resin), of glycoprotein nature, grouped in the 1st batch, delivered total protein and carbohydrate concentrations in 0.343 and 2.456 µg ml^{-1}, respectively. Fractions 8 and 9 (not attached to the resin), both from glicidic nature, were grouped in the 2nd batch, having as contents of total protein 0.0 and 0.0 µg ml^{-1} and as contents of carbohydrates 0.789 and 1.828 µg ml^{-1}, respectively. In addition to this, for all fractions tested at the phytoalexin assay, carbohydrate concentrations presented to be always higher in relation to the total protein values (Table 4).

The inducting standard treatment (*Saccharomyces* cerevisiae), known as phytoalexin inductor in soybean, produced a higher level of induction of gliceolin. Therefore, fractions 12, 8 and 9 also presented phytoalexin inducting effect, however with values 17.39, 56.52 and 60.87%, respectively, lower than the standard treatment. Total protein and carbohydrate contents found for fraction 8 were 0.000 and 0.789 µg ml^{-1}, respectively, and for fraction 9 were 0.000 and 1.828 µg ml^{-1}. Yet, it is verified that, for both of them, there were not registered total protein contents, but only carbohydrate content, probably for being glicidic in nature. In relation to fractions 11, 5 and 4, it was not observed gliceolin induction effect, these values being similar to the Tris HCl 0.05 M control treatment. Fractions 2, 7, 6, 10, 3, 1, suppressed the gliceolin phytoalexin induction in 39.28, 58.93, 62.50, 96.46, 96.43, 98.21%, respectively, when compared to the control treatment.

In soybean cotyledons, the GFC fractions also delivered to be efficient, as fractions 7, 17 and 26 were inserted in batch I, according the Scoot-Knott test, and presented gliceolin activity superior to the *S. cerevisiae* inducting standard treatment at 421.05, 410.53 and 289.47%, respectively (Table 5). Fractions 7 and 17 recorded molecular masses of 69.66 and 78.89 kDa and correspond to fractions 2 and 5 (not attached to the resin from IEC), which did not induct phytoalexin synthesis. Regarding fraction 26, of molecular mass of 176.91 kDa, it corresponds to fraction 9 from IEC, which was, initially, attached to IEC resin. The results indicate that for this fraction there was no elicitor compound loss during the dialysis by membrane phase, once its molecular exclusion limit is from 12 to 16 kDa, which usually may occur during the sample salt removing procedure (Table 5).

Table 2. Glycoprotein and glicidic fraction chromatograms from ion exchange chromatography (IEC) with their corresponding fractions obtained by gel filtration chromatography (GFC), from *Meloidogyne javanica*.

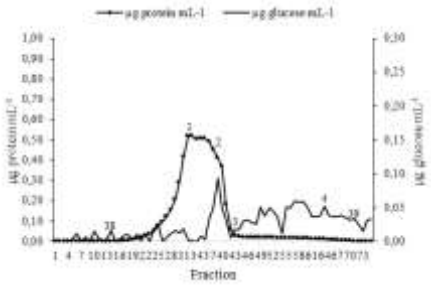
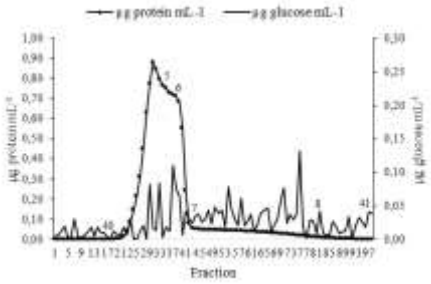
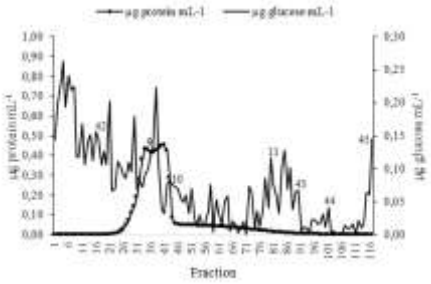
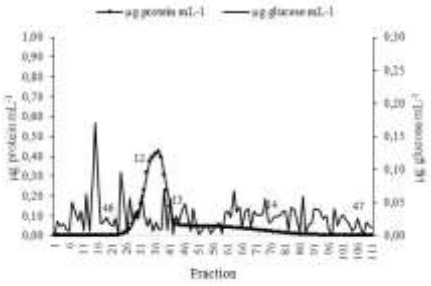
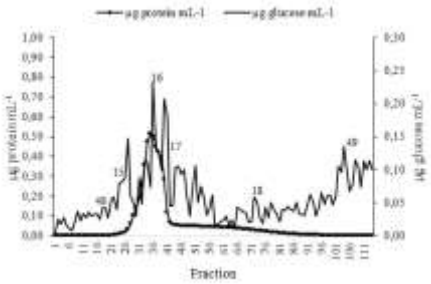
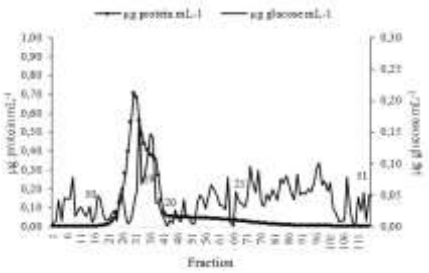
Fractions IEC	Fractions GFC	Profile
1	38 1 2 3 4 39	
2	40 5 6 7 8 41	
3	42 9 10 11 43 44 45	
4	46 12 13 14 47	
5	48 15 16 17 18 49	

Table 2. Contd.

	50 19 20 21	
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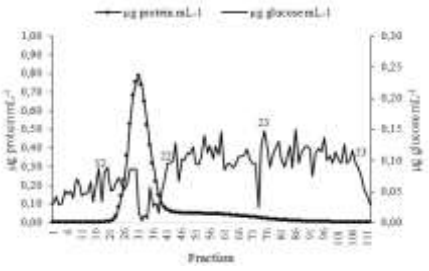
	51	
	52 22 23	
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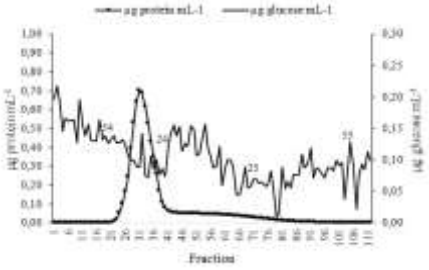
	54 24 25	
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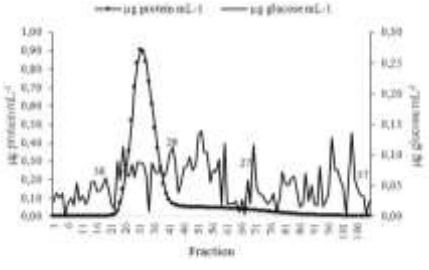
	56 26 27	
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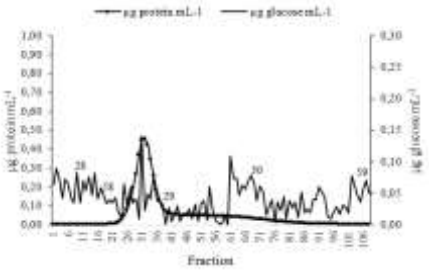
	28 58 29 30	
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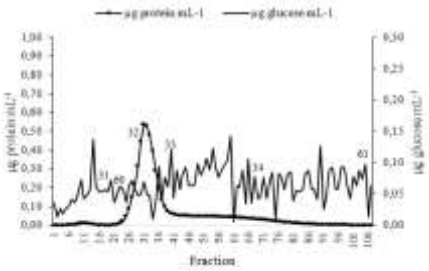
	31 60 32 33 34	
11		
	61	

Table 2. Contd.

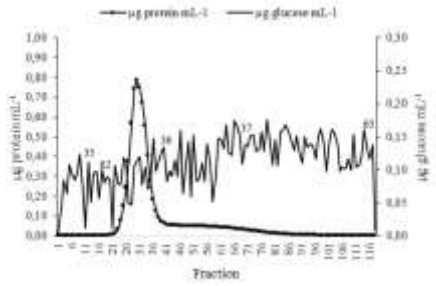
12	35	

Table 3. Fractions, nature, total protein content (µg mL^{-1}), molecular mass (kDa), carbohydrate content glucose (µg mL^{-1}), and the phaseolin accumulation in bean hypocotyls (nm $g.m.f^{-1}$), treated with the glycoprotein and glicidics fractions from gel filtration chromatography (GFC), from *Meloidogyne javanica*.

Fractions of GFC	Nature	Total protein content (µg protein ml^{-1})	Molecular mass (kDa)	Carbohydrate content (µg glucose ml^{-1})	Phaseolin (280 nm $g.m.f.^{-1}$)
6	Glycoprotein	1.142	114.51	3.229	13.61^{A}
15	Glycoprotein	0.159	329.31	3.882	12.04^{A}
17	Glycoprotein	10.873	78.89	3.326	11.76^{A}
2	Glycoprotein	0.505	107.62	2.626	11.28^{A}
22	Glycoprotein	3.869	188.25	4.220	10.76^{A}
7	Glycoprotein	1.280	69.66	3.471	8.79^{B}
56	Glicidic	0.000	--	3.471	8.75^{B}
3	Glycoprotein	0.067	61.52	2.457	8.42^{B}
33	Glycoprotein	7.102	166.25	3.085	7.44^{C}
20	Glycoprotein	4.848	101.13	3.930	6.69^{C}
10	Glycoprotein	0.820	57.81	4.002	6.44^{C}
27	Glycoprotein	0.659	37.42	4.147	6.41^{C}
34	Glycoprotein	0.159	54.33	3.519	6.21^{C}
16	Glycoprotein	1.392	114.51	3.664	6.19^{C}
26	Glycoprotein	6.833	176.91	3.374	6.13^{C}
29	Glycoprotein	0.113	176.91	3.302	5.94^{C}
36	Glycoprotein	4.614	213.16	3.423	5.93^{C}
55	Glicidic	0.000	--	2.940	5.84^{C}
1	Glycoprotein	0.243	156.24	2.698	5.69^{C}
54	Glicidic	0.000	--	3.205	5.58^{C}
45	Glicidic	0.000	--	3.278	5.55^{C}
24	Glycoprotein	1.964	176.91	3.205	5.52^{C}
42	Glicidic	0.000	--	3.060	5.43^{C}
40	Glicidic	0.000	--	2.795	5.39^{C}
41	Glicidic	0.000	--	2.867	5.30^{C}
21	Glycoprotein	0.947	47.98	2.988	5.27^{C}
38	Glicidic	0.000	--	3.109	5.26^{C}
25	Glycoprotein	0.059	47.98	3.471	5.24^{C}
46	Glicidic	0.000	--	2.795	5.21^{D}
49	Glicidic	0.000	--	2.360	5.21^{D}
39	Glicidic	0.000	--	3.205	5.09^{D}
8	Glycoprotein	0.455	42.37	4.002	5.02^{D}
37	Glycoprotein	0.059	54.33	3.616	4.99^{D}
12	Glycoprotein	0.021	107.62	3.036	4.99^{D}
47	Glicidic	0.000	--	3.616	4.99^{D}

Table 3. Contd.

