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Full Length Research Paper

Anti-atherogenic properties associated with the antioxidant activity from the hydrophilic extracts of *Halimeda incrassata* (Chlorophyta, Bryopsidales)

Alexis Vidal-Novoa[1], Ariadna Costa-Mugica[1], Yenisleidy Zulueta-Díaz[1], Daylín Diaz-Gutierrez[1], Ana Mara de Oliveira e Silva[2]*, Ana María Vazquez[3], Claudina Zaldívar-Muñoz[1], Dalva Assunçao Portari de Mancini[4] and Jorge Mancini-Filho[2]

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Seaweeds are a source of natural antioxidants having potential application in oxidative stress and associated diseases. In this work, anti-atherogenic properties associated with the antioxidant activity from the hydrophilic extracts of *Halimeda incrassata* were studied. The phenolic content assessed in the aqueous extract and fraction phenolic acids (FPA) was 0.13 ± 0.05 and 0.47 ± 0.09 mg of gallic acid equivalents (GAE)/g dry seaweed, respectively. In DPPH$^{\bullet}$, radical scavenging assay fractions exhibited a dependent concentration. The seaweeds extract inhibited the desoxirribose oxidation in the presence or absence of EDTA (IC_{50} = 1.91± 0.09 mg/mL) (IC_{50} = 2.95 ± 0.01 mg/mL). *In vivo* antioxidant properties of FPA-*H.incrassata* were investigated in rats with a CCl_4-induced liver injury. Pre-treatment with *H. incrassata* led to approximately 50% reductions in liver TBARS levels. The treatment with *H. incrassata* FPA also increased the activity of the CAT enzyme, which in turn resulted in an enhanced antioxidant defense. The expression of Catalase by PCR-RT technique demonstrated a higher gene expression when compared with that which was observed in the CCl_4-treated group. Antiatherogenic properties were studied in the inhibition of lipoprotein oxidation mediated by Cu^{2+} or HRP/H_2O_2, free radical scavenging, and metal ion chelation, and it was dose dependent with a higher concentration needed for the aqueous extract than for the FPA fraction. Antioxidant activity was also improved in macrophages as evaluated in the cell supernatant (by TBARS formation); and by luminol enhanced chemiluminescence after cell activation with zymosan; and a degree of cell lipoperoxidation was decreased by the *Halimeda incrassata* extract. The results of this work add to the antioxidant potential of the seaweed for its application in oxidative stress associated conditions.

Key words: Antiatherogenic properties, antioxidant activity, seaweeds, *Halimeda incrassate.*

INTRODUCTION

Oxidative stress is involved in a variety of pathologies and indeed it is presently considered a common factor in chronic non transmissible diseases as like atherosclerosis (Sanchez-Recalde and Kaski, 2001). Hence, among the most interesting alternatives for the modulation of oxidative stress as a target to halt the development of atherosclerosis, antioxidant effects have been sought at different stages of the disease: LDL oxidation, macrophage activation, foam cell formation, smooth muscle cell migration and advanced plaque remodeling (Kaliora et al., 2006); and then multiple mechanisms have been proposed for targeting an atherogenesis associated oxidative stress (Stocker and Keaney, 2004).

Seaweeds have combinations of highly developed antioxidant defense systems, allowing them to preserve structural integrity before different kinds of environmental stresses. However, they possess a wide diversity of bioactive compounds from amino acids, such as micosporines, polysaccharides, carotenoids, terpenoids, and an especially high phenolic content (Dutra-Rocha et al., 2007). Their unique composition makes them attractive candidates for their application in the antioxidant field. A correlation has also been found between the consumption of phenolic compounds in general, and seaweeds in particular, and the incidence of cardiovascular diseases (Bocanegra et al., 2009).

In the quest for more potent antioxidants from natural sources, our group has been especially interested in studying the beneficial properties of seaweed from the *Halimeda* genus for an application in biomedicine in hepato-, neuro- and athero-protection. Several lines of results have documented the ability of a hydrophilic extract from this seaweed to target free-radical mediated processes *in vitro* cell culture and *in vivo* experimental models (Rivero et al., 2003; Fallarero et al., 2003; Linares et al., 2004; Mancini-Filho et al., 2009; de Oliveira e Silva et al., 2012). In previous studies, *in vitro Halimeda* spp seaweeds have been described as having a relationship between antioxidant activity and antiatherogenic properties (Costa-Mugica et al., 2012). Additionally, it has been shown that *Halimeda* spp. has a high phenolic content (Vidal et al., 2009; Vidal et al., 2011), together with low amounts of other antioxidants, such as ascorbate, β-carotene, chlorophylls, and selenium, and that these compounds may be able to explain antioxidant properties.

Thus, in view of these previous considerations, the aim of this study was to evaluate the antioxidant properties of the hydrophilic fractions obtained from the green seaweed *Halimeda incrassata*, in free radical scavenging, and in animal models, and a possible relation to lipoprotein oxidation, and macrophage oxidative stress, as pathological stages of atherosclerosis.

MATERIALS AND METHODS

Seaweed collection and hydrophilic extract preparation

The seaweed *Halimeda incrassata* (Ellis) Lamouroux (Chlorophyta, Bryopsidales) was collected during December 2011 in the Bajo de Santa Ana, La Habana, Cuba. Voucher specimens were authenticated by Dr. A. M. Suárez from the Seaweeds Laboratory at the Marine Research Center of the University of Havana. Freshly collected specimens were washed with distilled water and dried at room temperature for 3-5 days. After milling and sieving, the dry powder was used to obtain the hydrophilic extracts. The dry seaweed powder was extracted with distilled water (1:5 w/v) at room temperature and centrifuged at 800 g and at 4ºC for 20 min. The supernatant was recovered, lyophilized, and kept at -20ºC until use. The weight yield of the final extract in terms of dry seaweed was 5%. The lyophilized material was dissolved in distilled water at known concentrations for the different studies. The lyophilized material was denominated as being an aqueous extract.

Polyphenolic rich fractions were obtained according to Krygier et al. (1982). Tetrahydrofurane extraction was performed on the dry seaweed in order to obtain a fraction rich in free phenolic acids (FPA). The weight yield of the final dry fraction in terms of dry seaweed was 0.8%.

Total phenolic concentration

The total phenolic content was determined by the Folin-Ciocalteau assay as previously described by Vidal et al. (2009) and expressed as µg of Gallic Acid Equivalents (GAE) /g of sample. The calibration curve was obtained in the range of 100-1000 µg gallic acid/ml.

DPPH$^{\bullet}$ radical scavenging assay

The free radical scavenging activity of the *Halimeda incrassata* extract was done similar to Goupy et al. (1999). Briefly, 0.6 ml of the extract (10 to 40 µg GAE) was mixed with 0.6 mL of a methanolic solution of DPPH$^{\bullet}$ (60 µM). Absorbance was measured in time. Radical scavenging activity was calculated relative to the reference absorption as a Percentage Inhibition (PI) (%) = $(1\text{-}A_{sample}/A_{reference}) \times 100$. IC_{50} was the antioxidant quantity needed (mg aqueous extract or fraction dry) to scavenge 50% of DPPH$^{\bullet}$.

Hydroxil scavenging and Fe^{3+} chelating capacity

Antioxidant protection in 2-desoxi-D-ribose oxidation by hydroxil radicals was quantified by the malondialdehyde formation according to Aruoma (1994). The reaction mix was 850 µL phosphate buffer (KH_2PO_4/KOH)

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10 mM, pH 7.4, with or without 50 µL EDTA 100 µM and 50 µl $FeCl_3$ 25 µM. After 1 min, 50 µl 2-desoxi-D-ribose 2.8 mM and H_2O_2 2.8 mM were added. Reaction was initiated with 50 µL 100 µM ascorbic acid and stopped after 1 h at 37°C. The degree of oxidation was assessed by a TBARS formation. Samples were incubated with 1 ml TBA 1%, 1mL TCA 2.8%, at 80°C for 20 min. Absorbance was monitored at 532 nm. Without EDTA, the assay indicated the Fe chelating capacity of the extract, whereas with EDTA, it measured the hydroxil radical scavenging activity.

Antioxidant activity protecting against liver damage in CCl_4 – induced Wistar rats

Animals and treatment schedule

Male Wistar rats from the University of São Paulo, Brazil, weighing 120 g–150 g, were maintained under a controlled diet, with cycles of 12 h of light/dark, at 25 °C and 60% humidity. The rats had free access to water and to a standard food diet according to the care guidelines for laboratory animals used in research. The animal studies were approved by the Institutional Ethical Committee for Animal Experimentation from the Faculty of Pharmaceutical Sciences (USP), Brazil.

Hepatic injury was induced in the rats by an intraperitoneal administration of a single dose of 3 mL CCl_4 (mixed 1:1 with olive oil) on day 21. Gallic Acid (GA) was used as a reference.
The animals were grouped as follows:

Group I: Control, treated daily with vehicle (1 ml, p.o.) for 21 days.
Group II: Treated daily with vehicle (1.0 mL, p.o.) for 21 days, followed by treatment with CCl_4.
Group III: Treated daily with an aqueous extract of *Halimeda incrassata* (300 mg/kg, p.o.) for 21 days, followed by treatment with CCl_4.
Group IV: Treated daily with ferulic acid (20 mg/kg, p.o.) for 21 days, followed by treatment with CCl_4.

At the end of the treatment (day 22), a blood sample and the liver of each animal was collected. ASAT and ALAT were determined by commercial laboratory kits (LABTEST).

TBARS assay

As a marker of lipid peroxidation, the TBARS contents were measured in the liver homogenates and serum of the animals using the method of Ohkawa et al. (1979). The results were expressed as nmol/mg protein.

Glutathione (GSH) analysis

The hepatic total of GSH content was measured using the method of Ellman (1959) as the change in absorbance was monitored at 410 nm for 5 min, and the GSH level was calculated by using pure GSH as standard.

Determination of superoxide dismutase (SOD) activity

The cytoplasmatic SOD activity was evaluated according to McCord and Fridovich (1969) by using 100 mM cytochrome C, 500 mM xanthine, 1 mM EDTA, and 200 mM KCN in 0.05 M potassium phosphate, pH 7.8. The xanthine oxidase (same volume in the blank) was placed in a glass tube along with 15 µl of the cytosolic fraction from each liver tissue. The results were expressed as U/mg protein. One unit (U) was the enzyme activity that induced 50% of inhibition of the xanthine reaction at 25 °C, pH 7.8.

Determination of catalase (CAT) activity

The activity was evaluated by the decomposition of hydrogen peroxide caused by the cytoplasmatic enzyme CAT according to Beutler (1975), through the decrement of the optic density at 230 nm (coefficient of the molar extinction 0.0071 mM^{-1} cm^{-1}) at 37 °C. One U of CAT corresponded to the enzyme activity that hydrolyzed 1 molecular weight of H_2O_2 per minute at 37 °C, pH 8.0. The activity was expressed as U/mg of protein.

Expression of hepatic enzymes in rats by RT/PCR

RNA Extraction: CAT and SOD gene evaluation

RNA was extracted from the rat liver utilizing a 100 mg sample and 1 mL of trizol reagent (Invitrogen). The extract was kept at room temperature for 5 min with the addition of 200 µl chloroform (Merck). The samples were mixed by vortexing for 15 s and kept at room temperature for 5 min. After this, they were centrifuged at 12,000 × *g* for 15 min at 4 °C. 400 µl of the supernatant was removed, avoiding the interphase, and mixed with 500 µl of isopropanol by vortexing for 5 s. These samples were then centrifuged at 12,000 × *g* for 5 min at 4 °C, discarding the supernatant. To the resulting pellet, 1000 µl of ethanol (75%) was added and gently mixed, followed by centrifugation at 7500 × *g* for 10 min at 4°C, discarding the supernatant. Finally, 20 µl of distilled water, RNAse-free, was added and incubated at 50 °C for 10 min. This material was then stored at -70°C.

Reverse transcription

2 µg of RNA were added to 1.0 µl of primers (SOD or CAT), 1.0 µl of 10 mM dNTP, and 4.0 µL of sterile distilled water. The reaction was started by heating at 65°C for 5 min; then it was quickly chilled on ice. Next, 4.0 µl of 5× first strand buffer (Invitrogen), 2.0 µl of 0.1 M DTT (Invitrogen), and 1.0 µl of RNAseOut Ribonuclease inhibitor (Invitrogen) were added and incubated at 37°C for 2 min. After that, 1.0 µl (200 U) of reverse transcriptase (M-MLV RT-Invitrogen) was added and incubated at 37°C for 50 min. The reaction was stopped by heating at 70 °C for 15 min. The PCR product (cDNA) was stored at -70°C.

PCR reaction for amplification

5 µl of cDNA was amplified in a volume of 50 µl containing 5 µl 20 mM Tris-HCl (hydroxymethyl aminomethane-hydrochloride) buffer, pH 8.4, 500 mM KCl, 1.5 µl 50 mM $MgCl_2$, 1 µl 10 mM dNTP, 35.1 µl diethyl pyrocarbonate (DEPEC), 1.0 µL of primers (SOD or CAT), and 0.4 µL (5 U/µl) Taq polymerase. This reaction mixture was warmed by a thermal cycler (Bio-Rad) at 94°C for 3 min and 35 cycles of 45 s at 94°C, 30 s at 55°C, 1.3 min at 72°C, and 72°C for 10 min. Finally, the mixture was cooled at 4°C for an indeterminate time. The PCR amplified product was analyzed by a 2.0% agarose gel (Sigma) electrophoretic run (60 V). The bands, stained with 0.5 µg/mL ethidium bromide, were documented by a fluorescent table (Vilber-Lourmat) and photographed by a digital camera (Sony). The bands were revealed with –262 bp (C to T) from the primers used to CAT genotyping and +242 bp (C to T) from the primers used to SOD genotyping (Promega, Madison/USA):

Primer SOD 1 – sequence (5' to 3'): TCT AAG AAA CAT GGC GGT CC.
Primer SOD 2 – sequence (5' to 3'): CAG TTA GCA GGC CAGCAG AT.
Primer CAT 1 – sequence (5' to 3'): GCG AAT GGA GAG GCA GTG TAC.
Primer CAT 2 – sequence (5' to 3'): GAG TGA CGT TGT CTT CAT TAG CAC TG.

Effect of hydrophilic fractions on the inhibition of LDL oxidation mediated by Cu^{2+} and HRP/H_2O_2

Oxidation experiments were conducted with heparin precipitated LDL (hep-LDL), a model of LDL that has interacted with extracellular matrix, and is, therefore, more prone to oxidation (Upritchard and Sutherland, 1999). Lipoproteins were isolated from normolipemic human serum by the method of Wieland and Seidel (Wieland and Seidel, 1983). 5 ml sodium citrate buffer (64 mmol/L, pH 5.12) containing heparin (50 000 UI/L) was added to 0.5 mL serum. After incubating for 10 min at room temperature the sample was centrifuged at 3000 rpm for 15 min. The Hep-LDL precipitate was washed 3 times with a Hepes buffer (5 mM Hepes, 20 mM NaCl, 4 mM $CaCl_2$ and 2 mM $MgCl_2$, pH 7.2), then by centrifuging at 3000 rpm for 15 min; the hep-LDL was was next dissolved in a 0.5 mL phosphate buffer, pH 7.4, with NaCl 4%. The Hep-LDL fraction was next divided into aliquots and kept at 4°C. The cholesterol content was determined by an enzymatic assay (Boehringer Mannhein Diagnostics) and the protein content by the Lowry method. In brief, for the oxidation, LDL (0.2 µmol cholesterol) was diluted in a phosphate buffer and incubated in the presence or absence, of hydrophilic fractions (aqueous extract and FPA fraction) for 6 h at 37°C, with 10 µM Cu^{2+} , or HRP (119 U)/H_2O_2 (12.9 µM). The maximum degree of oxidation was determined by TBARS as described in Frostegard et al. (1990) and expressed as nmoles MDA equivalents/ mg protein, using TMP as standard.

Antioxidant activity of hydrophilic extracts in macrophages

Cell experiments were done with the macrophage RAW 264.7 cell line. Cells were cultured in DMEM containing fetal bovine serum (FBS), 2 mM L-glutamine and streptomycin/penicillin in 5% CO_2.

TBARS formation by cells

For the assessment of antioxidant activity, cells were pre-incubated for 24 h with an aqueous extract of *Halimeda Incrassata*. Lipoperoxidation levels were evaluated in the supernatant by a TBARS assay as in Frostegard et al. (2003).

ROS production

ROS production by cells was determined in conditions similar to Kopprasch et al. (2008). Luminol 4 µM was added to cells in a 50 mM Hepes buffered DMEM. After adding seaweed, aqueous extracts cells were stimulated with opsonized zymosan (OZ) 1 mg/mL. Chimioluminiscent response was measured in time, and amplitude of the curve was taken as maximum ROS production. Experiments were done with a Lumi-Aggregometer from Chrono-Log Corporation with AGGRO/LINK software version 5.2.3.

Statistical analysis

Values are given as mean ± standard deviation (s.d.) of experiments that were done in triplicate, and performed at least two independent times. In studies of the antioxidant activity in the cell systems, statistical significance was determined by ANOVA with a Tukey posttest. Significant differences were concluded for $p < 0.05$. Data were processed using Microcal Origin and GraphPad Prism software.

RESULTS AND DISCUSSION

Over the last few years, seaweeds have been widely investigated as a source of bioactive compounds, with different attributes, and in this context, the genus *Halimeda* has been studied for different pharmacological properties, including antioxidant activity (Moo-Puc et al., 2008; Nor et al., 2010).

Phenolic content

The phenolic content, as assessed in the Aqueous Extract and by the Fraction Phenolic Acids (FPA), was 0.13 ± 0.05 and 0.47 ± 0.09 mg Gallic Acid Equivalents (GAE)/ g dry seaweed, respectively. Both fractions had a high phenolic content.

The phenolic content found is in the range of the one for seaweeds of *Halimeda* spp. worked by our research group (12-13) and higher than for other seaweeds informed in the literature like *Fucus vesiculosus* (Phaeophyceae) and *Caulerpa racemosa* (Chlorophyta) (Jimenez-Escrig et al., 2001).

The phenolic contribution to antioxidant activity was evaluated in the hydrophilic fractions. When comparing different fractions from the seaweed *Eisenia bicyclis* (Phaeophyceae), Kim et al. (2011) found that the highest phenolic content was associated with the antioxidant activity and the hepatoprotective effect, against tert-butyl hyperoxide damage (t-BHP) in hydrophilic fractions from the seaweed.

In our previous work, Vidal et al. (2009) identified 8 phenolic acids in *Halimeda opuntia* and *H. monile* (Chlrophyta) respectively. They reported that salicylic, cinnamic, gallic, pirogalic and cafeic acids were the principal polyphenolic compounds in both seaweeds. In *Halimeda incrassata*, it was identified that there were major polyphenolicqq compounds of salicylic and ferulic acids, and they suggested that their levels were related to the antioxidant activity of the seaweed (Vidal et al., 2011).

Likewise, the antiatherogenic activity of phenolic compounds has been studied when considering their antioxidant properties as being the main mechanism of action (Bocanegra et al., 2009; Jimenez-Escrig et al., 2001)

Antioxidant activity in vitro: $DPPH^{\bullet}$ radical scavenging and hydroxyl scavenging and Fe^{3+} chelating capacity

$DPPH^{\bullet}$ radical scavenging was determined for the Aqueous Extract and FPA. Both fractions exhibited a concentration dependent on free radical scavenging activity (Figure 1). The FPA fraction had an IC_{50} value of 0.46 mg of dry residue (27.1 µg of polyphenol); while the Aqueous Extract had an IC_{50} value of 5.75 mg of lyophilized substance (14.8 µg of polyphenol).

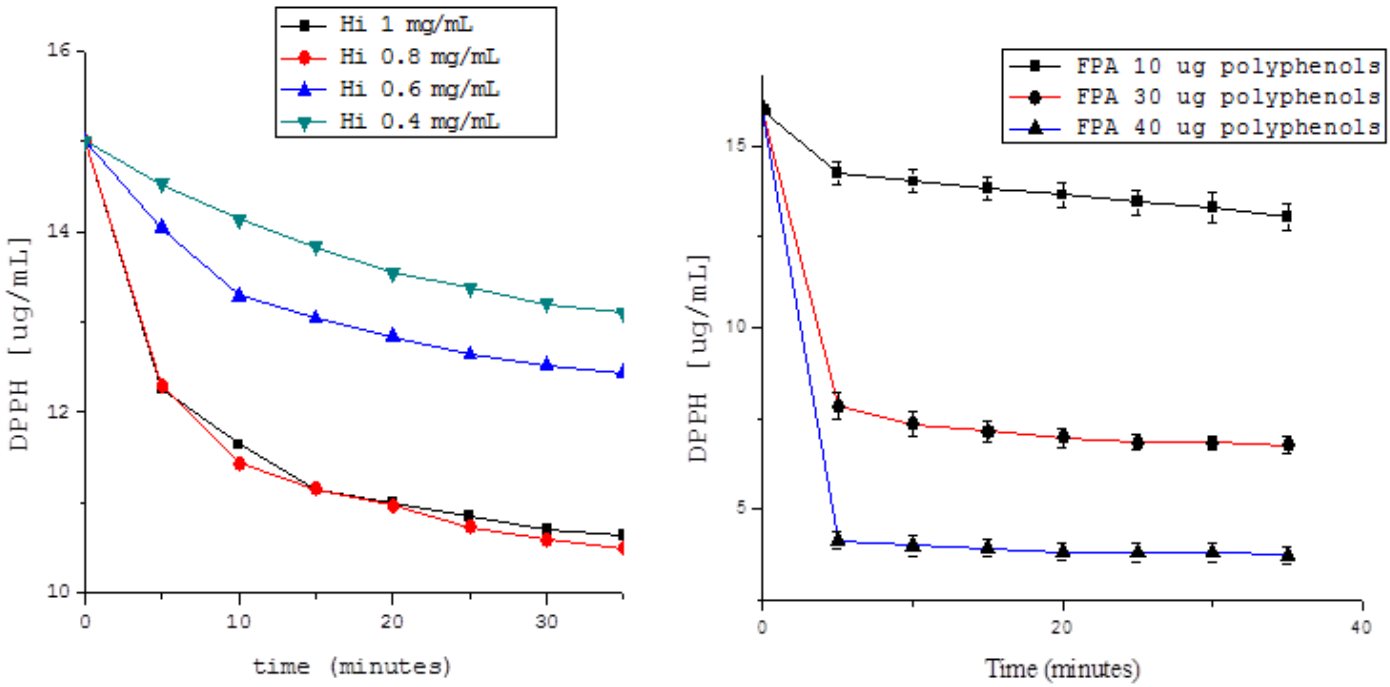

Figure 1. Scavenging activity of the DPPH• radical by hydrophilic fractions of *H. incrassate.* **(A)**: Scavenging of DPPH• radicals by aqueous extracts in time. **(B)**: Scavenging of DPPH• radicals by a free phenolic acid fraction (FPA) in time. The assay was performed according to Goupy et al. (1999). Values are given as a mean ± S.D. (n = 3). After incubating, increasing extract concentration (0.4-1 mg for aqueous extract or 10-40 µg total polyphenols for FPA fraction) with DPPH• solution, absorbance was measured at λ = 517 nm in time.

DPPH• radical scavenging has been widely used to study the activity of antioxidant molecules and plant extracts. It is considered, that presently, more than 90% of antioxidant studies use this method in combination with other assays (Goupy et al., 1999). In this work, free radical scavenging by DPPH• assay indicated a dose-dependent effect, and in comparison with *Halimeda incrassata*, a six-fold lower activity for *Caulerpa racemosa*, a more than 30-fold decrement in *Ulva lactuca*, and a similar activity in *Sargasum* spp.(Yangthong et al., 2009).

Antioxidant activity by DPPH suggests phenolic compounds are relevant to the effect. Different authors (Dutra-Rocha et al., 2007) have indicated an association between the phenolic content of seaweeds and DPPH• scavenging. Previous results from our group, regarding *Halimeda* genus, have indicated an association of antioxidant activity in DPPH scavenging with phenolic content (Vidal et al., 2009; 2011). Indeed, Katsube et al. (2004) found a tendency of higher antioxidant activity in the inhibition of LDL oxidation, and of DPPH• scavenging in plants with an increasing phenolic content. Hydroxyl radicals are a main initiator of lipid peroxidation. Thereafter, hydroxyl radical scavenging is a relevant indicator of the antioxidant activity of a natural compound, and in this area, plant polyphenols are compounds of interest, as they can react with these radicals to avoid oxidative damage (Stocker and Keaney, 2004).

Aqueous seaweed extract inhibited desoxiribose oxidation with a dose-dependent effect both in the presence of EDTA (Figure 2A) (IC_{50} = 1.91± 0.09 mg/mL), or in absence of EDTA (Figure 2B) (IC_{50} = 2.95 ± 0.01 mg/mL Fe chelating). Additionally, aqueous extracts had a concentration-dependent antioxidant effect, both with and without, EDTA, which is stronger than the one referred to for various antioxidant extracts (Vidal et al., 2006).

Antioxidants in *Halimeda incrassata* seaweed might offer protection from damage, by avoiding an attack by OH• radicals, generated by a Fenton reaction in the presence of EDTA. On the other hand, when Fe^{3+} is added to the reaction milieu (in the absence of EDTA), some ions might join desoxiribose sugar and take part in the Fenton reaction. Antioxidant activity in this assay would then show the Fe^{3+} chelating capacity of the extracts. Thus, our results also indicated that the extracts have the capacity to interfere with the site specific generation of OH•, catalyzed by Fe^{3+} ions, bound to desoxiribose. Indeed, other authors have remarked that the presence of phenolic compounds in seaweeds

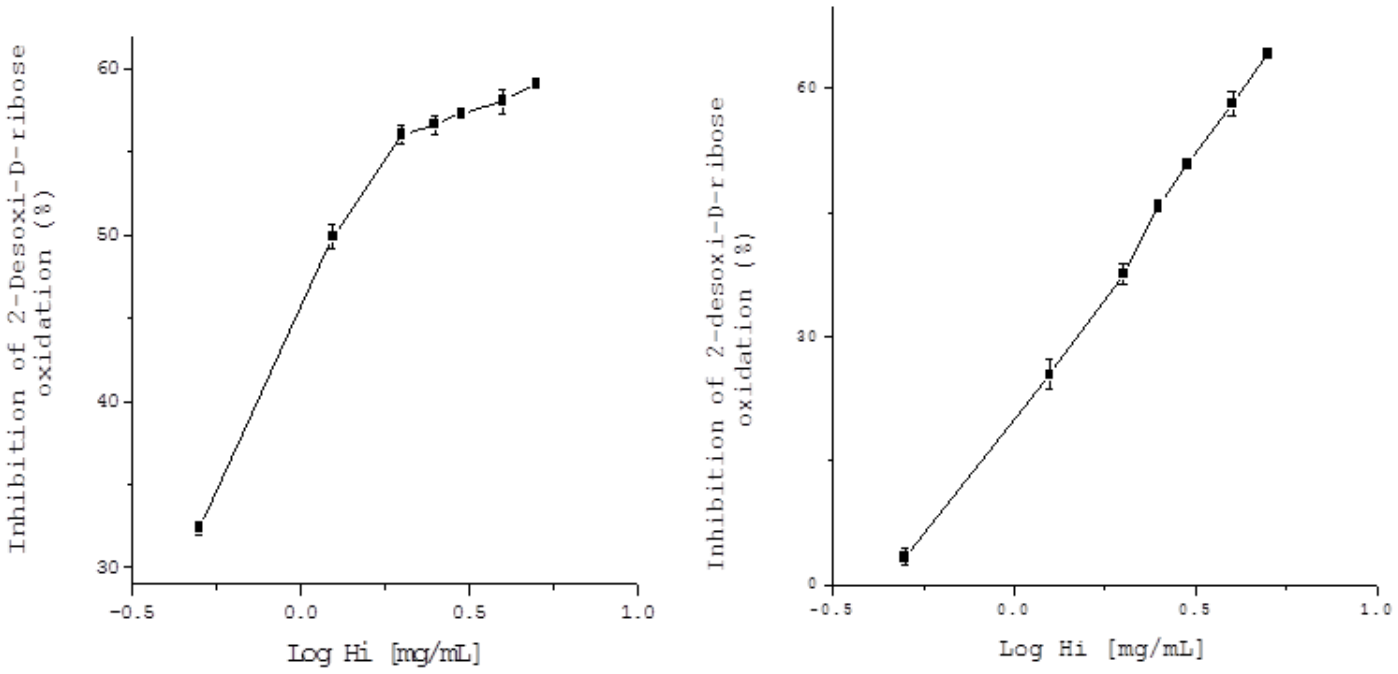

Figure 2. Inhibition of the oxidative degradation of 2-Desoxi-D-ribose by the aqueous extract of *Halimeda incrassata* (A) with EDTA, (B) without EDTA. The assay was performed as described by Aruoma (1994). Oxidation of desoxiribose was done in the presence, or absence of, increasing concentrations of *Halimeda incrassata*. Values are given as a mean ± S.D. (n=3).

confers them with a heavy metal chelating capacity, which is also related to $OH^{\cdot}$ radical scavenging (Vidal et al., 2006; Yuan and Walsh, 2006).

Antioxidant activity protecting against liver damage in CCl $_4$-induced Wistar rats

Antioxidant and hepatoprotective properties of the aqueous extract from *H. incrassata* were investigated in Wistar rats with a CCl_4-induced liver injury. Through the ASAT and ALAT activities (Table 1), it verified partial liver injuries caused by CCl_4. We observed that the rats treated with an aqueous extract from *H. incrassate*, or Ferulic acid, proved to be capable of attenuating the toxic effect produced by CCl_4. As will be appreciated, it was observed that a partial recuperation occurred with an aqueous extract from *H. incrassata*. In previous work, Mancini et al. (2009) reported similar results when investigating polyphenol fractions from *Halimeda monile*. These results are also in accordance with de Oliveira et al. (2012) whom investigated the hepatoprotective properties of polyphenolic fractions from *H. opuntia* under an experimental model CCl_4 injury. Tanigushi et al. (Tanigushi et al., 2004) reported that hepatic damage by CCl_4 may occur in a range of between 6 to 12 h after CCl_4 administration, and that restoration starts after 48 h. It is possible then to suppose that FPA- *H. incrassata*-treated rats had a faster recovery from liver injury at 24 h than expected, criterion that is concordant with our results.

In Table 1, it may be appreciated that TBARS levels in the liver tissues of CCl_4-treated rats increased, confirming the successful induction of oxidative damage, while pre-treatment with *H. incrassata* (300 mg/kg) led to an approximate 50% reduction in liver TBARS levels. A previous study from our laboratory showed that an aqueous extract from *H. incrassata* was effective in significantly reducing serum and brain TBARS levels and other parameters in rats with oxidative stress induced by methyl-mercury (2004). According to de Oliveira e Silva et al. (2012), a pre-treatment with polyphenol rich fractions from *Halimeda opuntia* led to reductions in serum and liver TBARS. Kim et al. (2011) also observed a comparable reduction in hydroperoxide levels in the liver, relative to the CCl_4-treated group, in a study of rats fed on Saengshik, a non-cooked food containing vegetables and seaweeds.

Animals with liver injuries caused by CCl_4 had GSH levels increased statistically in respect of all groups, while in an aqueous extract from the *H. incrassata* group was observed with only minor values. According to Chan et al.

Table 1. Values of parameters and enzymes in serum and liver tissue relative to oxidative stress.

GROUPS	Serum		Liver			
	AST	ALT	TBARS	GSH	CAT	SOD
	U/mL	U/mL	nmol/mg protein	µmol	U/mg protein	U/mg protein
Control	84.36 ± 0.61^{bc}	57.68 ± 2.70^{b}	0.19 ± 0.02^{b}	0.10 ± 0.02^{c}	219.91 ± 23.11^{a}	26.61 ± 2.71^{b}
CCl_4	116.30 ± 11.68^{a}	90.87 ± 20.08^{a}	0.87 ± 0.21^{a}	0.65 ± 0.11^{a}	144.24 ± 32.46^{b}	29.76 ± 3.85^{ab}
H. incrassata 300	99.86 ± 10.18^{ac}	67.76 ± 11.69^{ab}	0.43 ± 0.12^{b}	0.42 ± 0.10^{bd}	173.90 ± 23.86^{ab}	32.25 ± 1.40^{a}
Ferulic acid 20	112.25 ± 10.22^{a}	78.59 ± 13.11^{ab}	0.56 ± 0.29^{ab}	0.55 ± 0.12^{ad}	154.18 ± 40.77^{b}	33.22 ± 3.73^{a}

SD. Different letters indicate statistically significant differences, *p < 0.05.

(Chan et al., 2001), these increased levels may be explained as an adaptive response of the rats reacting against the oxidative stress introduced by CCl_4.

The CAT and SOD enzymes are considered to be as a fundamental antioxidant defense system in mammals, and it was demonstrated that CCl_4 treatment significantly reduced the activities of these enzymes. In this study, we observed the ability of CCl_4 to diminish the antioxidant enzyme activities.

As may be appreciated in Figure 3, treatment with the seaweed led to a significant increase in the activity of the CAT enzyme, which in turn resulted in an enhanced antioxidant defense. These results suggest antioxidant and hepatoprotective activities of the phenolic fraction of *H. incrassata*. Ozturk et al. (2003) observed in the CCl_4-treated group significant increases in kidney CAT activity. These results are in agreement with Mancini-Filho et al. (2009) that reported a considerable increase in the activity of CAT in rats treated with a polyphenol-rich fraction similar to that from *Halimeda monile.* High antioxidant enzyme activity has been reported through repeated administration of *Sargassum* spp. (Phaeophyceae) extracts (Raghavendran et al., 2005). Treatment with *Caulerpa prolifera* (Chlorophyta) and *Laurencia obtusata* (Rhodophyta) extracts also led to a rise in enzyme activity (Abdel-Wahhab et al., 2006).

Expression of CAT hepatic enzymes by PCR- RT

When considering results from antioxidant enzyme activities, only the expression of Catalase by PCR-RT technique was studied. As can be seen in Figure 4, the levels of CAT in liver tissues partially increased with a treatment of seaweed and a posterior CCl_4 administration, which shows alterations in the expression of catalase genes.

Treatment with *H. incrassata* aqueous extract (band 3) resulted in a higher catalase gene expression when compared with that observed in the CCl_4-treated group (band 2). A review by Stevenson and Hurst (2007) discusses recent evidence that polyphenols also have an indirect antioxidant effect through the induction of endogenous protective enzymes, and that these inductive or signaling effects may occur at concentrations much lower than those required for effective radical scavenging. Vidal et al. (2011) reported that a total phenolic contents of the hydrophilic fractions from *H. incrassata* were 255 µg of gallic acid equivalents/g of fresh seaweed, which more than half (63%) corresponds to free phenolic acids, and in this fraction, about 32% was identified as salicylic acid, while a small fraction was associated to ferulic acid. Yeh and Yen (2006) suggested that these three phenolic acids, including ferulic acid, modulate the phase II antioxidant enzymes and the phase II sulphate conjugative enzymes; and they seem to selectively induce hepatic mRNA transcripts for CAT, probably through the up-regulation of gene transcription, as well as the Nrf2 transcription factor. In previous results from our group, Mancini-Filho et al. (2009) reported an over-expression of CAT genes by treatment with FPA from *Halimeda monile*; while de Oliveira e Silva et al. (2012) showed that by using (RT/PCR) analysis increased the catalase (CAT) gene expression in the group treated with free phenolic acid (FPA) fractions from *Halimeda opuntia*, suggesting inducing effects on the enzyme.

Effect of Halimeda incrassata hydrophilic fractions on the inhibition of LDL oxidation mediated by Cu^{2+} and HRP/H_2O_2

The atheroprotective potential of *Halimeda incrassata* was determined through its effect on LDL oxidation. The inhibition of LDL oxidation by Cu^{2+}, or HRP/H_2O_2, was dose dependent, with a higher concentration needed for the aqueous extract than for the FPA fraction (Table 2). It was compared with the two oxidation systems relevant to LDL oxidation in the artery wall: by Cu^{2+}, or HRP/H_2O_2; and by studying the antioxidant effect in the mediated transition metal and independent LDL oxidation (Stocker

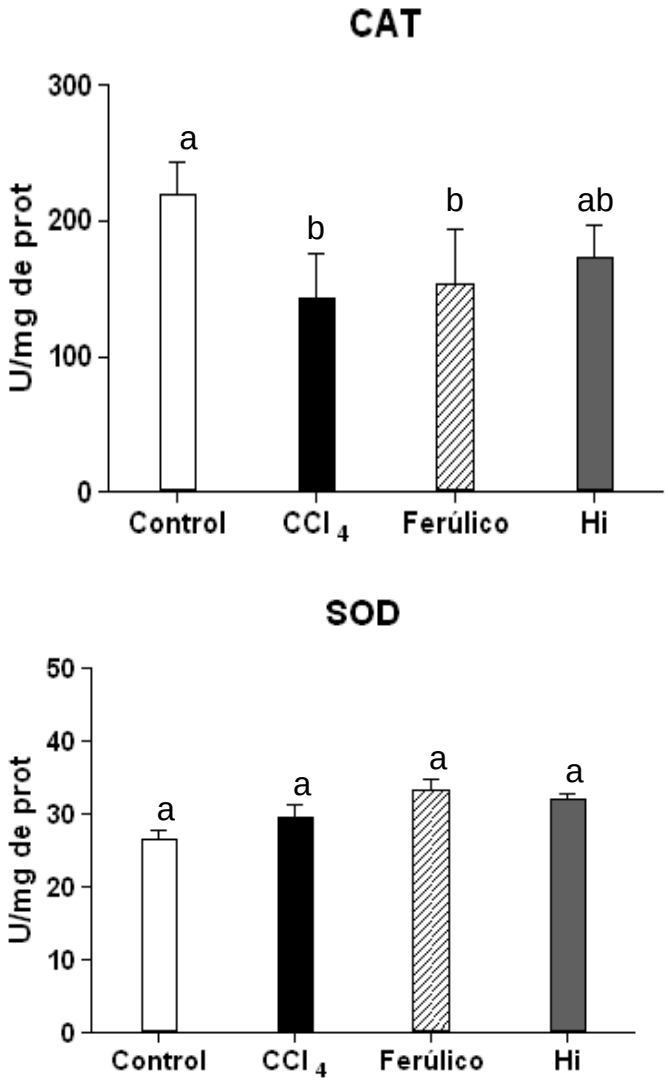

Figure 3. Activity of (A) SOD and (B) CAT liver tissues from control, CCl4-treated, Ferulic acid-treated, and *Halimeda incrassata*-treated rats. Different letters indicate statistically significant differences, *p < 0.05.

and Keaney, 2004). As shown, seaweed hydrophilic fractions had an inhibitory effect on both models of LDL oxidation, indicating its potential in atheroprotection.

The inhibition of LDL oxidation is considered a key target in atherosclerosis management, since oxidized LDL levels are presently one of the main emerging factors for cardiovascular risk (Kang et al., 2003; Levitan et al., 2010). *In vivo* is a complex process, and there is no certainty as to the precise mechanism of the initiation of lipoperoxidation in the vascular wall (2004). In the quest for the atheroprotective potential of natural compounds, different researchers have determined the antioxidant properties of seaweed extracts in the inhibition of LDL oxidation (Bocanegra et al., 2009; Jimenez-Escrig et al., 2001; Yang et al., 2011).

The activity of the inhibition of LDL-oxidation found in this work is promissory when compared to other natural extracts. Hseu et al. (2008) found 37% inhibition of TBARS formation in oxidation mediated by AAPH and 74% in peroxidation mediated by Cu^{2+} ions. In this study, the *Toona sinensis* extracts had 6.5 µg GAE, a phenolic content in the range of the one needed in our study for the 50% inhibition of TBARS formation by *Halimeda incrassata* extracts.