4	Glycoprotein	0.044	33.04	4.002	4.95^{D}
52	Glicidic	0.000	--	2.964	4.95^{D}
31	Glycoprotein	0.417	2.123.97	3.640	4.87^{D}
61	Glicidic	0.000	--	0.790	4.85^{D}
30	Glycoprotein	0.113	54.33	3.036	4.85^{D}
35	Glycoprotein	0.466	2.989.25	4.002	4.81^{D}
13	Glycoprotein	0.021	89.31	3.616	4.75^{D}
53	Glicidic	0.000	--	2.529	4.73^{D}
59	Glicidic	0.000	--	2.481	4.70^{D}
18	Glycoprotein	0.521	42.37	3.688	4.68^{D}
50	Glicidic	0.000	--	2.577	4.65^{D}
57	Glicidic	0.000	--	3.930	4.61^{D}
44	Glicidic	0.000	--	2.981	4.59^{D}
5	Glycoprotein	2.793	188.25	2.674	4.57^{D}
32	Glycoprotein	0.348	188.25	3.254	4.55^{D}
28	Glycoprotein	0.267	2.723.24	2.988	4.49^{D}
14	Glycoprotein	0.036	42.37	3.809	4.45^{D}
63	Glicidic	0.000	--	1.200	4.42^{D}
23	Glycoprotein	0.924	42.37	3.205	4.40^{D}
51	Glicidic	0.000	--	2.601	4.31^{D}
19	Glycoprotein	0.735	200.32	3.688	4.29^{D}
60	Glicidic	0.000	--	2.360	4.22^{D}
11	Glycoprotein	0.498	29.19	2.263	4.18^{D}
62	Glicidic	0.000	--	1.635	4.17^{D}
48	Glicidic	0.000	--	2.432	4.16^{D}
58	Glicidic	0.000	--	2.312	4.07^{D}
43	Glicidic	0.000	--	3.713	4.07^{D}
9	Glycoprotein	0.659	121.86	3.785	3.96^{D}
ASM*	--	--	--	--	6.06^{C}
TrisHCl*	--	--	--	--	4.75^{D}
CV %					16

*Acibenzolar-S-methyl (50 mg i.a. L^{-1}), used as inducting standard treatment; ** Buffer solution used as mobile phase in the chromatography (control treatment); Scoot-Knott test at 0.05%.

Fraction 3, from glycoprotein nature (61.52 kDa), grouped in the 2nd batch, also presented gliceolin inducting response, with average value 284.21% higher than *S. cerevisiae* standard treatment. It corresponds to IEC fraction 1, in which any phytoalexin inducting activity was not observed, but rather suppressing (Table 4). In relation to the 3rd group, there are fractions (all of them with their corresponding molecular masses): 2 (107.62 kDa), 33 (166.25 kDa), 13 (89.31 kDa), 6 (114.51 kDa), 36 (213.16 kDa), 10 (57.81 kDa), 22 (188.25 kDa), 24 (176.91 kDa), 20 (101.13 kDa), 5 (188.25 kDa) and 29 (176.91 kDa), which delivered gliceolin inducting activity, with 231.58, 221.05, 189.47, 184.21, 136.84, 78.95, 63.16, 42.10, 26.31 and 15.79%, respectively, greater than the *S. cerevisiae* treatment. IEC fractions corresponding to these ones are presented on Table 2. It is worth mentioning that GFC fractions 36 and 37 resulted in a lesser gliceolin activity when compared to fraction 12 initially obtained by IEC, which delivered the same level of induction as the standard treatment. Thus, the defense response provided after the separation process by GFC resulted in fractions of low efficiency at gliceolin production.

Fractions 16, 19, 25, 23, 97, 14 and 30 delivered the same level of gliceolin induction as the standard treatment. In addition, all fractions of phytoalexin induction mentioned presented glycoprotein nature as a characteristic. The other fractions, grouped in the 5th batch, proved to be inefficient at induction of phytoalexin synthesis in soybean cotyledons, these values being similar to Tris-HCl 0.05 M control treatment (Table 5).

DISCUSSION

Among the methods deployed on the molecule

Table 4. Fractions, nature, total protein content (µg ml^{-1}), carbohydrate content (µg ml^{-1}) and the gliceolin accumulation in soybean cotyledons (nm $g.m.f^{-1}$) treated with the glycoprotein and glicidics fractions from ion exchange chromatography (IEC), from *Meloidogyne javanica*.

Fractions of IEC	Nature	Total protein content (µg protein ml^{-1})	Carbohydrate content (µg glucose ml^{-1})	Gliceolin (285 nm $g.m.f.^{-1}$)
12	Glycoprotein	0.343	2.456	0.19^{B}
8	Glicidic	0.000	0.789	0.10^{B}
9	Glicidic	0.000	1.828	0.09^{B}
11	Glycoprotein	0.117	1.732	0.06^{C}
5	Glycoprotein	0.343	2.214	0.06^{C}
4	Glycoprotein	0.878	1.877	0.06^{C}
2	Glicidic	0.000	1.128	0.03^{D}
7	Glicidic	0.000	1.683	0.02^{D}
6	Glycoprotein	0.412	2.529	0.02^{D}
10	Glycoprotein	0.786	1.852	0.02^{D}
3	Glicidic	0.000	0.838	0.02^{D}
1	Glicidic	0.000	1.249	0.01^{D}
Tris HCl*	--	--	--	0.56^{C}
Saccharomyces cerevisiae **	--	--	--	0.23^{A}
CV %				15

Buffer solution used as mobile phase in chromatography (control treatment); ** *Saccharomyces cerevisiae* (Fleischmann Fresh Biological Yeast) (20 mg ml^{-1}), used as induction standard treatment; Scoot-Knott Test at 0.05%.

Table 5. Fractions, nature, total protein content (µg ml^{-1}), molecular mass (kDa), carbohydrate content (µg ml^{-1}), and the gliceolin accumulation in soybean cotyledons (nm $g.m.f^{-1}$), treated with the glycoprotein and glicidics fractions from gel filtration chromatography (GFC), from *Meloidogyne javanica.*

Fractions of GFC	Nature	Total protein content (µg protein ml^{-1})	Molecular mass (kDa)	Carbohydrate content (µg glucose ml^{-1})	Gliceolin (285 nm $g.m.f.^{-1}$)
7	Glycoprotein	1.280	69.66	3.471	0.99^{A}
17	Glycoprotein	10.873	78.89	3.326	0.97^{A}
26	Glycoprotein	6.833	176.91	3.374	0.74^{A}
3	Glycoprotein	0.067	61.52	2.457	0.73^{B}
2	Glycoprotein	0.505	107.62	2.626	0.63^{C}
33	Glycoprotein	7.102	166.25	3.085	0.61^{C}
13	Glycoprotein	0.021	89.31	3.616	0.55^{C}
6	Glycoprotein	1.142	114.51	3.229	0.54^{C}
36	Glycoprotein	4.614	213.16	3.423	0.45^{C}
10	Glycoprotein	0.820	57.81	4.002	0.45^{C}
22	Glycoprotein	3.869	188.25	4.220	0.34^{C}
24	Glycoprotein	1.964	176.91	3.205	0.31^{C}
20	Glycoprotein	4.848	101.13	3.930	0.27^{C}
5	Glycoprotein	2.793	188.25	2.674	0.24^{C}
29	Glycoprotein	0.113	176.91	3.302	0.22^{C}
16	Glycoprotein	1.392	114.51	3.664	0.21^{D}
19	Glycoprotein	0.735	200.32	3.688	0.21^{D}
25	Glycoprotein	0.059	47.98	3.471	0.18^{D}
23	Glycoprotein	0.924	42.37	3.205	0.17^{D}
37	Glycoprotein	0.059	54.33	3.616	0.17^{D}
14	Glycoprotein	0.036	42.37	3.809	0.16^{D}
30	Glycoprotein	0.113	54.33	3.036	0.16^{D}
8	Glycoprotein	0.455	42.37	4.002	0.15^{E}

Table 5. Contd.

21	Glycoprotein	0.947	47.98	2.988	0.14^{E}
39	Glicidic	0.000	--	3.205	0.14^{E}
41	Glicidic	0.000	--	2.867	0.14^{E}
18	Glycoprotein	0.521	42.37	3.688	0.14^{E}
42	Glicidic	0.000	--	3.060	0.14^{E}
27	Glycoprotein	0.659	37.42	4.147	0.14^{E}
12	Glycoprotein	0.021	107.62	3.036	0.13^{E}
43	Glicidic	0.000	--	3.713	0.13^{E}
34	Glycoprotein	0.159	54.33	3.519	0.12^{E}
11	Glycoprotein	0.498	29.19	2.263	0.12^{E}
40	Glicidic	0.000	--	2.795	0.12^{E}
44	Glicidic	0.000	--	2.891	0.12^{E}
32	Glycoprotein	0.348	188.25	3.254	0.12^{E}
53	Glicidic	0.000	--	2.529	0.11^{E}
59	Glicidic	0.000	--	2.481	0.11^{E}
51	Glicidic	0.000	--	2.601	0.11^{E}
48	Glicidic	0.000	--	2.432	0.11^{E}
55	Glicidic	0.000	--	2.940	0.11^{E}
63	Glicidic	0.000	--	1.200	0.11^{E}
9	Glycoprotein	0.659	121.86	3.785	0.11^{E}
49	Glicidic	0.000	--	2.360	0.11^{E}
4	Glycoprotein	0.044	33.04	4.002	0.11^{E}
28	Glycoprotein	0.267	2.723.24	2.988	0.10^{E}
52	Glicidic	0.000	--	2.964	0.10^{E}
50	Glicidic	0.000	--	2.577	0.10^{E}
38	Glicidic	0.000	--	3.109	0.10^{E}
57	Glicidic	0.000	--	3.930	0.10^{E}
46	Glicidic	0.000	--	2.795	0.10^{E}
54	Glicidic	0.000	--	3.205	0.10^{E}
61	Glicidic	0.000	--	0.790	0.10^{E}
62	Glicidic	0.000	--	1.635	0.10^{E}
31	Glycoprotein	0.417	2.123.97	3.640	0.09^{E}
1	Glycoprotein	0.243	156.24	2.698	0.09^{E}
58	Glicidic	0.000	--	2.312	0.09^{E}
45	Glicidic	0.000	--	3.278	0.09^{E}
35	Glycoprotein	0.466	2.989.25	4.002	0.09^{E}
56	Glicidic	0.000	--	3.471	0.09^{E}
15	Glycoprotein	0.159	329.31	3.882	0.09^{E}
47	Glicidic	0.000	--	3.616	0.09^{E}
60	Glicidic	0.000	--	2.360	0.08^{E}
Tris HCl*	--	--	--	--	0.09^{E}
Saccharomyces cerevisiae **	--	--	--	--	0.19^{D}
CV %					16

Buffer solution used as mobile phase in chromatography (control treatment); ** *Saccharomyces cerevisiae* (Fleischmann Fresh Biological Yeast) (20 mg mL^{-1}), used as induction standard treatment; Scoot-Knott Test at 0.05%.

purification from microorganisms, it is important to highlight the chromatography, a technique used in the present study, which allowed the separation of molecules present in females of nematodes, molecules with relevant characteristics for the study of resistance mechanisms in soybean and bean seedlings. It is worth mentioning that the majority of the fractions collected in this study presented a glycoprotein nature, that is, presence of

proteins and carbohydrates in the fractions. This information corroborates the description by Braga (2008), in which in the majority of the purified elicitor molecules, carbohydrates are found as a compound of purified fractions, together with proteins, forming thus, glycoprotein nature elicitors.

The *M. javanica* chromatographic fractions of IEC, tested in bioassays with bean hypocotyls, delivered different responses at the induction of plant defense mechanisms, as it was observed in the presence of eliciting molecules, which resulted in the accumulation of phytoalexin. Such result is tied to the molecule joint activity from the obtained peaks. Suppressing activity fraction was also found. Therefore, the IEC fractions, when submitted to GFC, were also efficient and resulted in components with individual activity capable of inducing phytoalexin into bean seedlings. Regarding the bioassay on soybean cotyledons, from IEC fractions, there was no observed presence of eliciting molecules capable of inducing a higher level than *S. cereviviae*, a treatment used as an induction pattern. Yet, the presence of suppressing molecules for gliceolin synthesis was reported. However, when submitted to GFC, there were obtained fractions of eliciting character. Therefore, the presence of molecules of differentiated characteristics is suggested as inductors and suppressors in females of *M. javanica* for tested condition.