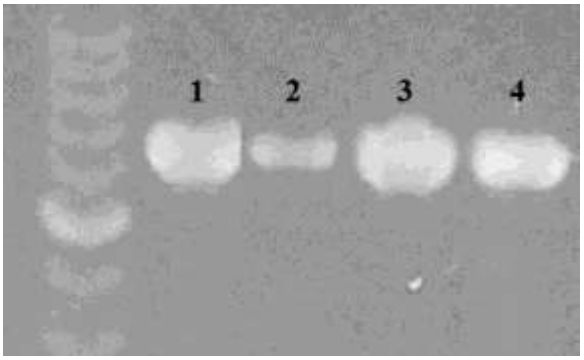

Figure 4. Electrophoresis of the expression of catalase hepatic enzyme in rats by RT/PCR. Experimental groups: Bands: (1) Control; (2) CCl_4; (3) *H. incrassata* 300 mg/Kg; (4) Ferulic acid 20 mg/Kg.

Table 2. Inhibitory effect of *H. incrassata* hydrophilic fractions on LDL oxidation induced by Cu^{2+} ions or peroxidase (HRP/H_2O_2), with or without increasing seaweed extract concentration. TBARS formation was determined according to Frostegard et al. (1990).

Fractions	50% inhibitory concentration (IC_{50}) of *H. incrassata*	
	Cu^{2+}	HRP
Aqueous extract (mg)	10.42 ± 0.295	1.09 ± 0.005
FPA (mg)	0.33 ± 0.04	0.66 ± 0.02

LDL oxidation studies were done with LDL that had interacted with heparin as a model of glycosaminoglycan since it has been reported that lipoproteins that have interacted with glycosaminoglycans are more susceptible to oxidation (Upritchard and Sutherland, 1999). In the case of peroxidase, this is associated with a change in the lipoprotein structure, giving an increased access of peroxidase to apo B, and forming free radicals from apo B and vitamin E that would mediate the oxidation of lipids by mieloperoxidase and HRP. The mechanism is different from Cu^{2+} mediated oxidation, where free radical formation and peroxidation takes place directly in the lipid phase.

Other authors have evaluated the effect of natural extracts in the inhibition of LDL oxidation by peroxidases. Wang et al. (2003) found a significant decrement in TBARS formation in extracts from *C. mukul* on LDL oxidation mediated by lypoxigenases. The extracts also had an antiatherogenic action in the inhibition of cholesterol uptake by macrophages and LDL oxidation mediated by Cu^{2+} ions.

Few studies approach the effect of antioxidants in LDL oxidation mediated by HRP. It has been shown that in this area, vitamin E acts by transferring radicals, from the aqueous to the lipid phase, and does not protect it from oxidation. However, in the presence of vitamin C, oxidation mediated by peroxidases is inhibited, as in this case, vitamin C acts as a co-antioxidant avoiding the formation of α-tocoferoxil (Upritchard and Sutherland, 1999). Other antioxidants, like some phenolic hydrophilic compounds, act synergistically in the scavenging of free radicals protecting vitamin E, licopene and β-carotene, contained in LDL from oxidation (Kaliora et al., 2006). The antilipoperoxidative activity of the hydrophilic fractions of *Halimeda incrassate*, in this model, indicates that the extracts are efficient in the inhibition of oxidation mediated by protein radicals, and add evidence to the antiatherogenic and antioxidant potential.

Other authors have obtained an excellent activity of antilipoperoxidation for *Halimeda incrassata* in β-carotene linoleate systems, with an inhibition of bleaching of 75% for *Halimeda incrassate*, while for *Bryothamnion triquetrum* seaweed , the activity was 20% at a dose of 2 mg (Rivero et al., 2003; Vidal et al., 2011).

Our results are also comparable to the work of Yuan and Walsh (2006) with a significant inhibition of conjugated dienes and TBARS formation, during the oxidation of linoleic acid for an aqueous extract from the *Palmaria palmate* seaweed, suggesting that the antioxidant activity was due to the complex mixture of

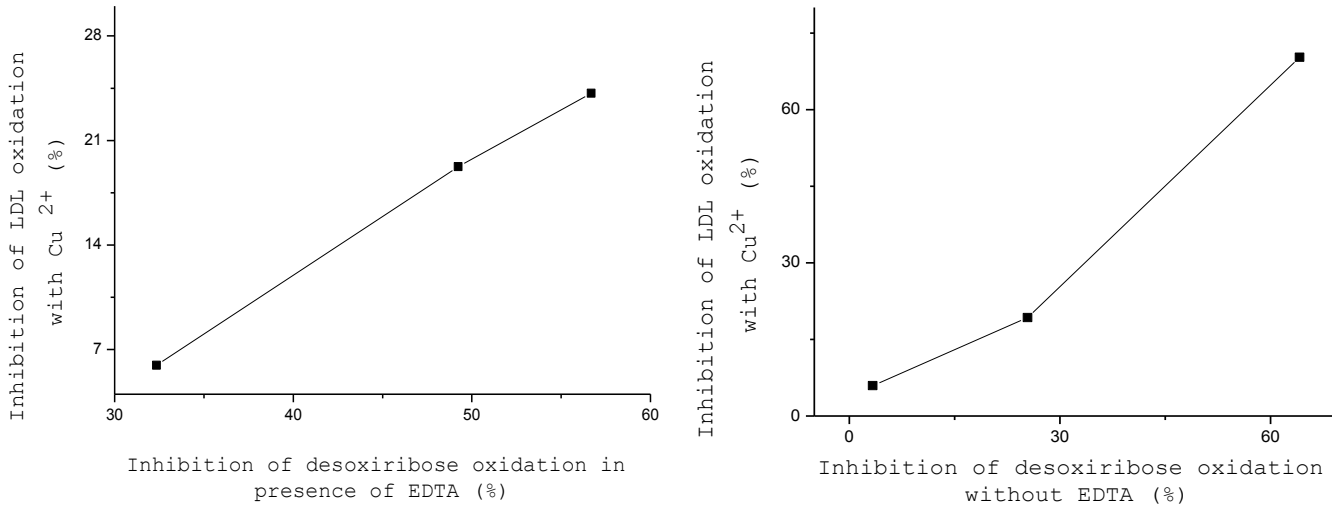

Figure 5. Correlation of antioxidant activity by different methodologies. A. Association of antioxidant activity in $OH^{\cdot}$ radical scavenging and the inhibition of LDL oxidation mediated by Cu^{2+} ions (r^2= 0.997). B. Association of antioxidant activity in Fe ion chelation and the inhibition of oxidation of LDL mediated by Cu^{2+} (r2= 0.943).

antioxidants present in the extract, with the presence of chlorophyll, polyphenols, carotene, and ascorbate.

Association of antioxidant activity in Fe Ions chelation and the inhibition of oxidation of LDL mediated by Cu^{2+}

The association of antioxidant activity and the inhibition of LDL-oxidation, evaluated by different methodologies in this paper, were performed to explain the antioxidant mechanisms of *Halimeda incrassata*. Figure 5A and B indicate the positive correlation between the inhibition of desoxiribose oxidation, with or without EDTA, and the inhibition of LDL oxidation mediated by Cu^{2+} (r^2= 0.997 and 0.943 respectively).

In this work, a positive correlation was found between the scavenging of $OH^{\bullet}$ radicals in the inhibition of the desoxiribose assay in the presence of EDTA (r^2 = 0.997), and the inhibition of LDL oxidation mediated by Cu^{2+}. A similar behavior was obtained for the chelating effect of metal ions in the inhibition of desoxiribose oxidation assay in the absence of EDTA (r^2 = 0.943) and the inhibition of LDL oxidation mediated by Cu^{2+}.

The results of the correlation of antioxidant activity in Fe ion chelation and the inhibition of oxidation of LDL mediated by Cu^{2+} suggest that the mechanism of action in the inhibition of LDL oxidation by hydrophilic fractions could be associated with the antioxidant properties of the seaweed in metal ion chelating and free radical scavenging.

Antioxidant activity of hydrophilic extracts of Halimeda incrassata in macrophages

As shown in Figure 6A, the degree of cell lipoperoxidation was decreased by about two-fold in the presence of *Halimeda incrassata* seaweed extract. The FPA fraction also inhibited TBARS production, indicating the contribution of phenolic compounds to the effect.

The improvement of antioxidant activity was also shown by the 1.2 fold decrement in ROS levels by the treatment with *Halimeda incrassata* aqueous extract, when aqueous extracts were added immediately before stimulation (Figure 6A), or by a nearly 50% decrement in ROS levels, when aqueous extracts were added 24 h before stimulation of the cells (Figure 6B).

The macrophages associated with oxidative stress are directly involved in the atherosclerosis progression, so when evaluating compounds of interest for atheroprotection antioxidant activity, it is frequently sought at the macrophage level (Kaliora et al., 2006).

A decreased peroxide basal content was found in the cell supernatant as a result of the preincubation with seaweed hydrophilic extracts. About a 3.3 fold higher concentration was required from the FPA fraction for it to reach a similar inhibition of MDA formation as to that of1.5 µg GAE/mL (0.5 mg/mL) for the aqueous extract. To evoke ROS production in macrophages, opsonized zymosan was added to the cells after the addition of aqueous seaweed extracts. About a 20% decrement in ROS production was found with the highest concentrations of seaweed used in the assay. Seaweed

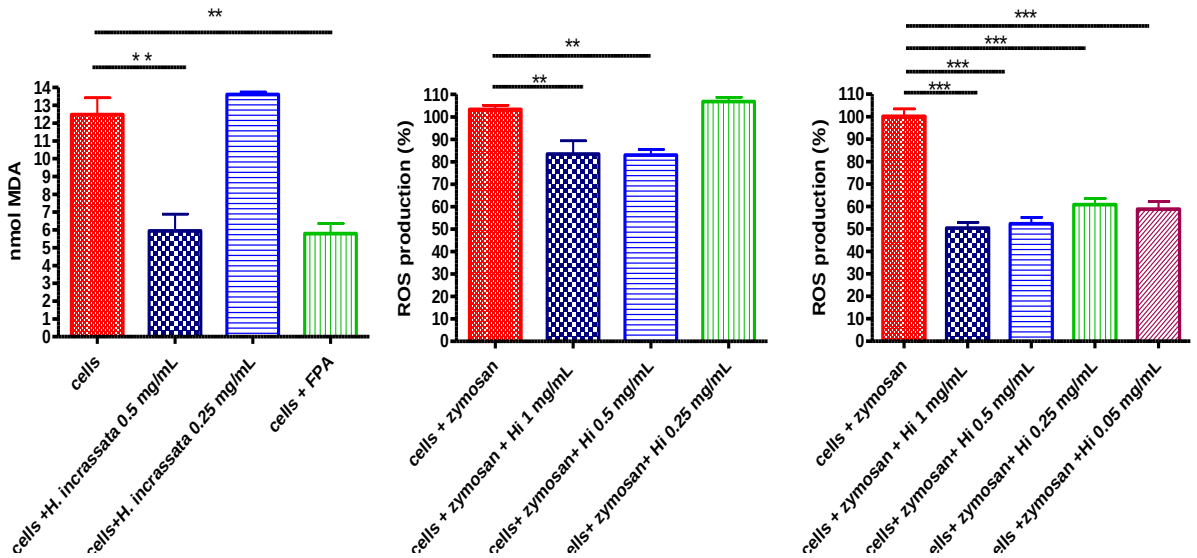

Figure 6. Antioxidant activity of hydrophilic extracts of *Halimeda incrassata* in macrophages. (A). TBARS levels in cell supernatant were determined as in Frostegard et al., 1990. (B) and (C) ROS production by cells after stimulation with zymosan was determined by luminol chemiluminiscence as in Kopprasch et al., 2003. Experiments were performed by adding aqueous extract immediately before cell stimulation (B) or by preincubating cells with extracts for 24 hours before stimulation (C). Significant differences were determined by ANOVA with a Tukey post test, ** $p < 0.001$.

preincubation with the cells improved the antioxidant capacity with a 50% decrement in ROS production with the highest concentration tested (1 mg/mL).

These results are in agreement with previous studies by our group for the aqueous extracts of *Halimeda incrassata* (Chlorophyta) and *Bryothamnion triquetrum* (Rhodophyta) seaweeds, and where basal and H_2O_2 elicited peroxides were decreased in the GT1-7 hypothalamic cells in the presence of the seaweeds (Fallarero et al., 2003).

Indeed, the antioxidant activity of natural extracts in macrophages has also been assessed by other authors that have found a decreased macrophage peroxidation and ROS production by incubation with phenolic rich extracts. Yang et al. (2011) found a 1.5% decrement in the peroxide levels in oxLDL stimulated macrophages, after preincubation with 1mg/mL mulberry leaf extracts (where quercetin, gallocatechin gallate and naringenin were the main polyphenolic constituents identified). The atheroprotective effect was related to an elevation of antioxidant enzymes GPx and SOD by the extracts.

Likewise, a significant inhibition of peroxidation was found in cells stimulated with oxLDL after preincubation with anthocyanin rich purple sweet potato extracts (0.5-0.6 mg/mL); that also inhibited the LDL uptake by macrophages and had antioxidant activity in DPPH$^{\bullet}$ scavenging (Park et al., 2010).

The antioxidant activity in macrophages has been studied by other groups that have found improved *in vitro* and *in vivo* antioxidant capacity, as a result of different phenolic rich extract supplementation (Aviram et al., 2008). Targeting oxidative stress as a cause of atherosclerosis progression, they have correlated phenolic content, *in vitro* antioxidant activity in DPPH$^{\bullet}$ radical scavenging, and the atheroprotective effect in macrophages (evaluated as the inhibition of LDL uptake, decreased macrophage peroxidation, and increased antioxidant enzyme activity) to be *in vivo* atheroprotection in apo E-/- mice.

Conclusion

In this work, the antioxidant activity in *in vitro* cell free systems and cell systems for the hydrophilic fractions from *Halimeda incrassata* was evaluated. Previously, it identified phenolic acids in hydrophilic fractions as being the main active components (salicylic and ferulic acid). It also tested the phenolic contribution to the antioxidant activity for a FPA fraction. A significant antioxidant activity was found in both cell free and cell systems. However, a higher concentration of FPA fraction was required to have a similar effect as for the one with the aqueous extracts. It is then possible to suppose that phenolic acids in the extract to be of relevance for the activity, since both ferulic and salicylic acids have been found to have antioxidant and antinflammatory properties that decrease the macrophage activation. Additionally, different authors

have shown a synergic effect in the antioxidant and antiatherogenic properties from seaweed crude extract, and this improved antioxidant activity could also be related to the synergism between the polyphenolic compounds and/or with other antioxidants. *Halimeda* spp. contains antioxidants like ascorbate, β-carotene, chlorophylls, among other compounds that might contribute to the effect.

In summary, this study adds further evidence about the beneficial properties of *Halimeda incrassata* as an antioxidant and as an antiatherogenic for a future phytotherapeutic application.

Conflict of Interests

The authors have not declared any conflict of interests.

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Determination of heavy metals in the roasted and ground coffee beans and brew

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Some compounds present in coffee beans can affect consumer health. The present study determines the content of heavy metal in coffee cultivated in the Cerrado Mineiro region (Alto Paranaíba – MG, Brazil), to compare the values found with the legal standards and check how these metals are extracted from the respective infusions. Fifty samples of coffee beans were analyzed, taken from the Alto Paranaíba region, MG, Brazil. Determination and quantification were done by recording the values from the atomic absorption spectrophotometer for the metals mentioned: cadmium (Cd), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), lead (Pb), and zinc (Zn). The Cr concentrations presented earlier the limit allowed by law in 66% of the coffee samples. And 74% of the samples contained Pb in higher than permissible concentrations. For all the infusions, the metals evaluated were found in lower concentrations and were less significant with respect to the maximum permissible daily intake, except for Pb were quantified very high levels. Only seven of the 50 coffee samples revealed results with levels that were quantified to be within the legally stipulated standards. The Pb and Cr metals were found to have the highest percentage of leaching in the coffee infusions.

Key words: Coffee powder, chemical contaminants, law, drink, leaching.

INTRODUCTION

Coffee culture greatly influences world trade. In 2015, the total coffee production was about 143 million bags (International Coffee Organization, 2016), whereas the world consumption in 2014 was 149 million bags, implying an enormous demand for coffee (International Coffee Organization, 2015). Coffee is consumed mostly for its sensory characteristics, besides various other social and economic factors (Carvalho et al., 2016).

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At present, Brazil ranks first in the world as a coffee producer and exporter and is the second largest consumer, after the United States (International Coffee Organization, 2015, 2016). The Brazilian output in 2015 reached 43 million bags (of 60 kg each) of processed coffee, nearly 31% of the global production (International Coffee Organization, 2016). It therefore becomes crucial to assess the quality of the bean and infusion Brazilian coffee.

Heavy metals are the most evaluated elements in food or any other product due to their ability to accumulate in the food chain (Silva et al., 2007). The maximum levels to which they are present therefore becomes the standard of quality across the world (Malik et al., 2008). As these elements are stable, they remain in the environment, accumulating in the soil (Hseu et al., 2010) due to the weathering process of rocks and soil formation, environmental conditions, technological practices and/or chemical usage (Ashu and Chandravanshi, 2011; Selinus, 2006).

Some metals are biologically crucial in low concentrations for living organisms, including copper (Cu), chromium (Cr), cobalt (Co), manganese (Mn), nickel (Ni), zinc (Zn); however, because elements such as arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg), titanium (Ti) and uranium (U) are not essential and exert harmful effects on different parts of the biosphere, they are termed toxic metals (Schmidt et al., 2009).

The coffee plants can absorb these metals and store them in the roots or transport them into the shoots and grains (Silva et al., 2007). The heavy metals vary in concentration in the different plant tissues, and normally, the grains contain lesser concentrations than the vegetative plant parts (Bettiol and Camargo, 2006). On reaching the coffee beans, these metals form the vehicles of contamination for humans inducing adverse health effects like severely decreased neurological and hepatic functions, as well as mutagenesis and carcinogenesis (Matés et al., 2010).

The leaching of each element present in the roasted and ground coffee samples and their infusions can differ (Stelmach et al., 2013), which makes it crucial to also assess the values of these elements in the beverage. Therefore, it is important to assess the dietary exposure for risk evaluation (Noël et al., 2012).

The present study provides a more detailed determination of the contents of cadmium, chromium, copper, manganese, nickel, lead and zinc in coffees cultivated in the Cerrado Mineiro region (Alto Paranaíba – Minas Gerais, Brazil), to compare the values found with the legal standards and check how these metals are extracted from the respective infusions.

MATERIALS AND METHODS

A total of 50 coffee Arabica (*Coffea arabica* L.) samples were collected from the farms and in the coffee marketing centers of the municipalities of Alto Paranaíba region, Minas Gerais, Brazil (Figure 1), including Carmo do Paranaíba (n = 21), Rio Paranaíba (n = 13), Serra do Salitre (n = 11) and Tiros (n = 5).

In each property, 10 samples from the same lot were collected with a coffee sampler. Then the material was homogenized and a 500 g portion was sent for heavy metal analysis. Analyses were performed in two replicate.

The coffee beans were put through a medium roasting process (120 to 150°C/7 to 8 min) utilizing a gas roaster (ROD-bel brand, TP2-L model, São Paulo, SP, Brazil). The samples were later crushed to 3.5 meshin the electric grinder (Probat Leogap brand, model M-50, Curitiba, PR, Brazil).

The coffee infusion was prepared as a beverage using roasted and ground coffee in boiling hot water (95 to 100°C) and filtering, in the ratio of 12 g of powder to 100 ml of water (Teixeira et al., 2016). Subsequently, 25 ml of the beverage prepared by volume was concentrated in a greenhouse with good circulation and air exchange (Tecnal brand, TE-394/2 model, Piracicaba, SP, Brazil) at 60°C, to make approximately 2.5 ml of the final volume.

The roasted and ground coffee samples and their infusions were mineralized by wetting, using a mixture of nitric and perchloric acids in a 3: 1 ratio. Then, the elements cadmium (Cd), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn) were analyzed in the samples (Gomes and Oliveira, 2011).

Readings were recorded from the fast sequential atomic absorption using the spectrophotometer (Varian brand, AA240FS model, Mulgrave, Vic, Australia) with atomization in flame air/acetylene flow at 13.3 L min^{-1}/2.9 L min^{-1} for Cr and 13.5 L min^{-1}/2.0 L min^{-1} for the other elements. A hollow cathode mono elementar lamp (HCL) was used as the radiation source. The electrical current intensities used were of the order of 7 mA (Cr), 5 mA (Mn, Pb and Zn), and 4 mA (Cd, Cu and Ni). Measurements were taken for the following wavelengths (nm): 228.8 Cd, 357.9 Cr, 324.7 Cu, 279.8 Mn, 232.0 Ni, 217.0 Pb, and 213.9 Zn (Onianwa et al., 1999).

The percentage of extraction of the method varies from 92 to 97%. The metal content found was compared to the standards established by Brazilian legislation in force (Brazil, 2013, 1965).

Statistical analysis

Standard curves of each of the white samples analyzed were drawn to determine the various concentrations. The elements of the reagents and samples were also analyzed the patterns of using the elements. Descriptive statistics were used to analyze the data.

Correlation analysis (Pearson) between cadmium, chromium, copper, manganese, nickel, lead and zinc concentrations in roasted and ground coffee samples and in the infusion prepared from these samples was performed.

RESULTS AND DISCUSSION

Manganese, copper and zinc are the heavy metals found in high concentrations in all the roasted and ground coffee samples (Table 1), concurring with the results of Santos and Oliveira (2001), Grembecka et al. (2007) and Ashu and Chandravanshi (2011).

The Southern Common Market Group (Mercosul) (Brazil, 2013) and European Commission (European Commission Regulation, 2008) have established regulations although not limited to these three elements in coffee. The maximum permissible amounts of 50 mg/kg for zinc and 30 mg/kg for copper in general foods

Figure 1. Location of the headquarters of the municipalities where samples of coffee were collected.

Table 1. Maximum concentration, mean and minimum metal roasted and ground coffee samples.

Metals	Cd	Cr	Cu	Mn	Ni	Pb	Zn
	Concentrations (mg/kg)						
Maximum	0.10	1.50	17.18	39.78	1.95	1.58	55.83
Minimum	0.03	0.05	0.70	9.808	0.03	0.03	5.53
Mean	0.01	0.34	10.38	19.44	0.70	0.75	6.62
Medium	0.00	0.23	11.09	18.16	0.64	0.78	6.42
Standard deviation	0.01	0.30	2.52	4.32	0.38	0.33	2.26

(Brazil, 1965) are according to Decree n° 55871 established on 26 March, 1965. Morgano et al. (2002) identified an average manganese content of 31.77 mg/kg in all the raw coffee samples and an average content of 30.33 mg/kg for only the coffee samples from the Alto Paranaíba region– MG, values more than those of the average concentration reported in this study.

One of the roasted and ground coffee samples, corresponding to 2% of the samples showed a higher concentration of zinc than the maximum (50 mg/kg) set by the Brazilian legislation (Brazil, 1965) (Figure 2).

Zinc concentrations for the remaining roasted and ground coffee samples ranged from 5.55 to 14.42 mg/kg. Morgano et al. (2002) reported average concentrations similar to those found this study, the average concentration of zinc raw coffee being about 8.33 mg/kg and that for the samples of the Alto Paranaíba region – MG of 7.04 mg/kg. Grembecka et al. (2007) also recorded values around these with the average concentrations of zinc 9.5 mg/kg for the Arabica coffee samples. Santos et al. (2009) estimated the metal content in two coffee farms in the state of Bahia, Brazil, and reported mean values of 25 and 45 mg/kg for zinc, greater than those found in most of the samples analyzed in this study. Ashu and Chandravanshi (2011) also reported zinc values higher than those in this study, (19 mg/kg) in the commercial roasted coffee samples.

All the roasted and ground coffee samples analyzed revealed copper concentrations below the maximum legal value (30 mg/kg) set by the Brazilian legislation (Brazil, 1965). These concentrations approximate the amounts reported by Santos et al. (2009) in the coffee produced in two places in Bahia (7.15 and 14.9 mg/kg). On comparison of all the samples, Morgano et al. (2002)

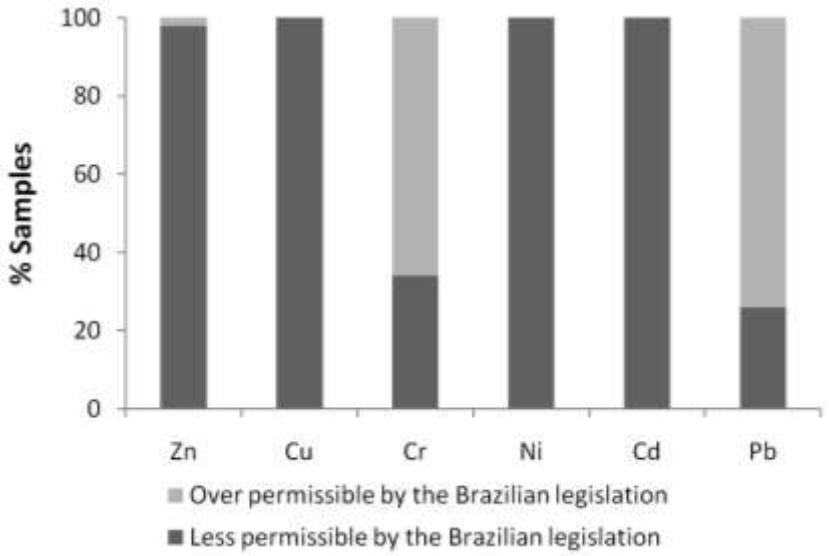

Figure 2. Percentage of roasted and ground coffee samples with metal content less than and higher than the permissible mount set by the Brazilian legislation (Brazil, 2013, 1965).

reported copper with medium values but higher than this study (29.86 mg/kg), although limited only to the samples from the Alto Paranaíba region – MG; the mean value (14.17 mg/kg) showed results similar to those of the current study.

The elements that occur in the lower concentrations or which are undetected in the samples include nickel, chromium, lead and cadmium, among which the latter were found in only 20% of the 50 samples of the roast and ground coffee analyzed. Lead and cadmium rank among the most toxic of the inorganic contaminants. The maximum permissible limit for cadmium set by the regulations of Mercosul (Brazil, 2013) and the European Union (European Commission Regulation, 2008) is 0.1 mg/kg. Lead has been established within the limits of 0.5 and 0.2 mg/kg in the Mercosul regulations (Brazil, 2013) and the European Union (European Commission Regulation, 2008), respectively

The cadmium element was identified in ten roasted and ground coffee samples, none of which exceeded the maximum limit specified by the European Union and Mercosul regulations. The cadmium concentrations in all ten samples ranged from 0.025 to 0.1 mg/kg. Santos et al. (2009) found cadmium concentrations higher than the limit drawn by the European Union and Mercosul regulations (0.1 mg/kg) in the coffee samples produced in the two different properties in Bahia state (0.70 and 0.75 mg/kg).

Lead as an element was absent only in one of the analyzed roast and ground coffee samples. Values ranging from 0.075 and to 1.575 mg/kg were detected in the samples. In fact, 74% of the 50 roasted and ground coffee samples analyzed contained lead concentrations higher than the maximum permitted under Brazilian law and Mercosul regulations (Brazil, 2013) (0.5 mg/kg), some containing almost three times the value (Figure 2). In 86% of analyzed samples of roasted and ground coffee, the element lead was in concentrations above the permitted by the regulations of the European Union (European Commission Regulation, 2008) (0.2 mg/kg). Lead is highly toxic and gets accumulated in the body. The main adverse effects of this metal on health are neurological, hematological, endocrinological, cardio-vascular, gastrointestinal and hepatic systems also affects growth, reproduction and development, and contains a carcinogenic potential (Moreira and Moreira 2004). Thus, to preserve consumer health, none of the high lead level samples could be sold in the market.

For the presence the chromium and nickel elements in coffee beans no maximum levels have been legally specified. Brazilian law permits a maximum of 0.1 mg/kg for chromium and 5 mg/kg for nickel in the general foods (Brazil, 1965). Chromium was absent in 14 of the 50 samples analyzed. However, in 66% of the roasted and ground coffee samples (Figure 2), the chromium concentration was over 0.1 mg/kg, the maximum set by the Brazilian legislation (Brazil, 1965), and the sample having the highest concentration contained greater than 15 times the maximum established. Santos et al. (2009) did not identify any chromium originating from the coffee samples of Bahia.

One sample not showed the presence of nickel while the other roasted and ground coffee samples contained nickel concentrations below 5 mg/kg, as per the requirements of Brazilian law (Brazil, 1965). Morgano et al. (2002) reported a similar trend for the raw coffee samples of the Alto

Table 2. Maximum, minimum and mean concentrations (mg / 50 ml) of metal present in a cup (50 mL) of coffee infusions.

Concentration	Cd	Cr	Cu	Mn	Ni	Pb	Zn
Maximum	0.0030	0.0025	0.0122	0.0373	0.0514	0.0120	0.1292
Minimum	0.0001	0.0001	0.0122	0.0132	0.0002	0.0002	0.0045
Mean	0.0001	0.0011	0.0002	0.0229	0.0011	0.0021	0.0131
Medium	0.0000	0.0011	0.0000	0.0221	0.0000	0.0000	0.1025
Standard deviation	0.0002	0.0005	0.0005	0.0047	0.0020	0.0024	0.0059

Paranaíba region – MG, in a concentration of 1.21 mg/kg. However, the overall average reported by Morgano et al. (2002), considering all the raw coffee samples, was 4.76 mg/kg, higher than those found in the coffee samples of the Alto Paranaíba region – MG and very near to the maximum permissible amount stipulated by Brazilian law (5 mg/kg) (Brazil, 1965). Metals are soil contaminants and are present in this resulting from atmospheric deposition or due to its incorporation, intentional or not, in the soil. Metals are non-biodegradable and due to their poor mobility in the soil they can remain in the superficial layers, in contact with the plant roots over longer time periods. When food crops absorb these metals, they easily enter the food chain, causing harmat all levels (Schmidt et al., 2009; Magna et al., 2013).

On comparing the results of this study with those in the literature, some differences in the concentrations were observed for certain elements. It is well known that rocks are the natural sources of all chemical elements existing on earth (Selinus, 2006). The elements released by rock weathering occur first in the soil and are then transported to the rivers and ground water. In the soil, plant roots absorb them and they thus enter the food chain (Silva et al., 2007). However, these elements naturally occur in equal distribution across the earth's surface and can cause problems when they occur either in very low concentrations (deficiency) or in very high amounts (toxic) (Selinus, 2006).

Studies on soils from the Alto Paranaíba region (MG, Brazil) show the presence of heavy metals (Fernandes et al., 2007; Neto et al., 2009). As there is no legal standard for acceptable limits of heavy metals for the State of Minas Gerais, for comparison purposes, we will use the guideline values of quality, prevention and intervention established by the Environmental Sanitation Technology Company of São Paulo (Fernandes et al., 2007). These studies showed that the levels of Cd (0 to 16.22 mg/kg) and Cr (175.13 to 960.00 mg/kg) were higher than the intervention value for agricultural activity (Cd: 3 mg/kg; Cr: 150 mg/kg). The other metals (Cu: 0 to 56.00 mg/kg; Ni: 9.18 to 32 mg/kg; Pb: 0 to 26.46 mg/kg; Zn: 16.00 to 34.20 mg/kg) were below or very close to the established value of prevention (Cu: 60 mg/kg; Ni: 30 mg/kg; Pb: 72 mg/kg; Zn: 300 mg/kg) (Fernandes et al., 2007; Neto et al., 2009).

The elements of copper, manganese, nickel and zinc are vital to the development of the coffee culture, being applied to the soil or to the leaf and are thus present in the beans, as evident in this study. Further, some elements are pesticide active ingredients used in cultivation. Copper, for instance, finds use as a fungicide in coffee culture as copper hydroxide, copper oxychloride, copper sulfate and/or copper EDTA, which facilitates the absorption of this element by plants, which justifies its presence in bean.

Water used in irrigation can be a source of heavy metal carriers. Silva et al. (2006) evaluated the levels of heavy metals in the waters of the Paranaíba River and found levels above that allowed by the legislation for Cu in 35.71% of the samples, for Zn in 28.57% of the samples and for the Pb in 68, 29% of the samples. The mean Pb content found (0.2611 mg/kg) was about 5 times higher than the value allowed by Brazilian legislation (0.05 mg/kg) (Silva et al., 2006). Rivers are accumulating points of pollutants, receiving pollution from landfills and various anthropogenic activities that develop along river basins (Ferreira and Rosolem, 2011).

The metal contamination also occurs due to human activities, either through waste mining, steel industry, cosmetics industry, or agriculture. The contamination that affects the agricultural areas is now a major problem because many pollutants somehow perform essential roles in economic activities, such as pesticides and fertilizers, and many of these products can remain in the soil and water, contaminating food (Souza et al., 2014).

It should be emphasized that the results were obtained from a single composite sample and had an exploratory and preliminary character. Definitive conclusions about these higher levels should be taken with caution and should be preceded by a more intense analysis of the collection points with problems, from a larger number of samples.

Reports on the maximum, minimum and average concentration of metals in coffee infusions made from the 50 roasted and ground coffee samples, are listed in Table 2. From the values reported for the metals in coffee infusion, the metal content (mg) in a 50 ml cup of coffee was calculated.

Table 3 shows the percentage of extraction (leaching) of the average of the metal in the ground and roasted coffee sample infusions, considering the ratio of the drink

Table 3. Average percentage of leaching metals from roasted and ground coffee samples for coffee infusions (6 g/50 ml infusion).

Metals	Cd	Cr	Cu	Mn	Ni	Pb	Zn
Extractions (%)	26.00	53.45	0.32	19.63	26.13	46.85	28.69

prepared. The elements leached showed higher chromium and lead content, with about 50% of the quantity present in the roasted and ground coffee being a leached infusion. These elements were found in most of the roasted and ground coffee samples in concentrations higher than the maximum established by Brazilian law (Figure 2). This justifies the high percentage of leaching, resulting in an increased concentration of these elements in the coffee infusions.

The element showing the least leaching was copper. Stelmach et al. (2013) reported an average of 6.3% of copper leaching. This low degree of extraction was most likely because of a complex formation of this ion with the strong coffee matrix (Stelmach et al., 2013). Cadmium, manganese, nickel and zinc showed leaching percentages between 20 and 30% (Table 3). Grembecka et al. (2007) reported that the element manganese had a leaching potential of 24% of the roast and ground coffee beans for infusion, similar to theresults of this study. Stelmach et al. (2013) identified an average leaching of 41.93% for the same element. Chromium and lead in this study revealed higher leaching percentages, because they possessed a lower interaction with the coffee matrix.

According to Padovani et al. (2006), the maximum tolerable intake - UL (Tolerable Upper Intake Level) of certain elements is calculated chiefly with respect to age and sex (also considering pregnancy and lactation). UL refers to the highest value of prolonged and everyday intake of a nutrient that apparently presents no risk of ill health effects to almost all the individuals irrespective of gender or the stage in life.

Considering, on average, a daily consumption of four cups of coffee (Arruda et al., 2009), the quantity of manganese ingested via the coffee infusion will be 0.0916 mg/day, comfortably less than the value set as the maximum tolerable limit for this metal, which is 11 mg/day (Padovani et al., 2006). The daily intake of four cups of coffee contributes 0.83% of the maximum daily intake set for manganese. Noël et al. (2012), in their evaluation of 30 coffee samples, arrived at an average manganese concentration of 0.662 mg/kg in the brewed coffee. When only one cup (50 ml) is considered, this value will be 0.0331 mg/50 ml, a little above than the average identified in this study, hovering close to the maximum values noted.

The daily zinc intake from the consumption of four cups per day of coffee from the coffee samples analyzed in the current study is about 0.0524 mg/day, indicating 0.13% of the maximum tolerable intake for this metal (40 mg/day) (Padovani et al., 2006). On analyzing the presence of metals in various products in France, Noël et al. (2012) arrived at a mean value of 0.01425 mg/50 ml for zinc in the coffee, almost identical to the findings of this study.

Copper metal was found in only one of the 50 coffee infusion samples at a concentration of 0.0122 mg/50 ml. The maximum tolerated limit for copper has been set at 10 mg/day (Padovani et al., 2006). Although, high copper concentrations are present in the roasted and ground coffee beans (Table 1), it was almost never detected in the coffee infusions, revealing an average of less than 1% leaching. Noël et al. (2012) found an average copper concentration of 0.0945 mg/50 ml on evaluating 30 coffee infusion samples.

Chromium however, revealed a unique pattern, quite different from that of the other elements. It occurred in higher concentrations of the brew when compared with the quantities present in the roasted and ground beans for a few samples. Ashu and Chandravanshi (2011) reported similar behavior for the elements of cobalt, zinc and manganese. In the coffee infusions, chromium was not identified in only two roasted and ground coffee samples. Santos and Oliveira (2001) detected chromium in only one of the 21 instant coffee samples studied by the authors. Noël et al. (2012) had earlier reported the chromium concentration to be 0.0023 mg/50 ml on average in coffee infusion. The permissible chromium intake for adults is 50 to 200 µg/day (0.05 to 0.2 mg/day) (World Health Organization, 2000). Although, most of the roasted and ground coffee samples analyzed showed a chromium concentration above the maximum set by the Brazilian legislation (0.1 mg/kg) (Brazil, 1965), the daily consumption of four cups of coffee would account for 4.4 µg chromium, corresponding to 2.2% of the allowable total intake (maximum of 200 mg/day).

According to Padovani et al. (2006), the maximum daily nickel intake is 1 mg/day. As it was identified in only eight coffee infusion samples, nickel was ingested in small amounts via the coffee intake. The nickel concentration on average in the coffee brewed in this study was less than that found by Noël et al. (2012), which was around 0.0041 mg/50 ml of the coffee infusion. Santos and Oliveira (2001) found no nickel at all in the 21 samples of soluble coffee analyzed.

Lead and cadmium are highly toxic elements; therefore, their consumption should be as minimal as possible. The maximum permissible limit for cadmium set by the "Joint FAO/WHO Expert Committee on Food Additives" is 7 µg/kg body weight/week (Food and Agriculture Organization/World Health Organization, 2004). Thus, for a 70 kg adult human being the maximum daily intake

Table 4. Pearson correlation matrix of analyzed metals present in roasted and ground coffee and coffee infusions.

Correlation	Mn r	Zn r	Cu r	Cr r	Ni r	Cd r	Pb r	Mn i	Zn i	Cu i	Cr i	Ni i	Cd i	Pb i
Mn t	1.000													
Zn t	0.040	1.000												
Cu t	0.510	0.104	1.000											
Cr t	0.024	0.015	0.027	1.000										
Ni t	0.240	0.169	0.284	0.706**	1.000									
Cd t	0.028	0.054	0.101	0.206	0.241	1.000								
Pb t	0.062	0.172	0.230	0.186	0.124	0.060	1.000							
Mn b	0.744	0.135	0.430	0.054	0.228	0.049	0.081	1.000						
Zn b	0.111	0.059	0.009	0.110	0.052	0.052	0.011	0.204	1.000					
Cu b	0.037	0.024	0.035	0.182	0.272	0.058	0.080	0.008	0.237	1.000				
Cr b	0.033	0.144	0.272	0.335	0.183	0.063	0.351	0.048	0.054	0.053	1.000			
Ni b	0.100	0.030	0.067	0.008	0.143	0.142	0.105	0.074	0.307	0.532	0.048	1.000		
Cd b	0.321	0.052	0.534	0.063	0.217	0.315	0.141	0.254	0.127	0.021	0.244	0.047	1.000	
Pb b	0.367	0.120	0.380	0.157	0.047	0.084	0.110	0.252	0.079	0.084	0.326	0.362	0.570	1.000

Correlation coefficients (R) followed by ** are greater than 0.60 and are significant by the t-test at the 1% probability level. r: Metals present in roasted and ground coffee; i: Metals present in the coffee infusions.

would be 70 mg. Therefore, the daily consumption of four cups of coffee will imply the ingestion of 0.4 µg of cadmium. This is a low value and accounts for 0.57% of the maximum daily cadmium intake, considering the overall average (0.0001 mg/50 ml). On analysis of the samples separately, the cadmium consumption may be much higher, achieving 17.1% (sample containing cadmium concentration of 0.003 mg/50 ml infusion of coffee). However, most of the samples analyzed (60%) did not show the presence of cadmium. Santos and Oliveira (2001) detected no cadmium in any of the 21 samples of instant coffee analyzed.