The results corroborate the description of Smith (1996), in which elicitors are constituted of a broad molecule chemical nature, and consequently there is not one single structural characteristic that can define its eliciting activity, similar to Dixon and Lamb (1990), there may be two or more eliciting molecules acting together and simultaneously. Therefore, the method used proved to be valid. The fact that some fractions purified in IEC did not deliver gliceolin and phaseolin inducting effect, but when subjected to GFC they did, indicates that, probably, for these molecules to be acknowledged as elicitors by the plant there is the need of individual activity by themselves. For IEC fractions, which were effective at the induction of phytoalexin and were purified by GFC they did not present the same response; it is believed that there is the need of the molecules to be acting together for such phenomenon to occur. Thus, the fractions resulting from IEC and their subsequent separations by GFC resulted in new fractions with differentiated molecule characteristics and groupings, not only for proteins but also for carbohydrates.

In a study conducted by Zanardo et al. (2009), supernatant fractions obtained from the ethanolic precipitation of the aqueous gross extract of the *Saccharimyces cerevisiae* presented resistance inducting activity in cucumber plant cotyledons. The presence of these fractions, separated by ion exchange chromatography, demonstrates the method efficiency as to the process of purification molecules with eliciting activity for studies of resistance induction. Yet, in a complementary manner, the authors verified that the fractions delivered a higher carbohydrate concentration in relation to the proteins in purified samples. These results confirm the observation in this study, in which the minority of the fractions purified by IEC and GFC presented a higher concentration of carbohydrates than of proteins. There is limitedr eports of obtaining purified fractions from females of phytopathogenic nematodes, but ion exchange chromatography and molecular exclusion techniques deserve to be highlighted for their importance and relevance regarding the research objective to purify and characterize molecules from other agents and, thus, study its resistance inducting potential in plants against diseases.

The recognition of these molecules by plants culminates in their reaction against pathogenic agents by the production of O_2 radicals, production of structural barriers and toxins that block the pathogen activity (Jones and Dangl, 2006). These defense responses may be induced when the plant recognizes molecular patterns associated to pathogens (PAMP's). PAMP's are molecules conserved by pathogens that use them to their own survival (Gheysen and Jones, 2013). According to Haegeman et al. (2012), the proteins present in nematode secretions are destined to protect the proteins from the host's defense responses. As an example, it is worth highlighting the gluatathione-S-transferase (GST) expressed in *M. incognita*s pharynx gland (Dubreuil et al., 2007). However, the authors also verified the GST acting on the detoxification of secondary metabolisms that the plant uses to prevent pathogen invasion.

In *Gobodera rostochiensis* it was identified the glutathione peroxidase, secreted and expressed in its hypoderm, however, its production in plants was also reported, which can be related to plant defense signaling pathways (Gheysen and Jones, 2013), as related in the present study, in which purified fractions posteriorly tested in assays of phaseolin and gliceolin phytoalexin induction, demonstrating phaseolin and gliceolin phytoalexin inducting potential. The hypothesis of the presence of elicitors in the female nematodes' cuticle composition and cell wall, as well as protein secretion by second-stage juvenile (J2) was based on the purified fractions capacity of inducing the gliceolin and phaseolin phytoalexin synthesis. Facing that, when performing the fraction partial purification from female nematodes of *M. javanica* via the IEC technique, followed by GFC, the action mechanism and the effect of these fractions on phytoalexin inducting activity on soybean cotyledons and bean hypocotyls were pointed out in the bioassays. Furthermore, there were elicitors, glycoproteins, on the anterior region of the nematode, capable of inducing the phytoalexin synthesis (Faria et al., 2003).

Phytoalexin synthesis play a fundamental role within the resistance induction studies, since this secondary metabolism, when synthesized by the plant, can affect the nematode functions, such as the rupture of

plasmalemma and vascular membranes, mitochondrial breathing inhibition and can also impair its own mobility (Giebel, 1973). Kaplan and Keen (1980) demonstrated that the oxygen absorption inhibition by J2 in *M. incognita* was due to gliceolin accumulation. Thus, the inducting characteristics of purified fractions may be tied to the molecules present in the nematodes, since for Ferraz and Monteiro (2011), the cuticle which involves the nematode body wall is a metabolically active structure and it is basically composed of proteins, therefore important to resistance induction studies, once they are still unknown.

Though nematodes have sub ventral esophageal glands, which produce proteins that act on the formation of feeding sites. During the eclosion period and during stage J2 these glands with highly functional cell are found, which play a fundamental role at the beginning of parasitism in the plant nematode, which in turn, also is worthy of attention as they act on the signaling paths of the plant defense mechanisms (Jones et al., 2003). The importance of knowing these proteins present in nematodes is due to the fact that during the infection phase, when penetrating the stiletto in the plant cell, the nematode injects proteins that culminate in cell physiological and morphological alterations and, as a result, originates giant cells that will produce more proteins to be used in the nematode feeding (Mitkowskli and Abawi, 2003). Therefore, the presence of these proteins, which constitute the females, and are part of their cuticle composition, as well as J2 glands, still in the egg interior, can justify the fact that some fractions selected in this study present the capacity of inducing cotyledons and bean hypocotyls to produce phytoalexins. Jones et al. (2000) and Robert et al. (2000) remark that a nematode cuticle is basically constituted by proteins, many of them important to the parasitism have already been identified and, besides that, carbohydrates and lipids were in the composition of their structures. These proteins may or may not result in the induction of the plant defense mechanisms, as to Temporal (2014) there are specific elicitors that activate signaling paths and, thus, induce the expression of specific genes. To Graham (1995), elicitors may induce precursors of plant tissue to produce phytoalexins, next to the infection area or where treatment was applied. The induction of genes associated to plant defense against pathogenic agents was reported by Manosalva et al. (2015), which aim to evaluate the plant perception and defense response as to the presence of signaling molecules, produced by plans parasite nematodes. These molecules, called ascarosides, are glycoside compounds that when undergoing hydrolysis generate sugars and, according to the authors, can be perceived by plants that is, receptors that activate defense response to several pathogens.

Still, for the present study, in the case of the fractions that have not induced phytoalexins, there are two ways in which the results can be based. The first is related to the volume of treatment used, because Hahn (1996) suggests that the low yield of purified fractions can mask the responses of defense or the activation of the routes of signalling. Although, Bonaldo et al. (2007) found that when the crude extract of eucalyptus was diluted, even so, there was induction of the phytoalexins, deoxiantocianidine in sorghum mesocotyls. Bonaldo (2005) also observed that the dilutions 10, 100, 1,000 and 10,000 times of preparation of *S. cerevisiae* induced phytoalexins in sorghum mesocotyls.

In the present study, it must be taken into consideration that during the dialysis procedure in reactions eluted in NaCl there may be compound losses by the dialysis membrane. According to Queiroz et al. (2001) the low molecular mass compounds can permeate the membrane and/or experiment dilutions. Based on this information, the possible lack of eliciting ability in purified molecule can also be highlighted. A second hypothesis would be fractions presenting phytoalexin suppressors, for in this study, beside inductors, inhibitors also were found, and that, according to Temporal (2014), the suppressing molecule grouping can result in fractions of low phytoalexin and enzyme inducting activity phytoalexin and enzyme induction. The suppression of plant defense responses may be related to the presence of proteins, called effectors, according to Kamoun (2006) and Kvitko et al. (2009) which are molecules released or associated to the organism and that can modify the physiology of another organism. These proteins can regulate the plant defense mechanisms, when inducing the plant cells to maintain or forge new feeding sites (Gheysen and Mitchum, 2011), for they interact with and affect the regulation of gene expression that culminate in alterations in the host cell functions (Gao et al., 2003).

When a purified fraction present elicitors able of inducing plant defense mechanisms and are recognized by cell receptors, many signals are forwarded, and then, converted into cell responses. At that point, activation genes induce a synthesis of many enzymes and among them there are some genes that produce phytoalexin. However, gliceolin and phaseolin synthesis was not always observed in the present study, and this result may be related to the activation of the mechanisms which were not analyzed in this study, but that do not discard the possibility of studying other host resistance activation paths.

In another study carried out by Gheysen and Mitchum (2011), the authors highlight that the nematode effecting proteins can avoid or suppress the defense responses, and that when interacting with the plant proteins they begin to control their development, such as cell growth and auxin transporting. Doyle and Lambert (2003) reported that the protein chorismate mutase, from which phytoalexins is derived, salicylic acid and auxins produced via the shikimate, was found in the *M. incognita* secretion and, as a result, there were alterations in the secondary metabolism of host cells. According to Davis et al. (2008), the majority of these proteins are produced

in the nematode esophageal glands (subventral and dorsal), and injected into the plant cytoplasm with the aid of a stiletto.

M. incognita proteins, involved in parasitism, have been purified and analyzed as to their capacity of suppressing the host defense responses and causing an infection (Bellafiore et al., 2008). The nematode effectors interact with the host proteins and manipulate the plant physiology (Quentin et al., 2013), as well as the signaling processes and hormonal and cellular balances (Wang et al., 2011), a fact which may be related to the suppressing responses found in the present study.

Proteins constitute a great percentage of mass of living organisms, and Castagnone-Sereno et al. (2011) reported that several protease genes were identified that compose the genome of gall nematodes. The authors affirm that, also, these proteins function as object of studies on phytopathogenic nematode control. This characteristic reinforces the importance of the present study and of new researches turned to the purification of eliciting molecules of plant defense inducing aspect from nematodes.

Therefore, while some effecting proteins suppress the plant defense response others can participate in the defenses signaling of host plants and/or fostering the formation of nematode feeding sites (Haegeman et al., 2012).

Conclusion

The chromatographic pattern indicated the presence of glycoprotein and glicidic elicitors capable of inducing phytoalexin synthesis. The suppression of gliceolin and phaseolin induction was verified for some of the fractions tested in the bioassays. Fractions obtained via ion exchange chromatography and posteriorly submitted to filtration chromatography influenced in the production of phytoalexins for some molecules shall act jointly, so there is eliciting effect and some others act separate, so that they may induce plant defense response.

Conflict of Interests

The authors have not declared any conflict of interests.

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Spatial variability of soil physical and hydraulic properties in the southern Brazil small watershed

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In order to provide an adequate usage to agricultural soils it is crucial to comprehend the soil physical attributes and their spatial variability. The variability of soil attributes is dependent on several formation processes and interactions. To properly describe such complex variability, the statistical methods used must incorporate the spatial and temporal influences. In this context, the present study was developed with the objective of evaluating the spatial variability of physical and hydraulic soil attributes, such as sand, clay and silt contents, soil macroporosity (Ma), soil microporosity (Mi), soil total porosity (TP), soil bulk density (BD), hydraulic conductivity (K), water contents at field capacity (tension of 10 kPa) and permanent wilting point (tension of 1500 kPa) (θ_{Fc} and θ_{PWP}), in a watershed, located in the Sul-Rio-Grandense Shield, in the South of Brazil, through the geoestatistics analysis based on Ordinary Kriging. It was found a better performance of exponential model for the semivariograms of most physical and hydraulic soil attribute in the 0-0.15 and 0.15 to 0.25 m layers. Spatial dependence was observed for all the soil physical and hydraulic attributes studied, classifying as high for the variables macroporosity (0 to 0.15 m) and clay (0.15 to 0.25 m), with the other variables classified as moderate spatial dependence. Besides, the variables K and macroporosity presented a high heterogeneity in relation to the average, with K in the surface layer and the variables clay, Ma, K and θ_{PWP} in the subsurface layer not presenting normality according to Shapiro and Wilk test ($p \leq 0.05$).