Lead was identified in 23 coffee infusions, corresponding to 46% of the samples, and in these samples the concentration of this element ranged of 0.0004 mg/50 ml to 0.0121 mg/50 ml. The maximum permissible lead intake is 25 µg/kg body weight/week (250 µg/day, for an adult human being weighing 70 kg). Consumption of the coffee samples would imply the ingestion of large quantities of this element, although absent in more than half the samples. Four cups of such coffee consumed could contribute to nearly 3.36% of lead ingestion, reaching 19.36% if the sample with the highest lead concentration of lead is consumed. This is a very crucial value, as coffee is a beverage consumed over a few days by volume when compared with other foods. Santos and Oliveira (2001) identified no presence of lead in 21 samples analyzed for soluble coffee.

Pearson correlation coefficients were estimated for the concentrations of metals present in roasted and ground coffee samples and infusions (Table 4). Among the same element, only the correlation for manganese was significant, that is, samples that contained higher concentrations of this metal in the roasted and ground coffee, also presented in their infusions, evidencing that this metal is quite soluble/leachable. In the analyses between metals, only the correlation between chromium and nickel was significant for roasted and ground beans. This indicates that the terrors of these two elements in the samples are directly proportional.

Conclusions

The chromium and lead elements in some samples are found in concentrations higher than the legal permissible extent. With respect to the maximum allowed, chromium and lead concentrations according to Brazilian law, only 14% of the samples analyzed are within the established norms. In the face of this contamination, new studies are needed to analyze the soil and the water used in the irrigation of these properties and in less disturbed areas to allow more adequate comparisons with the contents naturally present in soil and water.

There is a variation in the amount of extracted heavy metals for coffee infusions, due to differential interaction with the organic matrix. Most metals extracted chromium and lead, which were already in great amounts in roasted and ground coffee, contributing to a high content of these elements in infusions.

Conflict of Interests

The authors have not declared any conflict of interests.

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Sanitary analysis, transmissibility and pathogenicity of fungi associated with cashew nuts

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There are few reports of transmission of fungus associated with cashew nuts (*Anacardium occidentale* L.) in Brazil and worldwide. Thus, the aim of this study was to evaluate the incidence and severity of anthracnose on leaves and fruits (nuts) of cashew, nuts sanitary analysis, nuts-seedling transmission and pathogenicity of fungi associated with cashew nuts. To this end, leaves and nuts of cashew collected in the cities of Gurupi and Formoso do Araguaia, Tocantins state, Brazil, were used. The anthracnose average incidence and disease index were evaluated by observing the typical symptoms of the disease. The nuts were subjected to sanity analysis, by the filter paper method and transmission test via nuts for the seedlings. High anthracnose incidence and severity were observed in leaves and cashews nuts. *Aspergillus* sp. *Cladosporium* sp. and *Colletotrichum gloeosporioides* showed high incidence in all nuts samples analyzed. There was high anthracnose transmission by the nuts for the seedlings. Only *C. gloeosporioides* was pathogenic to cashew seedlings. Higher germination rates were observed with lower levels of lesions on the external surface. The fungal association with cashews nuts damaged generally normal seedlings development and quality.

Key words: *Anacardium occidentale*, *Colletotrichum gloeosporioides*, anthracnose, sanitary analysis.

INTRODUCTION

The cashew (*Anacardium occidentale* L.) is a tropical native species from Brazil spreading almost all-Brazilian territory (Coutinho et al., 2016). Constitutes an income source for the population and has great economic and

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social importance, especially in the Northeastern region. Although most of the cashew production is located in the country coastal region, a large increase in production area is occurring in Semiarid and Cerrados (Brazillian savanna) regions, probably due to favorable conditions for a higher fruits (nuts) and pseudo fruits (flower peduncle) quality than in any other country region. The cashew propagation is carried out through nuts (seeds) or vegetative parts of plant, the latter being the most recommended, as it ensures obtaining more uniform fields with desirable characteristics and more productive (Dendena and Corsi, 2014). The planting through nuts is still widely used in several regions. However, the use of contaminated and/or infected nuts by pathogens can result in lower plant population and lower production and pseudo fruits quality because they are vehicles of many pathogens that serve as initial inoculum and can cause epidemics in the orchard.

Anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. [anamorphic phase *Glomerella cingulata* (Stoneman) Sp. Schrenk], is the most widespread and destructive disease of cashews in Brazil. In most plants, it is mainly transmitted through seeds, but also through plant infected parts. Symptoms may appear on leaves, fruits and seedlings, occurring in all plant development stages and can cause seedlings necrosis and death (Dendena and Corsi, 2014). The fungus may attack during all the months in the year, but cause greater damaged in the rainy season and when issuing news hoots. The disease management was carried out using sanitary practices and chemical control (Figueirêdo et al., 2012). The use of resistant clones is also should be adopted, however, all commercially available clones are susceptible to disease (Figueirêdo et al., 2012).

Healthy seed must have a high germination rate and be free of seed-borne pathogens and healthy seeds are recognized as an important input in any agricultural production system. However, there is a little information available about the transmission of fungi through the cashew nuts in Brazil and worldwide, which makes this study of great importance. Based on the exposed, the aim of this study was to evaluate the incidence and severity of anthracnose on leaves and nuts of cashew nuts, sanitary analyzes, cashew nuts - seedlings transmission and pathogenicity of fungi associated with cashew nuts.

MATERIALS AND METHODS

Sample collection

The study was carried out from August, 2013 to October, 2014. An amount of leaves and nuts were collected from a total of 20 cashew trees, previously selected, from two cities of Northern region Brazil, Gurupi (11° 43' S; 49° 04' S) and Formoso do Araguaia (11° 47'45''S; 49° 31'52"W), State of Tocantins. After the collection, the nuts were mixed for performing the tests.

Pathogen identification

Cashew leaves fragments with anthracnose symptoms were subjected to sterilization by immersion in 50% alcohol solution for 30 s, 1% sodium hypochlorite for 40 s and three washes in sterile distilled water. After, the leaf fragments were placed on Petri dishes containing potato dextrose agar (PDA) culture medium, and maintained in a growth chamber at 25°C ± 2°C for seven days. After incubation, the fungi identification was carried out by analyzing the fungal structures, with the aid of an light microscope. The observed structures were compared with structures described in the literature (Barnett and Hunter, 1972).

The identified *Colletotrichum* sp. isolates were grown on PDA medium and incubated for seven days for further molecular identification at specie level. For DNA extraction, discs containing the mycelia were excised and inoculated into 60 mL of liquid medium Yeast extract (10 g yeast extract, 10 g dextrose, 1 L distilled water) with antibiotic (Cloranfenicol - 200mg/L). For mycelial growth, they were kept under agitation (120 g) for seven days at 27 °C. The mycelium was separated from the liquid medium by vacuum filtration. After washing with sterile distilled water the harvested mycelium was ground to powder in liquid nitrogen, with a pestle and a mortar. DNA was extracted from the powdered mycelium using a CTAB method adapted from Zolan and Pukilla (1986). Briefly, about 0.5 g of this powder mycelium was then transferred to a micro centrifuge tube, and 1 mL of preheated (65ºC) extraction buffer (2% p/v de CTAB; 2.5% p/v de PVP; 2M de NaCl; 100 mM Tris-HCl (pH 8.0), 25mM EDTA (pH 8.0), 2% (v/v) ß-mercaptoetanol) were added. The powder was mixed with the buffer, vortexed briefly, and stored at 60°C for 40 min and then centrifugate at 11,000 *g* for 10 min. After separation of the tissue from the aqueous phase, an equal volume of chloroform/isoamyl alcohol (24:1) was added. The RNA was degraded by treatment with RNase A (50 mg/mL) for 30 min at 37°C. The DNA was precipitated by adding 2.5 volumes of absolute ethanol and pelleted by centrifugation for 15 min at 12,000 *g*. The pellet was washed with 70% ethanol and resuspended in ultrapure water. The DNA concentration and purity were measured using a spectrophotometer.

The extracted DNA was subjected to the polymerase chain reaction (PCR) using a forward primer CaLac-f (5'-GAAGATCTCGGCACCATCAT-3') and a reverse primer CgLac-r (5'- AACAACAGGGACCAGGTCAG-3') to amplify the laccase gene of *C. gloeosporioides* (Shi et al., 2008). Each PCR reaction contained 2.5 ng of DNA, 13 µL of sterile water, 10 mM of Tris-HCl, 2 mM of $MgCl_2$, 0,1 mM of dNTP's, 1.25U Taq DNA Polymerase (Ludwig Biotec, Brazil) and 0.4 µM of each primer (final volume = 25 µL). This solution was submitted in thermal cycler with the following parameters: 4 min at 94°C, 40 cycles of 1 min at 60°C, 2 min at 72°C, and a final extension of 5 min at 72°C. After amplification the PCR products were subjected to electrophoresis on a 1.5 % agarose gel stained with Blue Green Loading dye (Promega, United States).

Incidence and anthracnose severity

Fifty cashews leaves and nuts were randomly obtained from 16 cashews tree to quantify the anthracnose incidence and severity. A completely randomized experimental design was used with 11

replicates. Average incidence of anthracnose on leaves and nuts was expressed in percentage using: % incidence = $[(\sum x/n) \times 100]$. Where x = number of infected leaves or fruits and n = total number of leaves or fruits observed. In the assessment of anthracnose average severity on leaves and nuts, we used rating scale 0-4 adapted from Santos et al. (2005), where 0 = no symptoms; 1 = symptoms at 10% of the affected area; 2 = 11 to 25% of the affected area; 3 = 26 to 50% of the affected area; and 4 = more than 50% of the affected area. Later, the disease index (DI) was determined using the McKinney index formula (1923) as shown: DI=$[\sum F(n) \times 100]/(TNL \times MN)]$. Where: ΣF(n) = the sum of notes frequency; TNL= total number of examined leaves; e MN = maximum scale note.

Sanitary analysis of cashew nuts

For this assay, the blotter test method was used, following the description in the Rules for Seed Testing – RAS (Brasil, 2009). The experimental design was completely randomized, with 16 replications. Initially, the nuts were washed in water and subjected to sterilization in 1% hypochlorite, 70% ethanol and distilled water. The nuts were placed in a transparent box (gerbox), with 25 nuts on each box, on two layers of germitest paper previously autoclaved and moistened with sterile water. The boxes containing the nuts were kept in incubation chamber at 25°C ± 2°C for seven days, with alternate light. After incubation, the fungi identification was carried out on the nuts as described above. The values of results were expressed in percentage of incidence of each fungus. When it was not possible the fungus genera classification using the structures present, the hyphae of these fungi were collected and placed on PDA medium, for later identification. For confirmation of Koch's principles, the fungi found in the nuts were isolated on PDA medium. To test the pathogenicity of these fungi to cashew plants, the conidia were inoculated by spraying at a concentration of 1 × 10^5 conidia mL using a manual sprayer in cashew seedlings at 40 days after emergence. Then the seedlings are stored for 24 h in a moist chamber until disease symptoms onset.

Cashew nut – seedling transmission

For the cashew nut – seedling transmission assay, 50 nuts in three replications, totalizing 150 cashew nuts were sowed without disinfestations, on plastic trays containing autoclaved sand. After the sowing, the trays were transferred to a greenhouse with temperature of 30°C ± 5°C. Daily irrigation was performed with the aid of a watering pot. The seedlings evaluation was made 15 days after the emergence by observation of the characteristic symptoms. The fungi transmission was assessed by considering the percentage of seedlings with anthracnose symptoms on leaves, steam and cotyledon. It is also evaluated the percentage of germinated and non-germinated nuts and the percentage of dead seedlings. To identify the fungi species in the seedlings lesions, the injured fragments of the primary root, stem, cotyledon and leaf were removed and submitted to the isolation process as described already.

Effect of anthracnose severity on the cashew nuts in the germination

The nuts were selection based on external surface visual observations and separated according to damage level using a rating scale adapted from Santos et al. (2005). We used a completely randomized design with three replicates; the treatments consisted of five different severity levels and each replicate was constituted by 50 cashew nuts. The sowing was made on polypropylene trays, with autoclaved sand as substrate. After sowing, the substrate was moistened and the trays were kept in a greenhouse with temperature of 30°C ± 5°C. The assessment was carried out over 30 to 50 days after the seedlings emergence. It's considered as germinated nuts, those that gave rise to normal seedlings with all the essential structures (Brasil, 2009). The transmission was observed in normal and not normal seedlings by count seedling with lesions on leaves. The pathogen identification was carried out as described above.

Statistical analysis

The data of anthracnose averange incidence, disease index and the relationship between infection severity and anthracnose transmission were subjected to analysis of variance (ANOVA) and differences between means were determined according to Tukey's test, at 5% probability. The data expressed as percentage were transformed into arcsin √x/100 for statistical purposes, when necessary. The graphics were plotted using the Sigma Plot (version 10.0) software and the statistical procedures were held with Sisvar (version 5.3) software.

RESULTS AND DISCUSSION

Pathogen identification

All isolates from cashew leaves with anthracnose symptoms were identified, based on morphological aspects, such as conidial morphology and presence or absence of setae, with similar characteristics to those described for species belong to the genera *Colletotrichum* sp. However, because definitive identification of *Colletotrichum* species based on morphology is difficult due to overlapping ranges of conidial and colony characteristics and the fact that variation in morphology is accepted for isolates within species, a number of molecular methods have been used to characterize species of *Colletotrichum* (Uaciquete et al., 2013).

Based on the analysis of laccase gene with the specific primers, this study was able to confirm that the cashew isolate were identified as *C. gloeosporioides.* The primer tested in our study generated amplification products of 505 bp (Figure 1) corresponding to *C. gloeosporioides* laccase gene described by Shi et al. (2008). Morphological and molecular characteristics performed in this study confirmed *C. gloeosporioides* as the causal agent of cashew anthracnose. These finds corroborate with the described by and Uaciquete et al. (2013) that *C. gloeosporioides* is commonly reported as the causal agent of anthracnose in cashew trees. The identification and confirmation of the *Colletotrichum* species pathogenic to cashews is crucial to the development of more efficient control strategies, besides contributing for a better

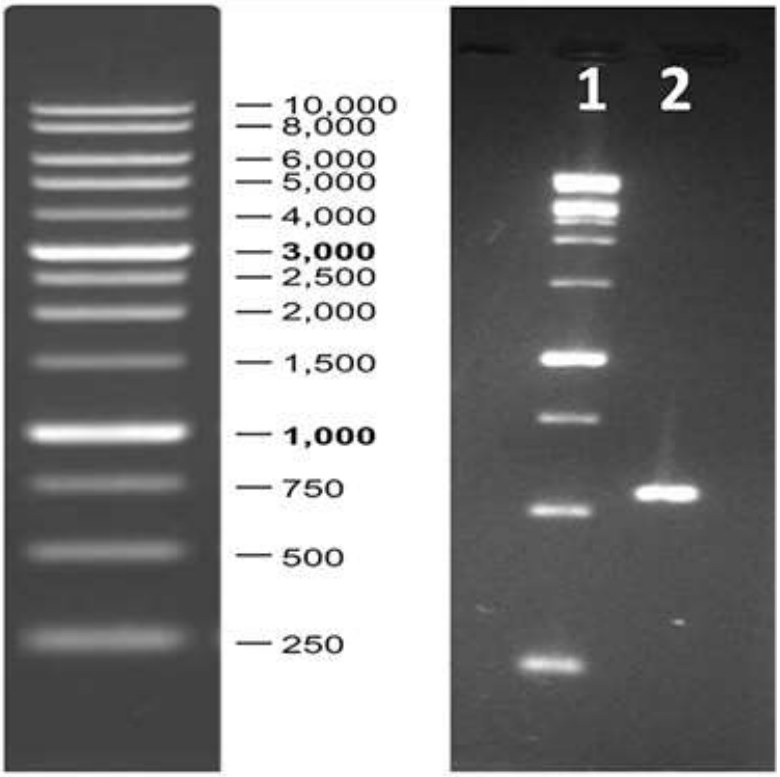

Figure 1. The amplification product obtained by PCR using the oligonucleotides CgLac-f and Calac-r specific to *Colletotrichum gloeosporioides*. Lane 1: molecular marker 1Kb; Lane 2: Cashew Isolated.

understanding of the anthracnose disease epidemiology.

Incidence and anthracnose severity

There was a high anthracnose incidence on the leaves (68.1%) in this study (Figure 2A). The anthracnose incidence on the leaves differed statistically (Tukey, P≤0.05) to the anthracnose incidence in nuts (31.1%). Distinct of the observed in anthracnose incidence, disease index was higher in the nuts than in the leaves, 34.7% and 31%, respectively (Figure 2B). Anthracnose of cashew is a disease known in several regions where it grows cashew, being its incidence always related to the region climatic conditions. The climatic conditions of the Brazilian Northern region are favorable to the growth and proper development of cashew tree. However, high temperatures and high relative humidity (around 85%) found here provide better pathogen development and increasing severity on leaves, stems and fruits, as observed in these study. Long-term exposure to disease promoting or predisposing factors can increase disease development in perennial hosts (Frare et al., 2016), as cashews trees. Host nutritional status is also an important abiotic factor that may be related to increased disease in orchards without proper maintenance, such as where the study was conducted.

Transport of fungi associated with cashew nuts

The results showed a high incidence of *Aspergillus* sp. (49.71%) in all tested samples (Figure 3). The incidence of the genus *Cladosporium* sp. (17.14%), *Penicillium* sp. (11.42%), *Pestalotia* sp. (0.5%) and *C. gloeosporioides* (26.85%) were also observed. Among all fungi found in the cashew nuts only *C. gloeosporioides* was pathogenic to cashew seedlings.

The fungi genus *Aspergillus*, *Cladosporium* and *Penicillium* found in large quantities in the assessed cashew nuts are involved in the decay process of the nuts at sowing stage, by interfering directly in germination rate and seed vigor. These fungi may attack different kinds of seeds, once they are able to survive on several

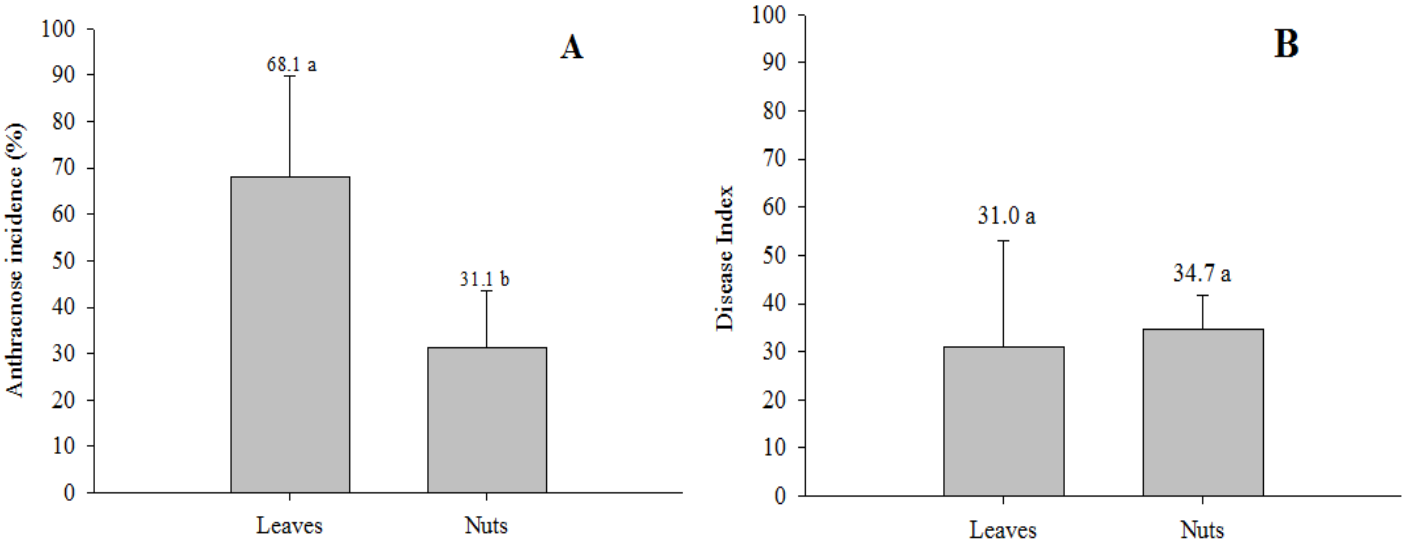

Figure 2. Anthracnose average incidence (A) and disease index (B) of cashew leaves and nuts (*Anacardium occidentale* L.). Percentage of anthracnose incidence data transformed in arcsin √x/100. Means by the same letter are not significantly different (Tukey, P≥0.05).