Key words: Ordinary kriging, semivariogram, physical and hydraulic soil attributes, Shapiro and Wilk test, exponential.

INTRODUCTION

In order to have an efficient use of natural resources, understanding soil properties, as well as its geographical distribution, mapping soil attributes becomes an important tool in the development of an adequate plan for soil management (Dessalegn et al., 2014). The study of the soil properties spatial distribution is a key issue to support sustainable land management, applying erosion control, crop choice, and possibilities of irrigation (Van de Wauw et al., 2008).

The variability of the soil attributes is a result of soil

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formation factors and processes interaction such as, the climate, the topography, the material origin and the vegetation (Rezaei and Gilkes, 2005a; Wang et al., 2009). According to the same authors, the interaction between the factors and processes occurs both in spatial and time scale, which can be altered locally mainly through soil erosion.

According to Gonçalves et al. (2001) the study of spatial and time variability is of extreme importance concerning the attributes that influence the soil water storage, such as: soil depth, infiltration capacity, topography and the regional climate. Li et al. (2002) mentions that the structural quality of the soil has been associated with the favorable conditions for the growth of the root system, aeration, infiltration and water movement in its profile. The attributes that present variability induced by management practices are, among others, the surface layer thickness, structure, bulk density, porosity and hydraulic conductivity of the saturated soil, are more dynamic in space and time (Rezaei and Gilkes, 2005b).

In this respect, the scientific knowledge advance in spatial variability studies of soil properties started to include, in addition to the classic statistic, the geoestatistics. The geoestatistics incorporates the spatial and temporal coordinates in the data process (Goovaerts, 1999; Webster and Oliver, 2007), presenting techniques that allows precise estimations of the studied variations (Scolforo et al., 2015). Among the geoestatistics methods utilized in spatial variability studies of soil attributes, kriging methodologies can be highlighted (Sun et al., 2003; Umali et al., 2012).

It should be noted that the studies carried out about the spatial variability of the physic and hydraulic properties are mostly applied in small scale areas, being inadequate or insufficient for the use in soil management in the catchment scale (Dessalegn et al., 2014; Tesfahunegn et al., 2011; Wang and Shao, 2013).

The soil properties are connected, having an important effect in the hydrology of the watershed, determining the water infiltration capacity into the soil, the groundwater flow, affecting the surface runoff and the baseflow (Price et al., 2010; Wang and Shao, 2013). Therefore, the comprehension of the attribute of soil behavior becomes relevant to help in the natural resources conservation, sustainable uses in watersheds and in the improvement of management practices of agriculture, as their effects on the environment (Cambardella et al., 1994; Pinto et al., 2016). According to Mello et al. (2011), the greatest concern in the studies in basin scale is in the comprehension on the environmental balance and the impact of the use and management of the soil in the process of runoff and erosion.

In this context, this study was developed with the objective of evaluating the spatial variability of physical and hydraulic soil attributes in a watershed, located in the Sul-Rio-Grandense Shield in the South of Brazil through the geoestatistic analysis.

MATERIALS AND METHODS

The Pelotas Stream watershed is located in the South of the State of Rio Grande do Sul - Brazil. It is an important water source for the region, once it is the main water public source in the city of Pelotas, where it is estimated around 342.873 residents (IBGE, 2015). The watershed is located in two geomorphologic provinces (Sul-Rio-Grandense Shield and Planície Costeira), the first is located in the higher altitude of the basin and the second in the portion near from the coast.

The study was carried out in the do Ouro watershed, a subbasin of the Pelotas watershed. The do Ouro watershed has an area of 17.17 km², located between the UTM coordinates 352243.02 and 346693.81 longitude E and 6506001.84 and 6500135.29 latitude S, with altitude ranging between 76 and 326 m (Figure 1A).

The regional climate, according to Köppen climate classification, is type “Cfa”, that is, humid temperate with hot summers. The annual average temperature, precipitation and relative humidity in the region are 17.5°C, 1276 mm and 83%, respectively.

The soils in the watershed of do Ouro Stream were classified as Argissolos and Neossolos based on the survey by Cunha et al. (1996) similar to Acrisols and Regosols as classified by World Reference Base WRB (FAO, 2014). The topography is mainly undulating (46.9% of the total area), followed by mildly undulating and strongly undulating with 21.7 and 19.4% of the area, respectively (Figure 1B). Regarding the soil use, according to Bartels (2015), native grass is de main land cover in 43.4% of the total area, being destined mainly for beef consumption and dairy cattle. The other areas as covered by native forests (34.1%), annual crop (13.9%), cultivated forests (4.2%) and orchards (3.1%). The soil samples were collected in 3 transects according to Figure 1C.

Each transect presents the same distance of 1500 m between each other, containing spaced points every of 150 m, totaling 67 samples. Thus, the sampled points represented the multiple uses present in the basin, as well as its different physiographical characteristics, such as slope and altitude. The soil samples were collected in the surface layer (0-0.15 m) and subsurface layer (0.15-0.25 m). Deformed samples were collected for particle size distribution analysis. Samples with preserved structure were also collected for determining the macroporosity (Ma), microporosity (Mi), total porosity (TP), soil bulk density (BD), hydraulic conductivity (K) and Field Capacity (θFC). This soil sampling was performed with Uhland soil sampler in cylinders with a diameter and height of 7.6 cm (approximately 344.1 cm³).

Afterward, soil samples were taken to Laboratory of Soils and Hydro-sedimentology of the Water Resources Engineering course - UFPel, in plastic bags previously identified. The particle size distribution was determined by the pipette method, according to what was proposed by Embrapa (1997), using chemical dispersion with NaOH solution 0.1 mol L^{-1} and mechanical agitation with high rotation for 15 min. The samples with preserved structures collected in the cylinders were saturated for 48 h through gradual water depth elevation.

The total porosity, macroporosity, microporosity and soil bulk density were determined according to methodology proposed in Embrapa (1997), with the TP determined by the difference between the saturated soil mass and the dried soil at 105°C for 24 h. For determining the microporosity, the tension table method was used, in which a -6 kPa tension was applied. The macroporosity was calculated by the difference between the total porosity and the microporosity.

The soil water contents at field capacity (θFc) were taken as those corresponding to potential at -10 kPa (Reichardt and Timm, 2012). After sample dried out at 105°C for 24 h, the soil bulk density was determined by the relation between the mass of dry soil and the cylinder volume. The hydraulic conductivity of the saturated soil was determined through a constant head permeameter, according

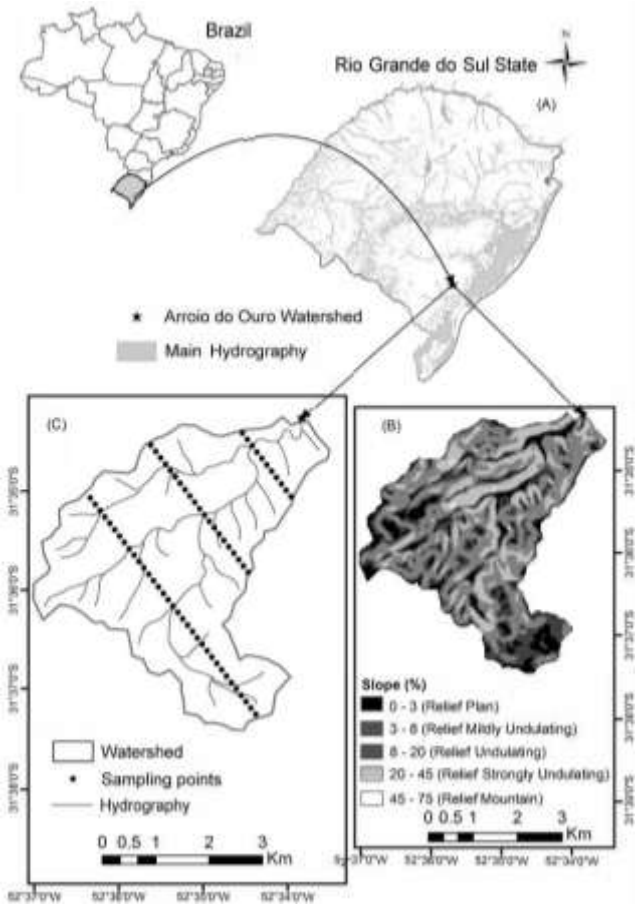

Figure 1. Location of watershed in do Ouro Stream (A), map of slope (B) and soil sampling points (C).

to Embrapa (1997). Soil water content at permanent wilting point (θPWP) corresponding to potentials of -1500 kPa (Reichardt and Timm, 2012), was determined in previously air-dried and sieved (2 mm sieve) soil samples, with a psychrometer (Gubiani et al., 2013).

Firstly, the obtained data were submitted to a descriptive analysis using the statistic software Assistat (Silva and Azevedo, 2009), where average, minimum, maximum, standard deviation (S), asymmetry, kurtosis, coefficient of variation (CV) and normality test, were obtained. In addition to this, boxplot graphics were analyzed for removing outliers. The coefficient of variation (CV) was classified, according to Wilding and Drees (1983) as: CV ≤ 15%; 15% < CV ≤ 35%; CV > 35%, as low, medium and high, respectively. For testing the hypothesis of normality of data distribution, the Shapiro and Wilk (1965) test was applied at a level of 5%.

With Geoest package (Vieira et al., 2002), the geostatistical analysis was carried out to characterize the spatial variability of the physical and hydraulic properties. Therefore, the calculation of an experimental and theoretical semivariogram and its respective adjustment parameters was performed (Equation 1).

$$\gamma(h)=\frac{1}{2N(h)}\sum_{i=1}^{N(h)}\left[Z(x_i)-Z(x_i+h)\right]^2 \quad (1)$$

Wherein: N(h) is the number of observation pairs Z(xi) and Z(xi+h) separated by a distance h. A theoretical model was adjusted to the experimental semivariogram, where C0 = nugget effect; C0+C1 = sill; A = range.

All the models of semivariograms were submitted to validation by the "Jack-Knifing" method (Vieira et al., 2002). The spatial dependence degree (SDD) was classified according to Zimback (2001), as: spatial dependence ≤ 25%; 25% < spatial dependence ≤ 75% and spatial dependence > 75%, in low, moderate and high, respectively.

RESULTS AND DISCUSSION

The values of asymmetry show an asymmetric distribution of the soil attributes (Table 1). Values of positive asymmetry show a tendency of concentration of values below the observed average, being this tendency more significant the higher the obtained value. An inverse situation occurs for negative values (Neves Neto et al., 2013). However the asymmetry in the data is not significant, once the asymmetry coefficients are inside the ±1 limit proposed by Kerry and Oliver (2007).

According to the classification of the coefficient of variation (CV) proposed by Wilding and Drees (1983), sand, BD and TP presented low variability (CV < 15%) for the two depths. It can be noted in Table 1 that the K was the attribute that showed the highest variability on both analyzed layers, followed by macroporosity. The high values for CV represent heterogeneity in relation to the average. The other attributes presented a moderate variability, with CV between 15 and 35%.

The results of the Shapiro and Wilk normality test (p≤0.05) indicate that only the variables K in the surface layer, and clay, Ma, K and (θPWP) in the subsurface layer, do not present normality. According to Paz-Gonzales et al. (2001), the kriging presents better results when the data normality is observed. However, the set of data normality is not a mandatory requirement for applying geoestatistics (Cressie, 1993).

The mathematical models of semivariograms are presented, as well as, its respective parameters of adjustment for the physical and hydraulic soil attribute in the 0 to 0.15 and 0.15 to 0.25 m layers (Table 2). On both layers, the variables sand, silt, Ma, Mi, TP, K and θPWP showed a better adjustment to the exponential model and the variable clay to the spherical model. The exponential model also adjusted the best to the variables BD and Field Capacity (θFc) in the 0.15 to 0.25 m layer. These same variables had a best fit to the spherical model in the surface layer (0-0.15 m). Mcbratney and Webster (1986) affirm that the spherical and exponential models are the ones most frequently adjusted to the soil attributes.