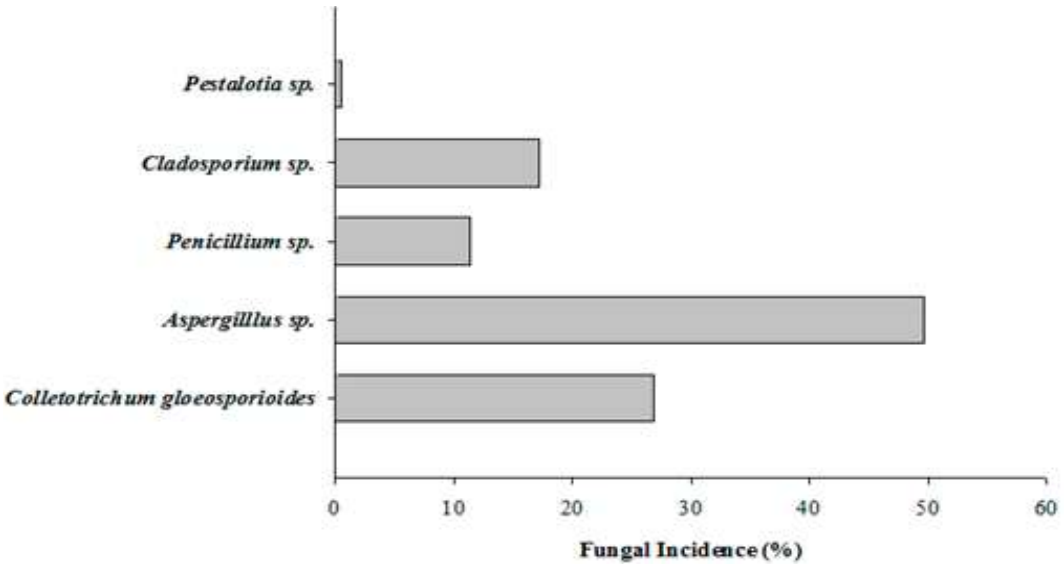

Figure 3. Fungal incidence associated with cashew nuts (*Anacardium ocidentale* L.) assessed by the sanitary test.

substrates, and are resistant to a variety of environmental conditions (Sharma and Kulshrestha, 2015). The high incidence of these fungi makes necessary the use of some control method, since it affects directly the final product quality, particularly cashew nuts intended for human feeding. It must be alert to contaminants fungi, *Aspergillus* sp. and *Penicillium* sp., which always occurs in higher percentages, besides being potentially mycotoxin-producing. Thus, they pose a potential hazard to consumers' health. The presence of some metabolites and traces of aflatoxin in cashew kernels has been demonstrated (Milhome et al., 2014). It is estimated that about 10% of the annual production of cashew nuts is unsuitable kernels for industrial processing and for

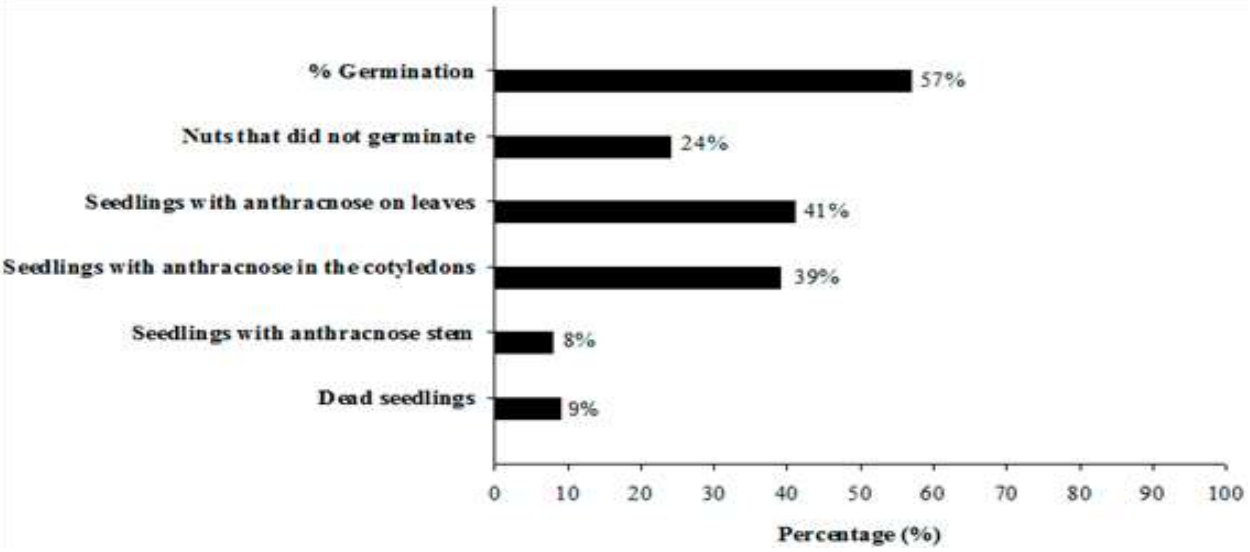

Figure 4. Percentage (%) of cashew seedling (*Anacardium occidentale* L.) with anthracnose symptoms, % dead seedlings and % germination in the transmission assay.

human feeding, due to the fungi presence, mostly (Adeigbe et al., 2015). Despite the well-known antibiotic properties of the cashew nut testa and shell (Schulze-Kaysers et al., 2015), a wide spectrum of fungi can survive saprophytically on cashew seeds. Following seed germination, young tissues may be susceptible to some of these fungi. The attack caused by fungi present in nuts on the cotyledons, causes seedling nutrients loss and healthy tissues destruction, resulting in a not normal seedlings development, with wrinkled and small size leaves and twisted stems.

The fungus *C. gloeosporioides* is commonly reported as causing rot in nuts (Sarkar, 2016), being found in the present study with an incidence of over 50% in the evaluated samples. However, the infection seeds not ensure the pathogen transmission, because in addition to the host, the factors linked to the environment and the pathogen must be considered. The fungus can survive in dead tissue and in the soil and can be spread by rain splash, wind, insects, etc. Nonetheless, when the pathogens find healthy tissue it can cause severe injury in favorable environmental conditions, and greatly decrease of nuts and peduncle production (Dendena and Corsi, 2014).

Cashew nut – seedling transmission

There are several factors limiting the efficiency of pathogen transmission by seeds. Each pathogen has specific requirements of temperature, humidity, oxygen, quantity of nutrients, mechanical injury, seed age and the presence of antagonistic microorganisms on the seed (Rego et al., 2012). In this study, anthracnose transmission of cashew nuts for the seedlings was verified through the percentage of seedlings with disease symptoms (Figure 4). In cotyledons was observed anthracnose incidence on 39% of seedlings and leaves symptoms were observed in 41% of the seedlings. For stem symptoms, there were few plants with anthracnose symptoms (8%). A similar result was found by Lopez and Lucas (2002) under laboratory conditions, they noted that 33% of seedlings emerging from non-treated cashew seeds had necrotic lesions on cotyledons, hypocotyls or epicotyl. *C. gloesporiodides* was detected in red pepper (*Capsicum annuum* L.) seeds and is associated with anthracnose in this crop. This pathogen can be transmitted from the endosperm tissue for hypocotyl and rootlets and is located generally on the surface of seeds, in the endosperm and embryo (Ali et al., 2016). The percentage of germinated nuts, with and without disease symptoms it was low (57%) and the quantity of nuts which do not germinate was 24%, while the number of dead seedlings was 9% (Figure 4). Seeds pathogen carriers may result in failures germination, seedling death or development of low-quality plants, thereby compromising production (Coutinho et al., 2016), as observed in this study.

Effect of anthracnose severity on the cashew nuts in the germination

There was a higher germination rates in nuts with lower

Table 1. Relationship between the infection severity in cashew nuts (*Anacardium occidentale* L.) and transmission of cashew anthracnose by *Colletotrichum gloeosporioides*.

Samples	Severity level[1]	% of emergency[2,3]	% seedlings with anthracnose[2,3]
	0 = (no symptoms)	78.5[a]	4[b]
	1 = (1-5.9% of LA)	62.5[a]	31[ab]
Cashew nuts	2 = (6-25.9% of LA)	79.5[a]	45.4[ab]
	3 = (26-50% of LA)	77[a]	47[ab]
	4 = (> 50% of LA)	57[a]	62.5[a]
CV (%)		16.25	27.04

[1]LA - lesion area. [2]Means by the same letter are not significantly different (Tukey, P≥0.05). [3]Data transformed arcsin√x/100.

lesions levels on the outside surface (Table 1). The nuts germination started at 19 days after sowing; however, it was found that there was a delay in some nuts and uneven germination. Nuts with a high degree of severity (note 3 and 4) gave greater number of plants with leaf spot and anthracnose symptoms. In a humid chamber, the presence of *C. gloeosporioides* in the injuries was found. The transmission in this case may be conditioned to the severity of the seed fungal infection, or be related to the lesion size and the amount present in the seed inoculum. Silva et al., (2013) using selected seeds with different levels of *C. lindemuthianum* incidence observed variation from 70 to 100% in the transmission rate of seeds with incidence of 1 to 5% the pathogen.

Germination, while above 50%, demonstrates the damaging effect of the fungi on the vigor of the nuts and possibly the onset of leaf spots. In nuts with higher levels of severity were observed higher rates of not normal seedlings, beside the highest number of nuts that did not complete germination, and seedlings that died after emerge. These effects may be related to the fact that most affected nuts having lower reserve level to assist the early seedling development, besides the high incidence of fungi that probably causes more damage to seedlings. It was also found that despite of the good external appearance in some nuts, not always indicate the absence of pathogens, since many of them may be in a latent state waiting for adequate moisture conditions for manifesting.

The difficulty of obtaining healthy seedlings, due to the number of pathogens that are associated with cashew nuts, might be one of the causes of low germination and reduced development of seedlings coming from seeds. To Marques et al., (2013) the dwarf cashews seed germination begins on the 10th day after sowing and continues until the 25th day, however normally 80% germination occurs between the 12th and the 20th day after sowing. Different from previously reported, the low vigor and the nuts germination delay observed in this study, may also be attributed to genetic uneven of the nuts, as these come from a heterogeneous population and uneven, which may have contributed to the results.

The results of this study showed that fungal associated to cashew nuts significantly damage the normal development and quality of seedlings, as well as serve as the initial inoculum for future disease outbreaks.

Conclusion

The presence of *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp. and *C. gloeosporioides* were observed in most of the analyzed nuts. The pathogen *C. gloeosporioides* can be transmitted efficiently to cashew seedlings via nuts. Cashew nuts with a high degree of anthracnose severity lead the emergence of a larger number of symptomatic seedlings.

Conflict of Interests

The authors have not declared any conflict of interests.

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Spatial variability of soil physical and chemical aspects in a Brazil nut tree stand in the Brazilian Amazon

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The Brazil nut is considered one of the noblest trees of the Amazon biome and contains social, ecologic and economic importance to this region. The study of the spatial variance of the edaphic properties in native nut trees can direct future researches about more efficient samplings. The Geostatistics is the methodology utilized for this type of study, once that it considers the structural and random characteristics of a variable spatially distributed. This work sought to get a higher knowledge about the distribution of the nutrients in the soil, verifying the relationship with the occurrence of this species, to thereby provide subsidies to future forest management and maintenance/enlargement of the productivity in these areas. The soil samples were collected from 30 × 30 m on the line, in all of the lines in part of the study, totaling 60 samples. All of the points were georeferenced. The preparation of the samples for the sample preparation for the chemical analysis and the methods and calculations to determine the physicochemical variables studied were described by Nogueira and Souza (2005). The statistical and geostatistical analysis were conducted using the R computational environment, version 3.2.2. Most of the studied variables presented defined level. For the physical variables, there was predominance of the adjustment to the model of the gaussian variogram, follower by the spherical model. In the case of the chemical variables, there were two occurrences for each adjustment model (spherical, exponential and gaussian). The variables that best presented spatial relation with the occurrence of Brazil nut trees were the silt, clay, macroporosity, pH, phosphorus, zinc and copper.

Key words: Geostatistics, Brazil nut, soil, physical properties, chemical properties.

INTRODUCTION

The soil is characterized by the heterogeneity and presents variations in its morphological, physical,

chemical and mineralogical properties (Oliveira et al., 2000). The diversity of the geomorphological aspects, the high temperatures and the elevated indexes of precipitation are the factors that justify these variations and the existence of different classes of soils of the Amazon Biome (Chig et al., 2008).

Spatial variation of edaphic aspects as it relates to the analysis of soil fertility could provide a basis for future research in order to conduct more efficient sampling in the field (Vieira, 2000). Detailed knowledge of variability in soil variables could provide a solid basis for making sound decisions with respect to choosing adequate management schemes (Silva et al., 2013). In managed systems, such as those of Brazil nut harvesting areas, there could be additional sources of soil heterogeneity besides those inherently natural (Camargo et al., 2010), and therefore quantifying soil spatial variation would permit better control over factors that control production and also over environmental monitoring (Silva Neto et al., 2012; Oliveira et al., 2013). According to Souza et al. (2009), the use of geostatistiscal techniques allows for interpretation of results using as a base the natural variability inherent in the system. For this reason there are a growing number of studies published that focus on spatial variation, especially in monoculture production systems (Machado et al., 2007; Lima et al., 2013) and agroforest systems (Campos et al., 2013; Oliveira et al., 2013).

The geostatistics is the methodology utilized for this types of study, once that it considers the structural and random characteristics of a variable spatially distributed (Moolman and Van Huyssteen, 1989). The main tool of geostatistics to describe and model the spatial pattern of a variable is a graph that associates distances with semivariances, named semivariogram (Seidel and Oliveira, 2014). To predict values in not sampled locations the geostatistics uses the kriging that allows the knowledge of continuity of the variable in interest in the study area. Kriging is realized through the interpolation in sites not sampled and it also produces variability (Santos et al., 2011). The geostatistics is one of the main tools of the MapCast project that studies spatial approach on Brazil nut in the Amazon forest.

The MapCast Project “Mapping of native nut trees and socioambiental and economic characterization of systems of production of the Brazil nut in the Amazon”, coordinated by the Brazilian Company of Agricultural Research (Embrapa) from 2014, searches to characterize the factor and systems of production of the *Bertholletia excelsa* Bonpl., Lecythidaceae family, also known as the Amazon nut and “Para nut”, through geospatial information to contribute to the management and adaption of good practices to the different realities of Amazon. One such site is localized in the Tapajós National Forest (FLONA) in the State of Pará, Brazil, which is a natural stand that serves as a food and income source for local families engaged in the extraction and sale of the Brazil nuts.

The Brazil nut is considered one of the noblest trees of the Amazon biome and contains social, ecologic and economic importance to the region. Its almonds are much very appreciated in the European, Asiatic and American continents (Salomão, 2014). Tonini and Pedroso (2014) consider it to be a key species for the management of the direct and indirect benefits of the forest. Due to these factors and also the indexes of the increasing deforestations, in 2008 this species was included in the list of endangered species of the Ministry of the Environment (MMA) and of the State of Para. Considering these aspects and the existence of extensive areas of primary and secondary forests in the Amazon, that house the chestnut tree, it has become urgent to advance in the ecologic, economic and social knowledge of this species (Salomão, 2014) and also provide regionalized informations for future actions of management that foment its production.

In 2011, the total Brazilian production of the Brazil nut was of 39,917 tons. In 2012 and 2013 the production presented reduction to 38,805 and 38,300 tons, respectively (IBGE, 2013). The gains with the Brazil nut can be expanded, in case the producing areas receive investments to develop its productive capacity.

The present work presents an geostatistic analysis of soil nutrient spatial distribution in a natural Brazil nut stand in the FLONA Tapajós. The aim was to obtain higher knowledge about the nutrients distribution in this environment, verifying the relation with the occurrence of Brazil nut trees, to thereby provide subsidies to future practices of forest management and maintenance/enlargement of the productivity of this area.

MATERIALS AND METHODS

Study area

The study site consists of a small stand (300 × 300 m) that was demarcated as part of the MapCast project, with six 50 m transects.The portion was installed in a native forest area with a natural density of Brazil nut, where every individual of the species were georeferenced (Figure 1). The soil was classified as a Clayey Dystrophic Yellow Oxisol (Oliveira Júnior and Corrêa, 2001) using the Brazilian Soil Classification System (EMBRAPA, 2001), and as a Typic Haplustox using the Soil Classification System of the United States (USDA, 1999). This forest fragment is inside the territorial limits of FLONA of the Tapajós, between the parallels 2°45’ and

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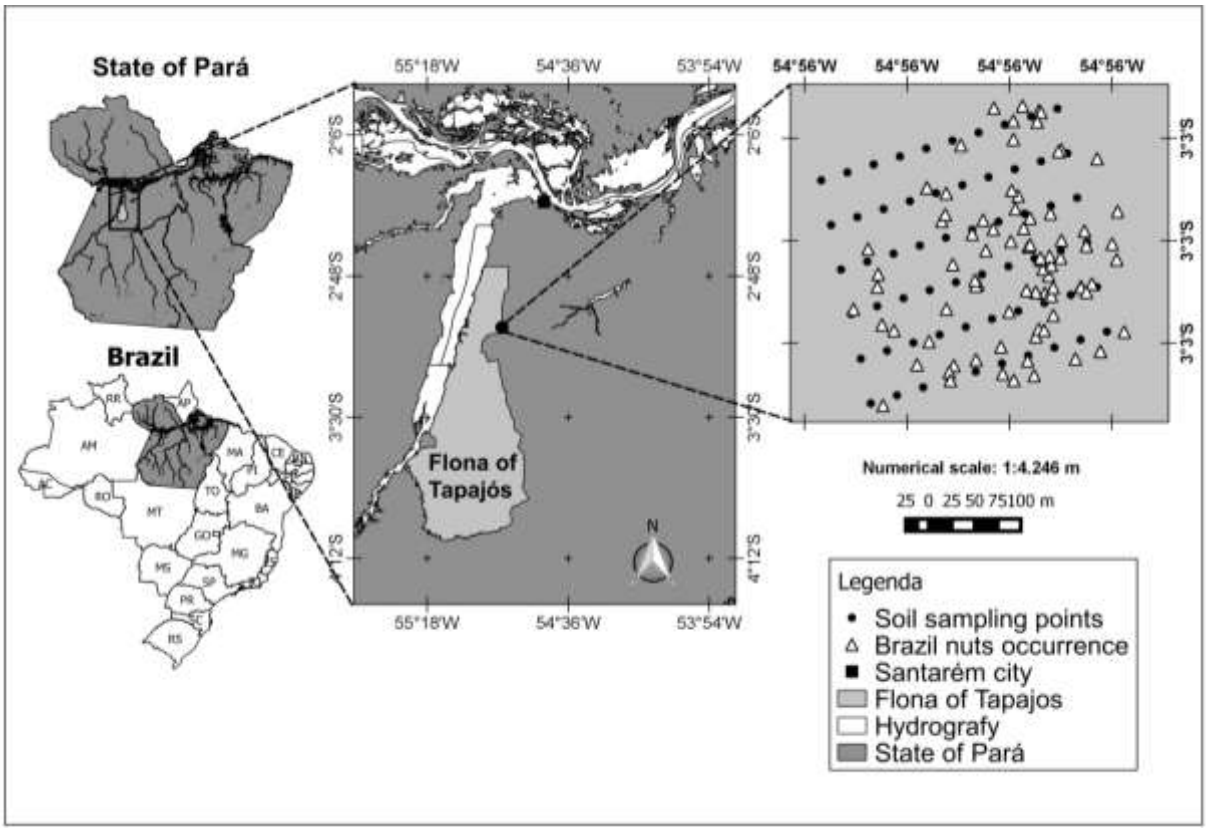

Figure 1. Localization of the study area in the FLONA Tapajós, Belterra-Para, with soil collection points following a systematic grid of 30 m × 50 m.