It was also observed that the range of spatial dependence found in 0 to 0.15 m depth presented the following values for the soil physical and hydraulic attributes: Sand, 700 m, clay, 600 m, Ma, 800 m, Mi, 850 m, TP, 548 m; BD, 480 m; K, 650 m, Fc, 560 and PWP, 670 m. For 0.15 to 0.25 m depth, however, the range (A) presented the following values: Sand, 870 m; silt, 840 m; Ma, 885 m; TP, 820 m, BD, 700 m, θFc 700 m and

Table 1. Parameters of descriptive statistic for physical and hydraulic attributes of the soil.

Variables	Mean	Median	Minimum	Maximum	SD	CV	Cs	Ck	SW (p-value)
0 - 0.15 m*									
Sand (g kg^{-1})	584.61	587.83	398.5	737.33	69.62	11.91	-0.17	0.28	>0.100$^{(N)}$
Clay (g kg^{-1})	166.87	166.87	98.53	263.47	38.59	23.13	0.44	-0.15	>0.100$^{(N)}$
Silt (g kg^{-1})	250.03	245.79	158.13	370.94	49.91	19.96	0.23	-0.34	>0.100$^{(N)}$
Ma (m^3 m^{-3})	0.14	0.13	0.04	0.29	0.06	40.52	0.59	-0.18	>0.100$^{(N)}$
Mi (m^3 m^{-3})	0.3	0.3	0.17	0.46	0.05	18.02	0.12	0.81	>0.100$^{(N)}$
TP (m^3 m^{-3})	0.44	0.43	0.35	0.56	0.05	10.59	0.36	0.27	>0.100$^{(N)}$
BD (kg dm^{-3})	1.42	1.42	1.15	1.61	0.1	7.36	-0.46	0.11	>0.100$^{(N)}$
K (mm h^{-1})	607	429.91	85.1	1773.59	449.6	74.07	0.95	-0.14	0.00002$^{(NN)}$
θ_{Fc} (m^3 m^{-3})	0.26	0.25	0.15	0.41	0.05	20.21	0.29	0.47	>0.100$^{(N)}$
θ_{PWP} (m^3 m^{-3})	0.08	0.09	0.05	0.13	0.02	20.66	0.08	0.02	>0.100$^{(N)}$
0.15 - 0.25 m**									
Sand (g kg^{-1})	556.58	561.75	289	731.83	83.04	14.92	-0.59	0.81	>0.100$^{(N)}$
Clay (g kg^{-1})	183.11	180.93	108.25	323.33	53.29	29.1	0.64	-0.26	0.00920$^{(NN)}$
Silt (g kg^{-1})	247.48	249.1	159.92	363.74	47.43	19.17	0.26	-0.25	>0.100$^{(N)}$
Ma (m^3 m^{-3})	0.13	0.12	0.04	0.28	0.06	44.21	0.83	-0.16	0.00037$^{(NN)}$
Mi (m^3 m^{-3})	0.29	0.29	0.14	0.43	0.05	18.33	0.06	0.39	>0.100$^{(N)}$
TP (m^3 m^{-3})	0.42	0.42	0.35	0.54	0.04	10.13	0.37	-0.06	>0.100$^{(N)}$
BD (kg dm^{-3})	1.43	1.45	1.11	1.63	0.1	7.03	-0.5	0.47	>0.100$^{(N)}$
K (mm h^{-1})	248.3	201.69	15.47	798.87	190.6	76.75	0.92	0.21	0.00077$^{(NN)}$
θ_{Fc} (m^3 m^{-3})	0.26	0.25	0.11	0.41	0.06	21.48	0.34	0.09	>0.100$^{(N)}$
θ_{PWP} (m^3 m^{-3})	0.09	0.09	0.05	0.16	0.03	28.03	0.61	0.35	0.02690$^{(NN)}$

Sand, Clay and Silt, sand, clay and silt contents; Ma, soil macroporosity; Mi, soil microporosity; TP, soil total porosity; BD, soil bulk density; K, hydraulic conductivity; θ_{Fc} and θ_{PWP}, soil water contents at field capacity (tension of 10 kPa) and permanent wilting point (tension of 1500 kPa), respectively; SD, Sample Standard Deviation, CV, Coefficient of Variation (%), Cs, Coefficient of Asymmetry, Ck, Coefficient of Kurtosis, SW, Shapiro and Wilk Test, Significance of 5%, N, Follows the Normal Distribution NN, Does not follow the Normal Distribution; * Surface layer; ** Subsurface layer.

θPWP, 680 m. According to Cora et al. (2004) the range values can influence the quality of estimates, as it determines the number of values used in the interpolation. Therefore, the estimates done with interpolation by ordinary kriging using values of higher range tend to be more reliable, presenting maps that better represent reality.

Physical and hydraulic soil attributes studied presented spatial dependence, as none has pure nugget effect (Table 2). The level of spatial dependence, according to the classification proposed by Zimback (2001), indicates that the variables sand, silt, Mi, TP, BD, K, θFc e θPWP in both layers presented spatial dependence classified as moderate (25% < spatial dependence ≤ 75%). The values of spatial dependence were high (spatial dependence > 75%) for Ma in the 0 to 0.15 m layer and clay in the 0.15 to 0.25 m layer (Table 2). The variations observed in the spatial dependence level of the physical and hydraulic soil attributes can be influenced by intrinsic factors, that is, soil formation factors, source material, relief, climate and organisms, and by extrinsic factors, that is, management practices such as fertilizing, plowing, harrowing, liming, etc. Figure 2 presents the spatial distribution maps of the attributes studied in the surface and subsurface layer.

The lower concentrations of sand are located in the southern part of the basin in both evaluated layers (Figure 2). The opposite occurs with the spatial distribution of clay contents. The spatial distribution of the variable soil bulk density in the layer 0.15 to 0.25 m is more heterogeneous when compared to its distribution in the surface layer. The higher values of BD can be considered normal throughout do Ouro watershed for the sand contents in the watershed. Reinert et al. (2008) analyzed the BD effect in the root growth in a soil with grain size similar and the observed in the study area. A normal root growth for bulk densities up to 1.75 kg dm^{-3} was found.

The values of total porosity observed in Figure 2 varied between 0.359 and 0.513 m^3m^{-3} in the experimental area, with the highest values found in the northern part where the higher concentration of sand was obtained. The total porosity and macroporosity maps show a opposite behavior in the 0.15 to 0.25 m layer, in other words, areas that present the lowest BD values have the highest TP values and Ma. The compaction caused by animal trampling can be linked to the increase in the soil BD. This basin is mainly covered by native grass which is

Table 2. Theoretical models of semivariograms and respective parameters of adjustment of physical and hydraulic soil attribute in the 0-0.15 and 0.15-0.25 m layers.

Variable	Model	C_0	C_1	C_0+C_1	A	SDD	$C_1/(C_0+C_1)$
0-0.15 m*							
Sand (g kg^{-1})	Exponential	2000	3100	5100	700	60.78	Moderate
Clay (g kg^{-1})	Spherical	450	1250	1700	600	73.53	Moderate
Silt (g kg^{-1})	Exponential	1100	1450	2550	650	56.86	Moderate
Ma (m^3 m^{-3})	Exponential	0.00057	0.0026	0.00317	800	82.02	High
Mi (m^3 m^{-3})	Exponential	0.0014	0.0013	0.0027	850	48.15	Moderate
TP (m^3 m^{-3})	Exponential	0.0009	0.00125	0.00215	548	58.14	Moderate
BD (kg dm^{-3})	Spherical	0.0042	0.0065	0.0107	480	60.75	Moderate
K (mm h^{-1})	Exponential	57000	140000	197000	650	71.06	Moderate
θ_{Fc} (m^3 m^{-3})	Spherical	0.0012	0.0014	0.0026	560	53.85	Moderate
θ_{PWP} (m^3 m^{-3})	Exponential	0.00012	0.00022	0.00034	870	64.71	Moderate
0.15-0.25 m**							
Sand (g kg^{-1})	Exponential	1870	5300	7170	870	73.92	Moderate
Clay (g kg^{-1})	Spherical	700	2300	3000	570	76.67	High
Silt (g kg^{-1})	Exponential	730	1750	2480	840	70.56	Moderate
Ma (m^3 m^{-3})	Exponential	0.00049	0.00144	0.00193	885	74.61	Moderate
Mi (m^3 m^{-3})	Exponential	0.0014	0.0016	0.003	790	53.33	Moderate
TP (m^3 m^{-3})	Exponential	0.0005	0.00138	0.00188	820	73.4	Moderate
BD (kg dm^{-3})	Exponential	0.003	0.0067	0.0097	700	69.07	Moderate
K (mm h^{-1})	Exponential	14000	23000	37000	650	62.16	Moderate
θ_{Fc} (m^3 m^{-3})	Exponential	0.0016	0.00166	0.00326	700	50.92	Moderate
θ_{PWP} (m^3 m^{-3})	Exponential	0.00028	0.0003	0.00058	680	51.72	Moderate

Sand, Clay and Silt: sand, clay and silt contents; Ma: soil macroporosity; Mi: soil microporosity; TP: soil total porosity; BD: soil bulk density; K: hydraulic conductivity; θ_{Fc} and θ_{PWP}: soil water contents at field capacity (tension of 10 kPa) and permanent wilting point (tension of 1500 kPa), respectively; C_0: nugget effect, C_1:structured variance, A: range (m);SDD: spatial dependence degree (%), macroporosity (Ma), microporosity (Mi), total porosity (TP), bulk density (BD), hydraulic conductivity of the saturated soil (K), volumetric field capacity ($\square_{FC}$), volumetric permanent wilting point ($\square_{PWP}$). * Surface layer; ** Subsurface layer.

destined to dairy cattle, which according to Menezes et al. (2016) is the main cause for the increase in soil BD followed by a decrease in TP in Oxisols.

Hydraulic conductivity of saturated soil maps present very similar pattern values with the maps of the variables macroporosity and sand, in both layers. Thus, it can be inferred that soils with higher sand content present higher quantity of macropores and as a consequence increase in water flow in the soil profile. The same cannot be stated about microporosity and soil density as they present an inverse relation to the saturated hydraulic conductivity, in both layers.

Conclusion

It can be concluded about the spatial variability of soil physical and hydraulic properites in the do Ouro watershed that: (i) The variables K and macroporosity presented a high heterogeneity in relation to the average, with K in the surface layer and the variables clay, Ma, K and θ_{PWP} in the subsurface layer not presenting normality according to Shapiro and Wilk test ($p \leq 0.05$). (ii) The theoretical model of the exponential semivariogram is the one that best describes the physical and hydraulic attributes in spatial variability structure. (iii) Spatial dependence was observed for all the soil physical and hydraulic attributes studied, classifying as high for the variables macroporosity (0-0.15 m) and clay (0.15-0.25 m), with the other variables classified as moderate spatial dependence. With the maps provided by the present study, it is possible to distinguish areas in the basin according to their soil and water attributes, enabling the development of conservation practices. However, some improvements in the map creation must be highlighted. Future studies may consider more sample points in order to improve the interpolation analysis. Moreover, a higher spatial density of soil samples is also recommended.

Conflicts of Interests

The authors have not declared any conflict of interests.