4º10' S and the meridians 54º45' and 55º30' W (Espirito-Santo et al., 2005), State of Para (north region of Brazil), in the central portion of the Amazon forest (Figure 1).

The region's climate, according to the classification of Köppen, is of the Type Ami (IBAMA, 2004). Through the common precipitation of the period from 1950 to 2000 of the station of Belterra-PA, Espirito-Santo et al. (2005) identified the presence of a rainy seasonal period (January - June) and other less rainy (July - December) well defined.

The area of the FLONA of the Tapajós covers part of the cities of Belterra, Aveiro, Ruropolis and Placa, extending through an area of approximately 545,000 ha (MMA, 2002). It was formed in the region of stratigraphic unit named Formação Barreiras (Barriers Formation), composed by rocks of fine sandstones and grey shales calciferous, being constituted mainly by red continental sediments and formed by intercalations of sandstones and mudstones with conglomerates subordinated (Damasceno, 2001; IBAMA, 2004). There are found Dystrophic Yellow Latosol, Argissolos Red-Yellow, Petric Plinthosols dealers and Entisol. The classes Argissolos Red-Yellow and Dystrophic Yellow Latosol occupy respectively, 37.1 and 25.34% of the FLONA (Espirito-Santo et al., 2005).

The region's vegetation is classified as Ombrophilous Dense Forest (Veloso et al., 1991), with abundance of large arboreal individuals of woody lianas, palmtrees and epiphytes. IBGE (1997) inform the predominance of the genus *Hevea*, *Bertholletia* and *Dinizia*. Guimarães (1999) and Gonçalves and Santos (2008) present more detailed floristic information of the FLONA Tapajós.

The anthropic actions in the area (extractive activities, hunting, fishing, wood extraction, family farm system in agriculture, and others) are promoted, in part, by the residents of communities. According to estimates, there are approximately 10,696 people living in the FLONA, distributed in 26 communities (IBAMA, 2004).

Collection, preparation and physicochemical analysis of the soil samples

The soil samples were collected in intervals of 30 m in line, in all of the lines from the grid of the study area, totalizing 60 sampling points (Figure 1). All of the points were georeferenced. For the determination of texture, and bulk and particle densities samples were collected at depths of 0-15, 15-30, 30-45 and 45-60 cm. For aggregate analysis and determination of the water retention curve, sampling was done in the two superficial layers. For the soil chemical determination, the sampling collection was conducted with a dutch auger at a depth of 0-20 cm. The preparation of the samples for the chemical and physical analysis and the calculation of the studied variables were done as in Nogueira and Souza (2005).

Soil texture was determined by the nugget method, and the density particle density (Dp), by the volumetric balloon method, utilizing ethyl alcohol, as the penetrating liquid, to measure the soil volume. The bulk density (Bd) was determined by the volumetric ring method. The total porosity (Tp) was calculated by the equation Tp = (1 - Bd/Dp). The microporosity was obtained by the curve of water retention in the soil, in tension equivalent to 6 kPa. The macroporosity resulted from the difference between total porosity and microporosity. The water retention in the tensions of -10, -33, -100, -500, -1,000 and -1500 kPa was determined with sampling, previously saturated with water, on a porous ceramic plate by the

application of these tensions, inside of a pressure cooker.

With these points (q, ym) determined, the adjustment of the retention curves of water was done according to the proposed model by Van Genuchten (1980). This adjustment was realized by the method that considers: qs = qmax, with ym = 0 and, qr = qmin, with ym = - 1,500 kPa. With these curves, the distribution of the size of the pores was calculated using the following system: (a) pores = 50 mm - by the difference between the total porosity value and the volumetric humidity obtained in the pressure of -6 kPa; (b) pores between 50 and 30 mm - difference of volumetric humidities between 6 and 10 kPa; (c) pores between 30 and 10 mm - difference of volumetric humidities between -10 and -30 kPa; (d) pores between 10 and 3 mm - difference of volumetric humidities between -30 and -100 kPa; (e) pores between 3 and 0.2 mm - difference of volumetric humidities between -100 and -1,500 kPa; (f) pores < 0.2 - value of the volumetric humidity in the pressure of -1,500 kPa.

The pH was determined using a suspension of soil: water with relation of 1:2.5, and the Walkley-Black method (redox volumetry) was used to determine the organic carbon. The total nitrogen was determined by sulfuric acid digestion, Kjeldhal distillation and titration, and potential acidity was determined through the neutralization volumetry method, using as an extractor a solution of acetate of calcium, pH 7.0. For the calcium, the manganese and exchangeable aluminium extraction a potassium chloride solution (pH 7.0) was used. The value of calcium and manganese were obtained through atomic spectrophotometric absorption and aluminium was determined by volumetry utilizing 0.025N sodium hydroxide. Phosphorus, the potassium, the sodium and micronutrients (copper, zinc, iron and manganese) were analyzed utilizing the Melich I extractor solution with phosphorus determined by colorimetry, the sodium and potassium by flame spectrophotometer and micronutrients by atomic absorption spectrophotometry (Nogueira and Souza, 2005).

Data analysis

The statistical data analysis was conducted using an exploratory analysis of the data to verify the central and dispersal tendency measures, aiming to improve the efficiency of the spatial analysis through the identification of discrepant values and the removal of outliers. Statistics used in this analysis were: Maximum, minimum, medium, standard deviation and the coefficient of variation. The normality of the variables was verified by the statistical test of normality Shapiro-Wilk, with a level level of significance of 5% (Zar, 1999).

To describe and model the spatial patterns geostatistical analysis was used with the adjustment of the semivariogram (Equation 1), which corresponds to a mathematical tool that allows study of the spatial dispersal of a variable in function of the distance (Isaaks and Srivastava, 1989; Vieira, 2000).

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

In which, $\hat{\gamma}$(h) = semivariance of the $Z(x_i)$ variable; h = distance; N(h) = number of pairs of measured points $Z(x_i)$ and $Z(x_i + h)$, separated by a h(lag) distance.

For the experimental semivariogram generated the theoretical models were adjusted that provided the Nugget effect, landing and reach parameters. These parameters were estimated by the adjustment methods of the theoretical models, by the Ordinary Least Square (OLS) method and Weighted Least Square (WLS) methods. The statistical theoretical models adjusted for the comparison were the spherical (Equation 2), the exponential (Equation 3) and the gaussian (Equation 4), according to Isaaks and Srivastava (1989) and Vieira (2000).

$$\hat{\gamma}(h) = Co + C1\left[\frac{3h}{2a} - \frac{1}{2}\left(\frac{h}{a}\right)^3\right], \text{Spherical} \quad (2)$$

$$\hat{\gamma}(h) = Co + C1\left[1 - e^{\left(-3\frac{h}{a}\right)}\right], \text{Exponential} \quad (3)$$

$$\hat{\gamma}(h) = Co + C1\left[1 - e^{-3\left(\frac{h}{a}\right)^2}\right], \text{Gaussian} \quad (4)$$

In which, $\hat{\gamma}(\boldsymbol{h})$= is the value of the estimated semivariance for the h distance; a = reach, corresponds to the distance after the one in which the spatial semivariance stabilizes; h = distance between measures; Co = nugget effect; C = landing, it is the value spatial semivariance that corresponds to its (a) reach; C1 = contribution, corresponds to the difference between the landing (C) and the nugget effect (Co).

In the Index of Spatial Dependency (ISD) of the variables, the classification of Cambardella et al. (1994) was used that proposed the following intervals to evaluate the index of spatial dependence of the phenomena: Values lower than 25% are considered as having strong spatial dependence; those between 25 and 75% indicated moderate spatial dependence, and values higher than 75% determine weak spatial dependence. The ISD value was obtained by Equation 5.

$$ISD = \frac{Co}{Co+C1}\ x\ 100 \quad (5)$$

From each one of the adjusted models, the interpolation by the simple kriging was conducted which generated the mapping of all of the soil variables in the area of the Brazil nut trees. The simple kriging implicitly evaluated the medium in the sampling space by neighborhood (Isaaks and Srivastava, 1989; Yamamoto and Landim, 2013), therefore, the estimated value in a random spatial x_0 position was obtained by the Equation 6.

$$\hat{z}(x_0) = \sum_{i=1}^{n} \lambda_i z(x_i) \quad (6)$$

In which: $\hat{z}(x_0)$ = is the estimated value for the point x_0; λ_i = are to the weight of the kriging defined according to the semivariogram's parameters; $z(x_i)$ = are the values observed in the sampled points (sampling space by neighborhood).

Definition of the best model utilized the technique of cross-validation, that consists in prediction of the known value $z(x_i)$ of the random variable, comparing to the observed value. The errors of the observed and predicted values were analyzed through the statistics: Mean error (ME), root mean square standardized (RMSS) and absolute error (AE) described by Vieira (2000).

The analysis were realized in the computational environment R (R-Development-Core-Team, 2015), associated to the outliers packages (Komsta, 2006) for the identification and removal of the outliers, nortest (Ross and Ligges, 2015) for the test of the normality and geoR (Ribeiro and Diggle, 2001) for the geostatistical analysis with the semivariogram adjustment and realization of the simple kriging.

RESULTS AND DISCUSSION

The results of the descriptive statistics and the geostatistics analysis for the physicochemical properties of the studied soil are presented in Table 1.

The obtained values for the variables presented a normal distribution by the Shapiro-Wilk (significance of 5%) test except for the phosphorus, potassium, sodium, calcium, zinc, manganese and copper. According to

Table 1. Descriptive statistics and results of the geostatistical analysis of the physicochemical properties of the soil under native Brazil nut trees in the FLONA Tapajós, Para.

Variable	Descriptive statistics			Geostatistical analysis				
	Average	SD	CV (%)	Model	Nugget effect	Landing	Practical reach (m)	ISD (%)
Physical								
Total sand ($g.kg^{-1}$)	372.4	23.2	11.1	Spherical	77.82	544.72	115.09	14.3
Silt ($g.kg^{-1}$)	102.7	16.9	17.3	Gaussian	180	304	71.71	59.2
Clay ($g.kg^{-1}$)	524.9	27.2	7.4	Spherical	240.56	740.66	92.12	32.5
TP ($g.cm^{-3}$)	0.559	0.04	7.3	Gaussian	0.0009	0.00177	111.31	50.9
Macrop. ($g.cm^{-3}$)	0.208	0.03	23.5	Gaussian	0.0005	0.00101	132.02	49.5
Microp. ($g.cm^{-3}$)	0.352	0.04	10.2	Gaussian	0.001	0.00144	48	71.4
CC	0.329	0.03	10.7	Gaussian	0.00093	0.00117	72.47	80.6
PWP	0.235	0.02	11.2	Spherical	0.00038	0.00059	55.29	63.8
Chemical								
pH (H_2O)	4.08	0.18	4.5	Exponential	0.0245	0.00335	179.74	73.1
Carbon ($g.kg^{-1}$)	13.4	2.01	19.0	PNE	-	-	-	-
Nitrogen ($g.kg^{-1}$)	1.13	0.12	13.8	PNE	-	-	-	-
C/N	11.92	1.46	14.1	PNE	-	-	-	-
Phosphorus ($mg.dm^{-3}$)	2.75	0.38	18.0	Gaussian	0.078	0.142	79.26	54.9
Potassium ($mg.dm^{-3}$)	21.27	6.7	32.6	Spherical	76.28	92.38	54.16	66.0
Sodium ($mg.dm^{-3}$)	3.29	1.29	53.0	PNE	-	-	-	-
Calcium ($cmol_c.dm^{-3}$)	0.175	0.12	84.8	PNE	-	-	-	-
Magnesium ($cmol_c.dm^{-3}$)	0.209	0.08	46.8	PNE	-	-	-	-
Alumínio ($cmol_c.dm^{-3}$)	1.59	0.35	22.1	PNE	-	-	-	-
Iron ($mg.dm^{-3}$)	225.2	44.3	23.7	PNE	-	-	-	-
Zinc ($mg.dm^{-3}$)	0.727	0.16	35.2	Exponential	0.0193	0.0283	272.16	68.7
Manganese ($mg.dm^{-3}$)	3.14	2.06	80.1	Spherical	3.33	4.98	180.3	66.9
Copper ($mg.dm^{-3}$)	0.341	0.18	75.3	Gaussian	0.024	0.034	227.30	70.8

TP, Total porosity; Macrop., macroporosity; Microp., microporosity; CC, camp capacity; PWP, permanent wilting point; C/N, relation carbon/nitrogen; ISD, index of spatial dependency; SD, standard deviation; CV, coefficient variation; m, meters; PNE, pure nugget effect.

Isaaks and Srivastava (1989), normality is not the determinant factor for the realization of the geostatistical analysis, being more important the existence of sills well defined in the semivariograms. Rachid Junior et al. (2006), and Souza et al. (2010), realize the geostatistical evaluation of the variables that did not present normality and obtained well defined sills.

The coefficient of variation (CV) obtained for the physical variables of the soil samples was considered low (CV < 12%), as proposed by Warrick and Nielsen (1980), except for the silt and macroporosity, that presented moderate variation (12% < CV > 60%). The silt presented more expressive variability due to its greater mobility in the soil and deposition in the floodplain (Santos et al., 2012). The soil chemical variables showed a more heterogeneous behavior, with only pH in the interval of low variation; calcium and manganese presented high variability (CV > 60%) and the remaining variables presented moderate variation. Aquino et al. (2014), studying the spatial distribution of chemical properties of soil in the Amazon forest, also registered moderate and high variation. For Carvalho et al. (2003), it is common that the variability of the soil properties presents moderate to high values, because there are many environmental factors that interfere in its dynamics of such. Souza et al. (2014) highlights that the mapping of the soil properties with higher variability can be less precise.

After the exploratory analysis, the values obtained for the physicochemical variables were submitted to the geostatistical analysis with the aim to verify its spatial dependence. The variables: carbon, nitrogen, C/N, sodium, calcium, magnesium, aluminium and iron presented a Pure Nugget Effect (Figure 2), meaning that, they are spatially independent and it was not possible to determine the variogram components. For Silva et al. (1989) that happens when the spacing adopted in the sampling is higher than needed to reveal the spatial

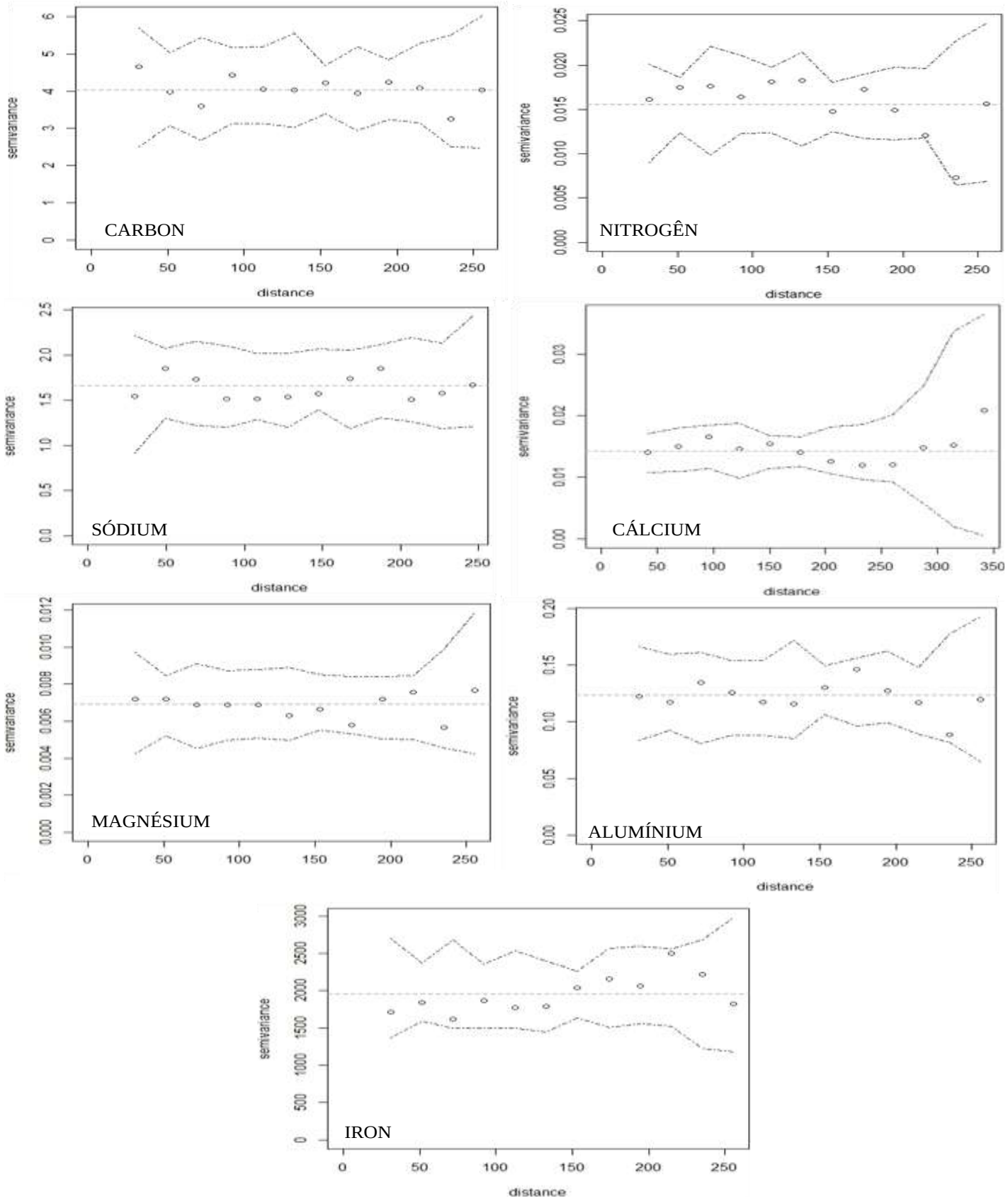

Figure 2. The spatial dependence analysis presenting only the sill (pure nugget effect), for the variables: Carbon, nitrogen, sodium, calcium, magnesium, aluminium and iron.

dependence. The nugget effect indicates the variability not explained or the variation not detected and occurs due to measurement errors and or when the sampling configuration was not robust enough (Cambardella et al., 1994). Therefore, the nugget effect can show the nature of spatial dependence (Chaves and Farias, 2008), the continuity of the phenomena, and the confidence in the estimate (Vieira, 2000; Yamamoto and Landim, 2013).

For the other studied variables, the sampling configuration was sufficient to determine the spatial dependence. The variables that presented a more expressive nugget effect were microporosity, phosphorus, zinc, manganese and copper. Cerri et al. (2004), studying the variables of a soil in the state of Rondonia, adopted sampling with distance between the points of 25 m and also obtained an elevated nugget effect in most of the variables. That indicates that there is high variability inside a small space, in a shorter distance to that practiced in the collections (Novaes Filho et al., 2007).

The majority of the studied variables presented good semivariogram behavior, allowing for semivariogram adjustment for the gaussian, spherical and exponential models. Thus, the choice of the model was determined by the best adjustment of the line to the points located in the contribution band of the semivariogram, by the cross-validation methodology (through the relation: predicted/observed). For the physical variables (Figures 3 to 5), there was predominance of the adjustment to the gaussian semivariogram model, followed by the spherical model. In the case of the chemical variables (Figures 6 and 7), there were two occurrences for each model of adjustment (spherical, exponential and gaussian).

The gaussian model was the most prevalent model in the works of Machado et al. (2007) and Souza et al. (2008) that studied physical variables of a Red Latosol and a Fluvisol, respectively. This model has a large reach and its landing presents equal values to the exponential model; it is a transitive model, appropriate for modelling continuous phenomena (Isaaks and Srivastava, 1989). The spherical and exponential models describe properties with high spatial continuity, or less erratic in short distance (Isaaks and Srivastava, 1989) and are considered the most common when working with soil and plant variables (Carvalho et al., 2003; Lima et al., 2013).