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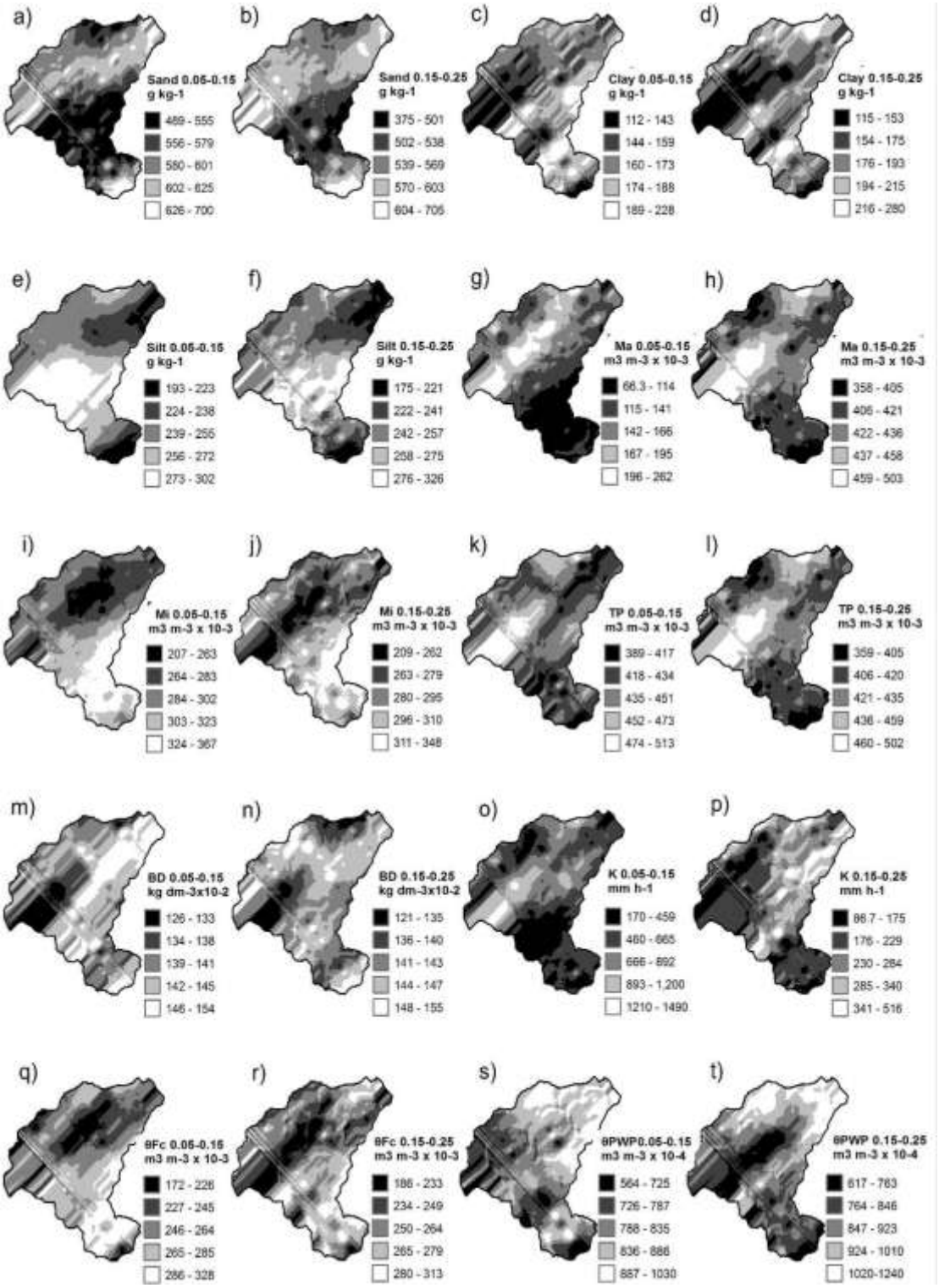

Figure 2. Spatial distribution of sand, clay and silt (g kg^{-1}), TP (m^3 m^{-3}), Ma (m^3 m^{-3}), Mi (m^3 m^{-3}), BD (kg dm^{-3}), K (mm h^{-1}), θ_{Fc} (m^3 m^{-3}) and θ_{PWP} (m^3 m^{-3}), in the 0-0.15 and 0.15-0.25 m layers, in the do Ouro watershed.

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Effect of nitrogen and potassium fertilizations on elephant grass genotypes used for energy purposes in Northern Rio de Janeiro State, Brazil

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Similar to many other human activities, the energy sector has a global concern with environmental issues. The use of renewable energy sources such as biomass is an alternative to the use of fossil fuels. Eight elephant grass genotypes showing energy production potential were herein assessed. The genotypes were grown from February 2014 to March 2016 in Campos dos Goytacazes County – Rio de Janeiro State, Brazil. They were fertilized with three different nitrogen doses (400, 1000 and 1600 kg N ha^{-1}) and two potassium doses (200 and 500 kg K_2O ha^{-1}). The experiment followed a randomized block design, with three repetitions, using a split-plot factorial scheme. The aim of the current study was to assess the effect of different nitrogen and potassium doses in fertilizing different genotypes of elephant grass by analyzing the morphoagronomic traits. The lowest K dose (200 kg ha^{-1}) was enough to generate the best outcomes in characteristics presenting significant effects. The N increase in the fertilization process did not promote dry matter production gains. The lowest N dose (400 kg ha^{-1}) was enough to promote the highest values. As for the other traits assessed in the current study, although there was a genotype that showed statistically significant difference from any other genotype at a particular dose, the increasing N doses in the fertilization did not influence the performance of the genotypes.

Key words: Renewable energy, biomass, mineral nutrients, *Pennisetum purpureum* Schum.

INTRODUCTION

Fossil fuels such as coal, oil and natural gas are non-renewable, as well as environmentally damaging energy

sources due to their formation (millions of years), consumption rates and polluting potential. They release CO_2 in the atmosphere during their extraction process. The price raise and the negative environmental impacts of the use of fossil fuels have reinforced the interest in using renewable forms of alternative energy. Extensive studies on bioenergy indicate that biomass conversion into energy is an important alternative, since it is a renewable source. Biomass is considered a CO_2 neutralizer because the carbon dioxide released during its combustion is recaptured by the new biomass growth. Thus, biomass is the only alternative energy source able to produce liquid, solid and gaseous fuels to replace the fossil ones (Simacek, 2008; Ohimain et al., 2014; Oliveira, et al., 2015b).

Elephant grass is a perennial C4-metabolism poaceae acknowledged as energy crop due to its advantages: fast growth, disease resistance, adaptability, little handling and easy propagation (Tsai, 2009). In addition, it is highly efficient in fixing the CO_2 from the atmosphere and has the potential to be used in biofuel production or in direct combustion (Rossi et al., 2014).

Nitrogen (N) and potassium (K) stand out among the macronutrients because of the key role they play in plant nutrition. Nitrogen is an essential constituent of proteins and it directly interferes in the photosynthetic process due to its participation in the chlorophyll molecule. Potassium is the cation showing the highest concentration in plants; this nutrient has relevant physiological and metabolic functions such as enzyme activation, photosynthesis and translocation of assimilates, as well as nitrogen uptake and protein synthesis. Thus, nitrogen and potassium are limiting factors for systems that make intensive use of cultivated soils (Taiz and Zeiger, 2012).

Nitrogen fertilization is the main N addition vehicle and one of the most important inputs in agricultural systems due to its increased performance in plant productivity (Lopes, 2007). Since elephant grass is a high productivity species, it is worth considering that its nutrient requirements are related to the production potential of cutting-system crops. According to Mistura et al. (2006), elephant grass responds to increasing nitrogen levels. The authors also highlight that potassium fertilization is of great importance, mainly when the plant is grown in cutting systems. Potassium removal due to plant cutting and transportation to other areas, other than the production site, often results in major nutritional imbalance issues in the soil.

The aim of the current study is to assess eight genotypes of elephant grass (Cubano Pinda, Vruckwona, IAC-Campinas, Capim Cana D'África, Cameroon, CPAC, IJ 7139 and BAG-86) with energy potential subjected to three nitrogen doses (N1= 400, N2 = 1000 and N3 = 1600 kg N ha^{-1}) and to two potassium doses (K1 = 200 and K2 = 500 kg K_2O ha^{-1}), under the soil and climate conditions of Campos dos Gotacazes in the Northern region of Rio de Janeiro State, by analyzing the morphoagronomic traits.

MATERIALS AND METHODS

Location and experimental design

The experiment was conducted at the Agro-Energy and Waste Utilization State Research Center of PESAGRO located in Campos dos Goytacazes County-RJ, Brazil (latitude 21° 44' 43'' S; longitude 41° 18' 29'' W; 10 m altitude; datum WGS84). The climate in the region is hot and humid, and the mean annual temperature is 22.7°C. The results of the soil analysis conducted in the experimental site on January 8th, 2014, were: water pH = 5.7; P = 7 mg dm^{-3}; K = 121 mg dm^{-3}; Ca, Mg, Al, H + Al and Na = 3.8, 2.5, 3.6 and 0.1 cmol dm^{-3}; CEC = 10.2; and organic matter = 26.5 g dm^{-3}.

The experiment was arranged in a randomized block design with three repetitions, according to a split-plot factorial scheme comprising three factors: Factor 1 (plots): genotypes - 8 clones; Factor 2: N - three doses (400, 1000 and 1600 kg N ha^{-1}); and Factor 3: K - two doses (200 and 500 kg K_2O ha^{-1}) (sub-plots). Each block comprised of 8 plots. The plots corresponded to parallel rows (12 m-long) spaced 1.5 m from each other. Each row was planted with one of the eight genotypes. The plot was divided into 6 sub-plots (2 m each) wherein the treatments corresponding to six N and K doses (400 x 200, 1000 x 200, 1600 x 200, 400 x 500, 1000 x 500 and 1600 x 500 kg ha^{-1} of N x K) combinations were applied. An area of 1.5 m^2 was used to collect the samples to be analyzed through laboratory procedures, the central length (1.0 m) of each sub-plot was taken into account.

The experiment was conducted on February 12th, 2014. The soil was conventionally prepared using a harrowing grid and cultivation furrows (10 cm-deep) were opened. Culm fragments were arranged with 100 kg ha^{-1} of simple superphosphate (P_2O_5) within the furrows, in a single row. Thirty days after the planting, 25 kg ha^{-1} of urea (CH_4N_2O) and potassium chloride (KCl) were applied to the furrows.

The genotypes used in the experiment came from the Active Elephant Grass Germplasm Bank (AEG GB) of Norte Fluminense Darcy Ribeiro State University, located in Campos do Goytacazes County – RJ, Brazil. The genotypes were selected through visual analysis based on late flowering. Such property is desirable for long-term elephant grass cultivation aiming at dry matter production. The following genotypes were selected: Cubano Pinda (G1), Vruckwona (G2), IAC-Campinas (G3), Capim Cana D'África (G4), Cameroon (G5), CPAC (G6), IJ 7139 (G7) and BAG-86 (G8).

The standadized cut was performed in the culm base on March 29th, 2014 (45 days after planting) in order to enable the uniform growth of the shoots. Two assessment cycles were held (two cuts after a year of growth). The fertilization was split in 6 applications in each assessment cycle, according to the rainfall. The first

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assessment cut took place on March 10th, 2015 and the second one, on March 15th, 2016. The experiment was conducted under natural conditions, without irrigation.

The following morphoagronomic traits were assessed before each cut: number of tillers per meter (NT), which were found by counting the number of tillers in the 1.5 m² area of the sub-plot; mean plant height (H), based on the mean height of the plants in the sub-plot, expressed in m and measured using a graduated scale; mean culm diameter (CD), expressed in cm and measured 10 cm from the ground level, using a digital caliper; mean leaf blade width (LBW), expressed in cm, based on the mean width of the center of three leaves.

The collected samples were fractionated, packed in paper bags and weighed after cutting. Subsequently, they were dried in a forced air circulation oven at 65°C. Seventy-two (72) hours later, the samples were removed in order to be weighed and to obtain air-dried samples (ADS). The ADSs were ground in Wiley mill (2 mm sieve). Two grams (2 g) of it were dried in an oven at 105°C, for 16 h, and then weighed in order to obtain the oven-dried samples (ODS). The dry matter rate DMR was estimated through the ratio between the ADS and ODS values. The dry matter production (DMP) resulted from the product between fresh matter production and DMR, and it was converted to t ha^{-1}.

Statistical analysis

The statistical analyses were performed in the Genes software (Cruz, 2013). The variance analysis of the traits assessed in the statistical randomized block design used the following statistical model, according to a split-plot factorial design:

$$Y_{ijkl} = \mu + B_l + G_i + \varepsilon_a + N_j + G_iN_j + K_k + G_iK_k + N_jK_k + G_iN_jK_k + \varepsilon_b$$

Where, Y_{ijkl} = value of the i^{th} genotype, at the j^{th} nitrogen dose, at the k^{th} potassium dose and in the l^{th} block; μ = overall mean of the experiment; G_i = effect of the i^{th} genotype; B_l = effect of the l^{th} block; ε_a = effect of the error a, associated with the i^{th} genotype in the l-th block; N_j = effect of the j-th nitrogen dose; G_iN_j = effect of the interaction between the i^{th} genotype and the j^{th} nitrogen dose; K_k = effect of the k^{th} potassium dose; G_iK_k = effect of the interaction between the i^{th} genotype and the k^{th} potassium dose; N_jK_k = effect of the interaction between the j^{th} nitrogen dose and the k^{th} potassium dose; $G_iN_jK_k$ = effect of the interaction between the i^{th} genotype and the j^{th} nitrogen dose and the k^{th} potassium dose; ε_b = effect of the error b, associated with the i^{th} genotype, with the j^{th} nitrogen dose and with the k^{th} potassium dose in the l^{th} block.

A multiple comparison test was conducted in order to study the contrasts between the means of the N factor within the K factor after the individual variance analysis, according to Tukey's method, at 5% significance level.

RESULTS AND DISCUSSION

The G factor had no effect (P > 0.05) on any of the traits assessed in the two cuts. Only the N factor had effect on the DMP trait in the first year of the experiment, whereas all factors (except for the G factor) and interactions had effect on the DMP trait in the second year of the experiment. Only the N factor had statistically significant effect on the DMR trait in the first year of experiment. Only the interaction between K and N had effect on the NT trait, in the second cut. Only the N factor had effect on the H trait, in the first cut; and on the CD trait, in the second year of experiment. Only the interaction between K and G had significant effect on the LBW trait, in the first year of experiment.

High coefficients of variation (CV) were found in most variables. According to Pimentel-Gomes (2000), the CV values cannot be standardized for all cultures since each culture has its own peculiarities, depending on the assessed traits. Thus, these values may be acceptable since the studied traits are ruled by many genes, as well as strongly influenced by the environment.

There was also the option of conducting a study focused on the effects of the N levels within K as it is a tipical procedure in experiments based on the factorial scheme. According to such scheme, a factor must be assessed in constrast to the other one. However, with regard to the characteristic wherein K has presented significant effect, either alone or in interaction, it was observed that the lowest K dose (200 kg ha^{-1}) was enough to genetrate the best outcomes.

Table 1 shows that, according to the Tukey's test (P < 0.05), there was statistically significant difference between at least two genotypes in traits such as DMP, DMR and CD at doses N3, N1 and N3, respectively, in the second cut; and between genotypes of the LBW trait, at doses N1 and N3, in the first cut. There was no statistically significant difference between genotypes in the other traits, in their respective cuts.

As for the DMP trait, at dose K2N3, only the G2 genotype (Vruckwona) differed from the lowest mean observed in the second cut. Most genotypes showed DMP values at minimum N dose (600 kg ha^{-1}) higher than those found at the maximum dose (1600 kg ha^{-1}) in both cuts. Similar results were found by Novo (2015), who applied increasing N and K_2O doses to 3 genotypes of elephant grass for two years in Bom Jesus do Itabapoana and found high DMP estimates at minimum N dose (100 kg ha^{-1}). By analyzing both cuts, it was observed that the means ranged from 26.70 to 59.81 t ha^{-1}. These values were similar to those found by Oliveira et al. (2014), who used the same genotypes used in the current study and observed variation from 27.38 to 47.70 t ha^{-1}, in the first cut, and from 25.64 to 51.81 t ha^{-1}, in the second one.

The PMS values found in the present study, which varied from 24 to 48.15 (Cubano Pinda); from 23.63 to 59.81 (Vruckwona); 28.38 to 51.86 (IAC-Campinas); 22.65 to 51.94 (Capim Cana D'África); 26.91 to 46.04 (Cameroon); 22.93 to 50.70 (CPAC); 24.45 to 57.78 (IJ 7139) and 21.22 to 43.48 (BAG-86) kg ha^{-1}, were similar to or higher than the outcomes found by Oliveira et al. (2015a). They worked with 6 elephant grass genotypes grown under increasing N doses (cut at 9 month old) in Campos dos Goytacazes – RJ, Brazil and found mean PMS values varying from 17.61 to 57.65 t ha^{-1}.

Table 1. Mean values of the morphoagronomic traits assessed in eight genotypes of elephant grass grown for energy production purposes for two years and treated with nitrogen (400, 1000 and 1600 kg ha^{-1}) and potassium (200 and 500 kg ha^{-1}) doses; Campos dos Goytacazes – RJ.

	GEN	Cut 1						Cut 2					
		K1			K2			K1			K2		
		N1	N2	N3	N1	N2	N3	N1	N2	N3	N1	N2	N3
DMP	G1	31.18^{a}	27.43^{a}	27.36^{a}	39.22^{a}	28.96^{a}	23.99^{a}	47.26^{a}	32.85^{a}	31.85^{a}	30.40^{a}	48.15^{a}	40.38^{ab}
	G2	30.80^{a}	39.63^{a}	24.19^{a}	35.91^{a}	42.65^{a}	23.63^{a}	49.55^{a}	55.13^{a}	35.28^{a}	55.13^{a}	36.29^{a}	59.81^{a}
	G3	40.49^{a}	28.38^{a}	35.91^{a}	38.68^{a}	31.02^{a}	34.07^{a}	51.86^{a}	48.63^{a}	41.64^{a}	43.54^{a}	45.45^{a}	29.36^{b}
	G4	28.72^{a}	30.73^{a}	22.65^{a}	33.70^{a}	27.46^{a}	33.11^{a}	41.55^{a}	30.26^{a}	41.80^{a}	51.94^{a}	41.54^{a}	35.02^{ab}
	G5	31.25^{a}	32.73^{a}	27.70^{a}	44.23^{a}	26.91^{a}	28.90^{a}	31.62^{a}	29.32^{a}	46.04^{a}	29.20^{a}	42.05^{a}	42.44^{ab}
	G6	35.05^{a}	31.09^{a}	24.31^{a}	37.77^{a}	22.93^{a}	29.20^{a}	34.70^{a}	47.70^{a}	46.74^{a}	50.70^{a}	45.74^{a}	29.81^{b}
	G7	35.46^{a}	31.14^{a}	24.45^{a}	36.97^{a}	33.56^{a}	44.61^{a}	57.78^{a}	35.78^{a}	49.24^{a}	55.10^{a}	29.70^{a}	26.02^{b}
	G8	26.70^{a}	36.32^{a}	26.24^{a}	39.22^{a}	28.96^{a}	23.99^{a}	40.06^{a}	43.34^{a}	21.22^{a}	36.07^{a}	29.22^{a}	38.43^{ab}
DMR	G1	29.66^{a}	27.98^{a}	26.89^{a}	29.38^{a}	28.80^{a}	23.31^{a}	41.53^{a}	33.39^{a}	32.64^{a}	27.73^{b}	36.09^{a}	35.63^{a}
	G2	31.57^{a}	28.23^{a}	27.23^{a}	29.54^{a}	27.39^{a}	25.83^{a}	41.54^{a}	36.99^{a}	38.05^{a}	36.99^{ab}	30.50^{a}	35.66^{a}
	G3	30.05^{a}	28.29^{a}	29.17^{a}	31.02^{a}	27.76^{a}	29.73^{a}	37.86^{a}	39.02^{a}	35.01^{a}	37.70^{ab}	34.65^{a}	32.55^{a}
	G4	32.68^{a}	23.69^{a}	24.00^{a}	27.50^{a}	27.28^{a}	25.32^{a}	38.75^{a}	31.16^{a}	40.11^{a}	38.66^{ab}	33.42^{a}	35.57^{a}
	G5	28.75^{a}	25.26^{a}	27.30^{a}	33.22^{a}	27.21^{a}	25.07^{a}	33.49^{a}	35.11^{a}	36.06^{a}	30.52^{ab}	37.98^{a}	35.16^{a}
	G6	30.59^{a}	26.85^{a}	26.61^{a}	31.58^{a}	24.14^{a}	24.19^{a}	33.79^{a}	39.22^{a}	37.93^{a}	35.74^{ab}	33.58^{a}	35.61^{a}
	G7	32.30^{a}	24.38^{a}	23.78^{a}	26.27^{a}	27.67^{a}	37.97^{a}	35.28^{a}	35.85^{a}	36.68^{a}	45.59^{a}	29.90^{a}	31.90^{a}
	G8	28.66^{a}	29.81^{a}	29.51^{a}	29.30^{a}	28.78^{a}	28.43^{a}	40.05^{a}	34.19^{a}	30.75^{a}	38.52^{ab}	34.37^{a}	33.58^{a}
NT	G1	27.66^{a}	25.66^{a}	30.66^{a}	34.00^{a}	35.00^{a}	26.66^{a}	35.00^{a}	33.33^{a}	35.33^{a}	38.33^{a}	50.00^{a}	36.66^{a}
	G2	22.33^{a}	40.66^{a}	29.66^{a}	30.00^{a}	40.66^{a}	34.00^{a}	47.66^{a}	48.66^{a}	34.66^{a}	48.66^{a}	42.33^{a}	47.66^{a}
	G3	41.00^{a}	29.66^{a}	39.33^{a}	32.33^{a}	31.33^{a}	28.66^{a}	37.66^{a}	38.66^{a}	43.66^{a}	42.66^{a}	40.33^{a}	29.33^{a}
	G4	23.66^{a}	37.33^{a}	34.00^{a}	32.66^{a}	31.66^{a}	34.66^{a}	39.66^{a}	36.66^{a}	47.33^{a}	47.33^{a}	46.33^{a}	36.00^{a}
	G5	33.00^{a}	37.66^{a}	29.33^{a}	31.66^{a}	30.33^{a}	31.33^{a}	33.66^{a}	29.33^{a}	38.66^{a}	30.00^{a}	44.00^{a}	28.66^{a}
	G6	37.00^{a}	31.33^{a}	23.66^{a}	38.00^{a}	30.66^{a}	40.00^{a}	24.33^{a}	38.66^{a}	48.00^{a}	39.33^{a}	49.00^{a}	35.66^{a}
	G7	27.66^{a}	40.33^{a}	30.66^{a}	33.00^{a}	35.00^{a}	35.66^{a}	40.66^{a}	33.66^{a}	44.33^{a}	42.00^{a}	33.33^{a}	32.00^{a}
	G8	24.66^{a}	42.00^{a}	27.66^{a}	31.33^{a}	46.33^{a}	29.66^{a}	37.33^{a}	38.33^{a}	28.33^{a}	36.00^{a}	37.00^{a}	38.33^{a}
H	G1	3.25^{a}	3.43^{a}	3.35^{a}	3.20^{a}	3.35^{a}	3.43^{a}	3.08^{a}	2.96^{a}	2.98^{a}	3.06^{a}	2.90^{a}	3.06^{a}
	G2	3.40^{a}	3.33^{a}	3.31^{a}	3.35^{a}	3.31^{a}	3.40^{a}	2.96^{a}	3.06^{a}	2.93^{a}	3.06^{a}	3.06^{a}	3.10^{a}
	G3	3.13^{a}	3.23^{a}	3.28^{a}	3.30^{a}	3.43^{a}	3.36^{a}	3.08^{a}	3.05^{a}	3.16^{a}	3.05^{a}	3.06^{a}	3.06^{a}
	G4	3.26^{a}	3.31^{a}	3.35^{a}	2.83^{a}	3.33^{a}	3.41^{a}	3.05^{a}	3.00^{a}	2.86^{a}	3.10^{a}	3.03^{a}	3.05^{a}
	G5	3.30^{a}	3.23^{a}	3.50^{a}	3.33^{a}	3.41^{a}	3.41^{a}	3.06^{a}	3.06^{a}	3.26^{a}	3.15^{a}	3.03^{a}	3.05^{a}
	G6	3.13^{a}	3.43^{a}	3.46^{a}	3.30^{a}	3.23^{a}	3.38^{a}	3.00^{a}	2.96^{a}	2.96^{a}	2.98^{a}	3.06^{a}	2.96^{a}
	G7	3.10^{a}	3.21^{a}	3.40^{a}	3.36^{a}	3.30^{a}	3.38^{a}	3.03^{a}	3.08^{a}	3.06^{a}	3.13^{a}	3.00^{a}	2.96^{a}

Table 1. Contd.

	GEN	Cut 1						Cut 2					
		K1			K2			K1			K2		
		N1	N2	N3	N1	N2	N3	N1	N2	N3	N1	N2	N3
CD	G8	3.25^{a}	3.31^{a}	3.46^{a}	3.26^{a}	3.26^{a}	3.58^{a}	3.00^{a}	2.86^{a}	2.88^{a}	3.01^{a}	2.98^{a}	3.01^{a}
	G1	1.57^{a}	1.87^{a}	1.66^{a}	1.70^{a}	1.67^{a}	1.76^{a}	1.76^{a}	1.73^{a}	1.64^{b}	1.80^{a}	1.82^{a}	1.63^{a}
	G2	1.63^{a}	1.91^{a}	1.76^{a}	1.56^{a}	1.53^{a}	1.52^{a}	1.84^{a}	1.86^{a}	2.23^{a}	1.86^{a}	1.98^{a}	1.75^{a}
	G3	1.72^{a}	1.69^{a}	1.73^{a}	1.68^{a}	1.59^{a}	1.65^{a}	1.69^{a}	1.67^{a}	1.69^{b}	1.58^{a}	1.71^{a}	1.68^{a}
	G4	1.61^{a}	1.61^{a}	1.79^{a}	1.65^{a}	1.62^{a}	1.71^{a}	1.73^{a}	1.59^{a}	1.63^{b}	1.70^{a}	1.46^{a}	1.67^{a}
	G5	1.68^{a}	1.67^{a}	1.62^{a}	1.67^{a}	1.68^{a}	1.73^{a}	1.55^{a}	1.63^{a}	1.61^{b}	1.45^{a}	1.62^{a}	1.53^{a}
	G6	1.43^{a}	1.71^{a}	1.80^{a}	1.69^{a}	1.75^{a}	1.71^{a}	1.61^{a}	1.52^{a}	1.79^{b}	1.69^{a}	1.76^{a}	1.49^{a}
	G7	1.61^{a}	1.60^{a}	1.70^{a}	1.59^{a}	1.66^{a}	1.58^{a}	1.67^{a}	1.77^{a}	1.62^{b}	1.90^{a}	1.77^{a}	1.40^{a}
	G8	1.58^{a}	1.71^{a}	1.84^{a}	1.75^{a}	1.63^{a}	1.72^{a}	1.73^{a}	1.56^{a}	1.55^{b}	1.59^{a}	1.70^{a}	1.73^{a}
LBW	G1	6.60^{ab}	6.00^{a}	6.03^{ab}	5.80^{a}	6.20^{a}	6.30^{a}	5.03^{a}	5.26^{a}	5.16^{a}	5.10^{a}	5.00^{a}	5.20^{a}
	G2	6.06^{ab}	5.50^{a}	6.00^{ab}	6.03^{a}	6.50^{a}	6.00^{a}	4.96^{a}	4.93^{a}	5.13^{a}	4.93^{a}	5.16^{a}	5.26^{a}
	G3	7.06^{a}	6.33^{a}	6.13^{ab}	6.33^{a}	5.50^{a}	6.16^{a}	5.30^{a}	5.10^{a}	5.06^{a}	5.20^{a}	5.10^{a}	5.13^{a}
	G4	6.50^{ab}	6.33^{a}	7.00^{a}	6.16^{a}	5.90^{a}	5.83^{a}	5.10^{a}	5.03^{a}	5.03^{a}	4.96^{a}	5.00^{a}	5.06^{a}
	G5	5.96^{ab}	5.66^{a}	5.83^{ab}	6.16^{a}	5.66^{a}	5.83^{a}	5.13^{a}	5.06^{a}	5.13^{a}	5.06^{a}	5.16^{a}	5.00^{a}
	G6	5.83^{b}	6.16^{a}	6.00^{ab}	5.66^{a}	5.66^{a}	6.13^{a}	4.96^{a}	5.13^{a}	5.20^{a}	5.10^{a}	5.10^{a}	5.23^{a}
	G7	5.83^{b}	5.83^{a}	6.00^{ab}	6.10^{a}	6.16^{a}	6.00^{a}	5.06^{a}	5.16^{a}	5.16^{a}	5.10^{a}	5.23^{a}	5.23^{a}
	G8	6.00^{ab}	6.50^{a}	5.66^{b}	5.36^{a}	6.06^{a}	6.33^{a}	5.06^{a}	5.10^{a}	5.23^{a}	4.90^{a}	5.23^{a}	5.30^{a}

Means followed by the same letter in the column do not statistically differ from each other, according to the Tukey's test, at 5% probability. DMP = total plant dry matter production in t ha^{-1}; DMR = total dry matter rate; NT = number of tillers linear m^{-1}; H = mean plant height in meters; CD = mean culm diameter in centimeters; LBW = mean leaf blade width in centimeters.

Only the G7 genotype (IJ7139) at dose K2N1 differed from the lowest mean observed (G1) in the second assessment cut in the DMR trait. The general mean of such trait in the two cuts was 28.13 and 35.67%, respectively, and the values ranged from 23.31 to 37.97% and from 27.73 to 45.59% in the first and second assessment cycles, respectively. The herein observed values were above those reported by Santos et al. (2014), who assessed three genotypes of elephant grass used for energy purposes, 180 days after fertilization with nitrogen (500 and 1000 kg ha^{-1} of N) in Alegre County - ES, Brazil. They found mean DMR 24.8%, values ranging from 23.29 to 25.77%. Oliveira et al. (2015a) found mean values ranging from 25.77 to 35.36%. These values were corroborated by the present study wherein mean values from 26.75 to 36.63% were found between the two assessment cycles.

The general mean of the NT trait was 32.74% in the first cut, and 38.85% in the second one. The lowest mean of the G2 genotype (Vruckwona) at dose K1N1 in the first assessment cycle was 22.33%, and the highest mean of the G1 genotype (Cubano Pinda) at dose K2N2 was 50.00% (Table 1). There was no statistically significant difference between genotypes in the N level assessment within K1 and K2; however, the outcomes were equal to or even higher than those found in studies that have demonstrated the high

productive potential of elephant grass. Oliveira et al. (2013) assessed the morpho-agronomic traits of the energy biomass from 73 genotypes of elephant grass in two cuts, after six months of growth in Campos dos Goytacazes - RJ. They found mean NT values of 29.5, 28.5, 26.5, 32 and 23 tillers per linear meter' in genotypes such as Cubano Pinda, Vruckwona, Cameroon, IJ 7139 and Capim Cana D'África, respectively. According to Daher et al. (2004), the NT trait has direct effect on DMP, and it is highly desirable in genotypes used in bioenergy production. Menezes et al. (2014) found direct positive correlation between NT and DMP, and indirect positive correlation between H and DMP. Thus, when the aim is to increase the DMP, as in the case of the elephant grass used to increase biomass energy, it is possible to select high-tillering plants, because they may have increased H and, consequently, high DMP.

With respect to H, the genotypes did not differ from each other when they were subjected to different N levels within K. The general mean height was 3.32 and 3.03 m in the first and second assessment cycles, respectively. The lowest height (2.86 m) was found in the G8 genotype (BAG-86) at dose K1N2 in the second cut. The highest height (3.50 m) was found in the G5 genotype (Cameroon) at dose K1N3 in the first cut. The H trait was important because it was directly correlated with dry matter production (Xia et al., 2010; Menezes et al. 2014). These values are different from those found by Oliveira et al. (2015a), who found mean H 3.54 m when they assessed six genotypes of elephant grass grown under increasing N doses and cut at 9 months of age in Campos dos Goytacazes – RJ.

However, these results are similar to those found by Novo (2015), who obtained general mean H 3.04 m, and individual H means ranging from 2.79 to 3.19 m in elephant grass genotypes subjected to fertilization using increasing N and K_2O doses.

With respect to the CD trait, the G2 genotype (Vruckwona) at dose K1N3 differed from the other genotypes in the second cut, and presented 2.23 cm CD. The general means were 1.68 and 1.69 cm in the first and second cuts, respectively. These values corroborate those found by Rossi et al, (2014), who assessed the morphoagronomic traits of 40 genotypes of elephant grass in Campos dos Goytacazes - RJ. The authors found general CD genotype mean of 1.72 cm in 11-month-old plants, in the third cut. However, Santos et al. (2014) reported higher CD values in the second assessment cut in 300 day-old plants. They found means of 1.80 and 1.78 cm in genotypes subjected to N doses of 500 and 1000 kg ha^{-1}, respectively. On the other hand, the CD outcomes in the present study are above those found by Oliveira et al. (2015a), who found mean value of 1.59 cm, ranging from 1.27 to 1.83 cm for the herein described characteristic in 6 genotypes of elephant grass assessed for energy purposes at the age of 9 months.

The culm diameter showed positive correlation with dry matter production (Xia et al., 2010) and it had direct effect on such trait (Daher et al., 2004).

The LBW trait is concerned the leaf area used to capture sunlight and, consequently, its photosynthetic capacity. The general LBW means observed in the first and second cuts were 6.06 and 5.11 cm, respectively. Two genotypes shown values above the average. The G3 genotype (IAC-Campinas) has shown mean LBW 7.06 cm at dose K1N1. The G4 genotype (Capim Cana D'África) has shown mean LBW 7.00 cm at dose N3K1. These values are different from those found by Rossi et al. (2014), who reported mean LBW 3.95 cm in 11-month-old genotypes; and also from those observed by Oliveira et al. (2015a), who reported mean LBW 4.71 cm in genotypes fertilized with increasing N doses.

Conclusions

Overall, the genotypes did not differ from each other when they were assessed for biomass production potential. The lowest K dose (200 kg ha^{-1}) was enough to generate the best results in characteristics that presented significant effect. The fertilization with increased N amounts did not promote DMP gains, and the lowest N dose (400 kg ha^{-1}) was sufficient to promote the highest values.

Likewise, as for the other traits assessed in the current study, although there was a genotype that showed statistically significant difference from any other genotype at a particular dose, the increasing N doses in the fertilization did not influence the performance of the genotypes. However, the results were consistent and met the expectations of energy production potential, since they assured the use of eight elephant grass genotypes as alternative biomass production sources.

Conflict of Interests

The authors have not declared any conflict of interests.

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