As in the established intervals by Cambardella et al. (1994), the physical variables presented moderate spatial dependence (25% < ISD > 75%), except the total sand and the field capacity, which obtained the values of 14.3 and 80.6%, being categorized in the strong (< 25%) and weak (> 75%) classifications, respectively. The percentual values obtained for the chemical variables did not present high discrepancy and are considered moderate. The strong spatial dependence demonstrates that the semivariograms explain the greater part of the variance of the experimental data with high confidence in the estimate (Souza et al., 2010) and generally are more influenced by factors of formation of the soil. According to Cambardella et al. (1994), values of weak spatial dependency can indicate sites that are suffering higher external pressure and moderate spatial dependency occurs when there is homogenization of the soil. In this study, 86% of the variables presented moderate spatial dependence. Campos et al. (2013) and Aquino et al. (2014) also found moderate spatial dependence for the majority of the physical variables of their soil sampling in the Amazon.

The range is another important parameter in the study of the semivariogram, because it corresponds to a maximum distance (influence zone) in which a variable is spatially correlated, meaning that, the model establishes a maximum distance to where the value of a variable demonstrates spatial dependence with its neighbors (Santos et al., 2012; Oliveira et al., 2013). Therefore, analyses done at higher distances than the established range will have random distribution and, because of that, they are independent between themselves. In a practical way, the range of a variable guarantees that all of the neighbors are so similar that they can be used to estimate values for any point (Machado et al., 2007). A lower interval than the range provides soil samples with superposition of the spatial characteristics; on the other hand, a higher interval than the range does not comprise the spatial variability, while the medium value obtained does not reflect the area studied (Motomiya et al., 2011).

In the current study, the higher ranges for the physical variables were registered for macroporosity (132.02 m), total sand (115.09 m) and total porosity (111.31 m) and the lower range (48 m) for microporosity. Within the chemical variables, the higher ranges were identified for zinc (272.16 meters) and copper (227.30 meters) and lower for the potassium (54.16 m). A future experiment that will be conducted in the same area under the same conditions will generate Geostatistical results (Table 1) that will serve to better estimate the necessary number of samples necessary for each variable. According to Carvalho et al. (2002), in order to guarantee spatial dependence, sample points should be collected at a distance equal to half the reach value and in a systematic grid arrangement.

Relation of the variables with the Brazil nut trees

Through simple kriging it can be estimated the concentration of the studied nutrients for the area of the sampling grid (interpolation), was delimited due to the expressive occurrence of Brazil nut trees. All of the individuals of this species were georeferenced and their coordinates were plotted in the simple kriging maps, in order to visualize their occurrence in relation to the concentration of the variables of the soil. A higher concentration of Brazil nut trees was identified in the areas with higher values for the silt and clay variables

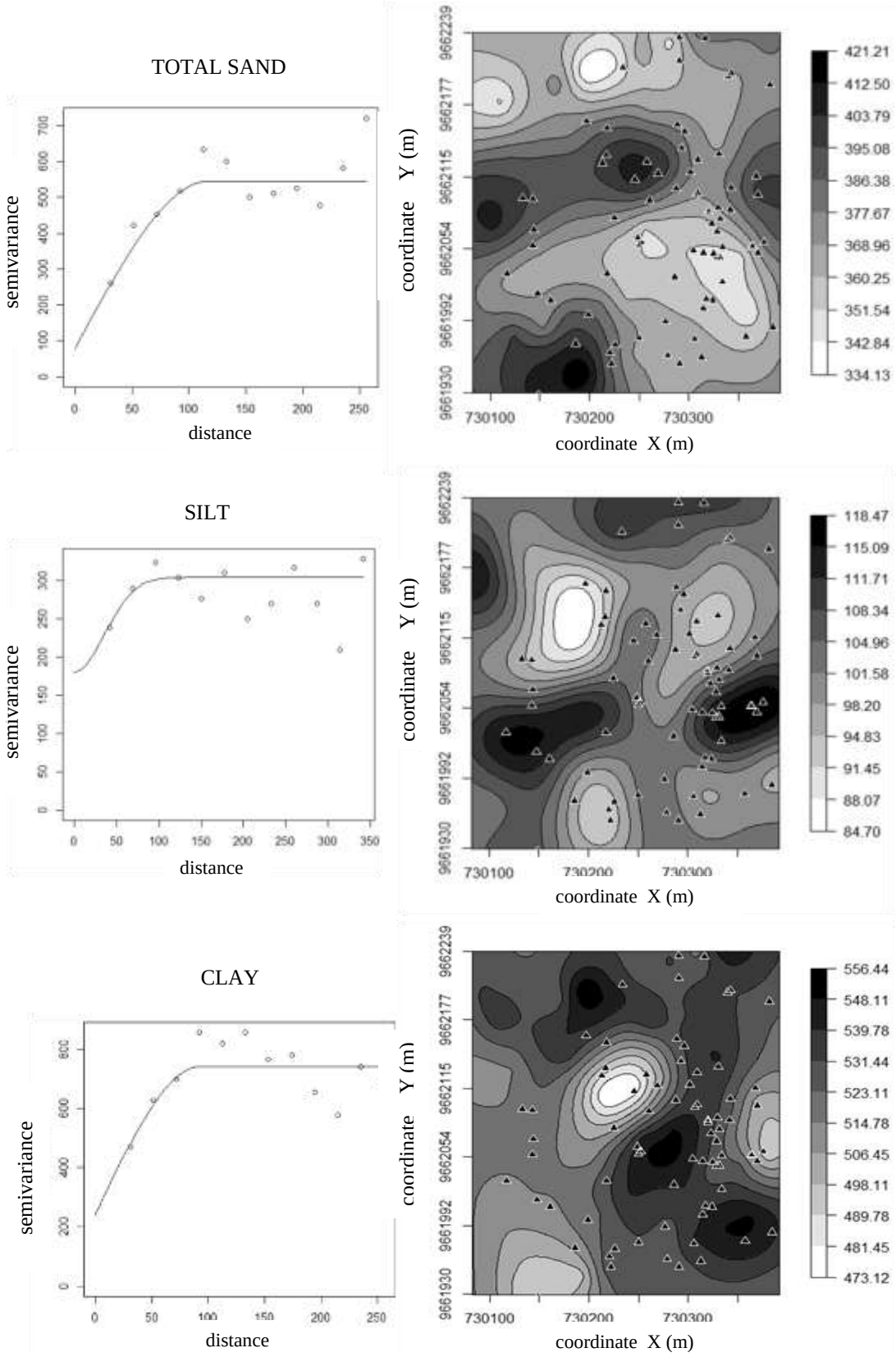

Figure 3. Semivariograms adjusted for the spherical (total sand and clay) and gaussian (silt) models and their related maps obtained through the simple kriging process. The symbol in the shape of a triangle represents the georeferenced Brazil nut trees in the study area.

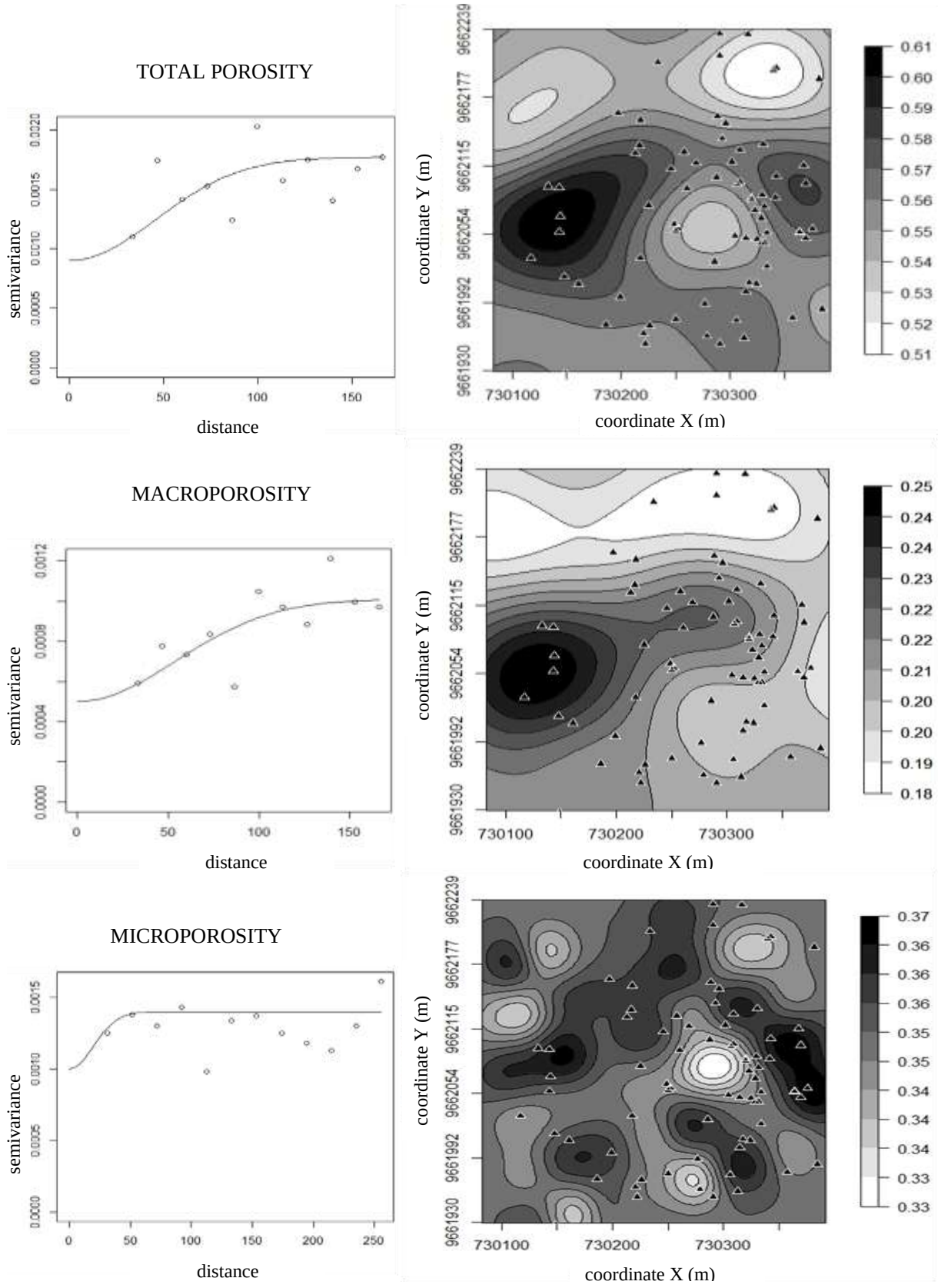

Figure 4. Semivariograms adjusted for the gaussian model (total porosity, macroporosity and microporosity) and its related maps obtained through the process of simple kriging. The symbol in the shape of the triangle represents the georeferenced Brazil nut trees in the study area.

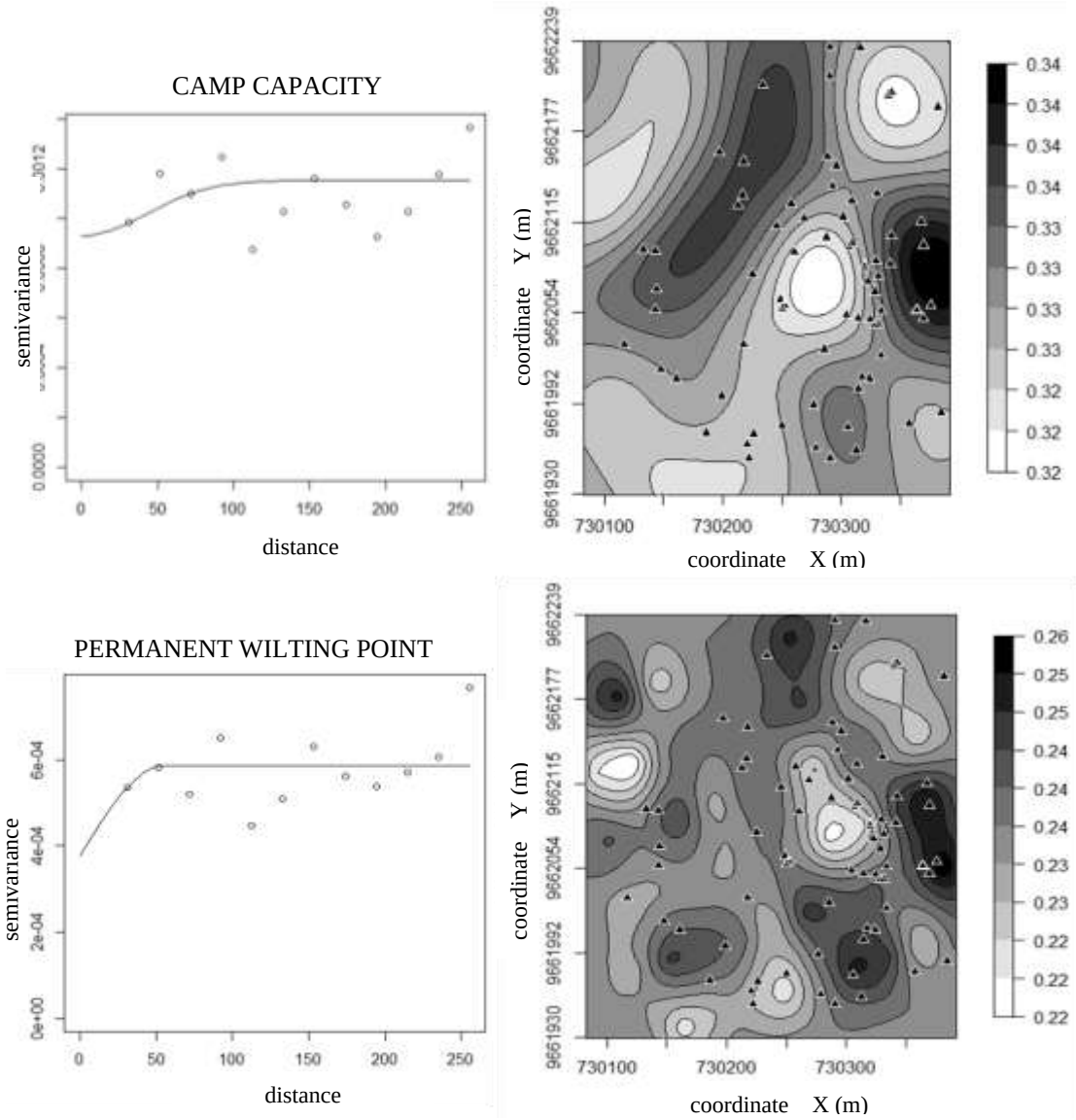

Figure 5. Semivariograms adjusted for the gaussian (camp capacity) and spherical (permanent wilting point) models and their related maps obtained through the simple kriging process. The symbol in the shape of a triangle represents the georeferenced Brazil nut trees in the study area.

(Figure 3) and smaller values for the macroporosity (Figure 4), pH, phosphorus (Figure 6), zinc and copper (Figure 7).

The predominance of individuals in the more clayey part in study area corroborates with the studies of Fernandes and Alencar (1993), Muller (1995) and Espirito-Santo et al. (2005). The results of these authors indicated that this species presents better production in soils with clayey to very clayey texture and soils of sandy texture are unsuitable to maximize the growth potential of this species.

The lower values obtained for phosphorus, zinc, and copper levels in the area of higher Brazil nut tree population indicates the demand that this species has for these nutrients. Lima and Azevedo (1996), studying the Brazil nut tree in agroforestry systems on a yellow Latosol, in the State of Amazonas, identified around 200% greater growth of three-year old individuals after the incorporation of nitrogen, phosphorus, potassium, magnesium and micronutrients. Lima et al. (2004), evaluated the growth response of Brazil nut trees with respect to the soil physicochemical variables and concluded that the variables total sand, silt, available water, phosphorus, zinc, sodium, aluminium and magnesium were the ones that influenced its growth.

As there is generally predominance of individuals in areas with lower pH values, the study of Locatelli et al. (2002) analyzed the chemical variables of an Argisol in a

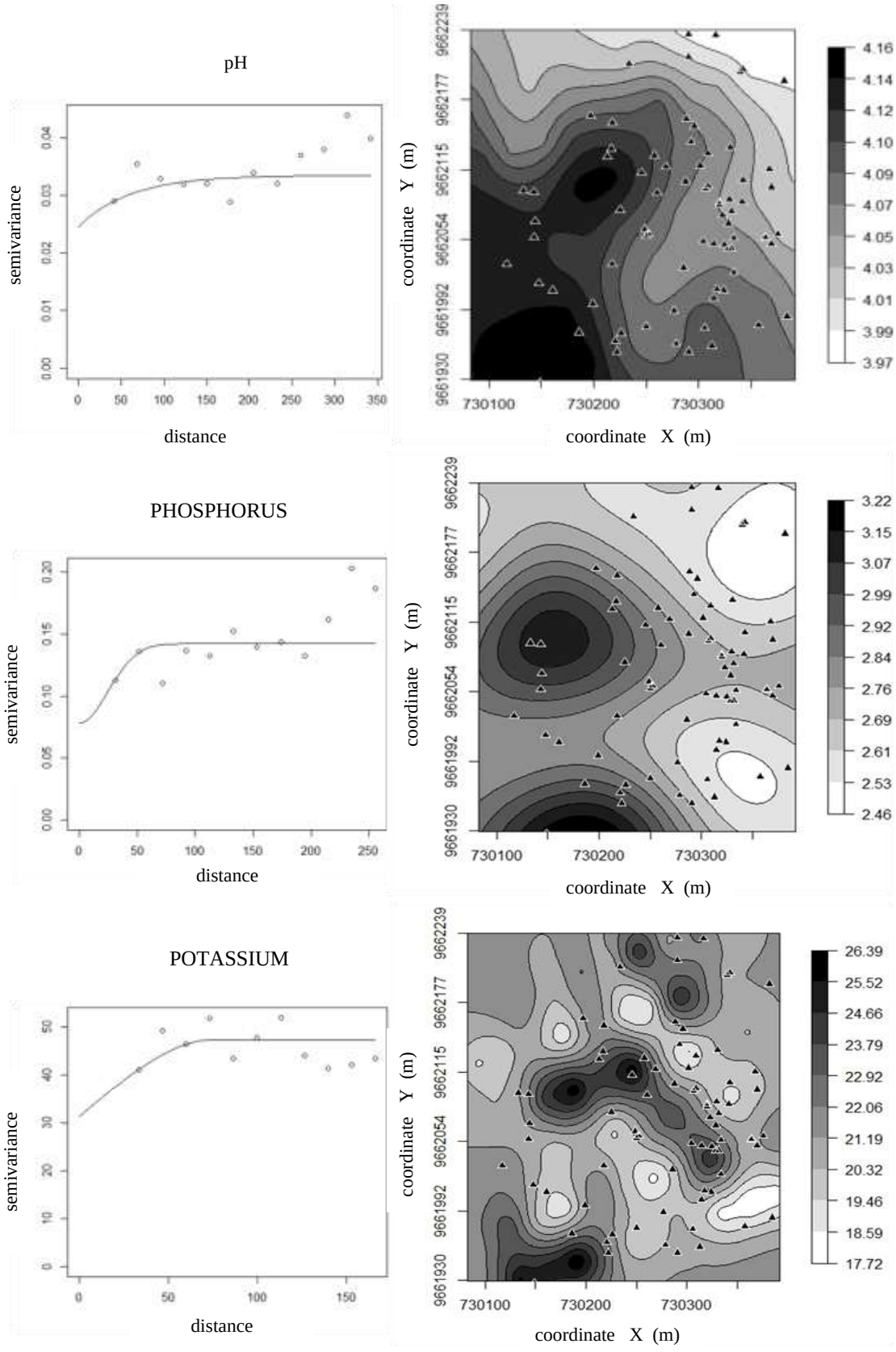

Figure 6. Semivariograms adjusted for the exponential (pH), gaussian (phosphorus) and spherical (potassium) models and their related maps obtained through the simple kriging process. The symbol in the shape of a triangle represents the georeferenced Brazil nut trees in the study area.

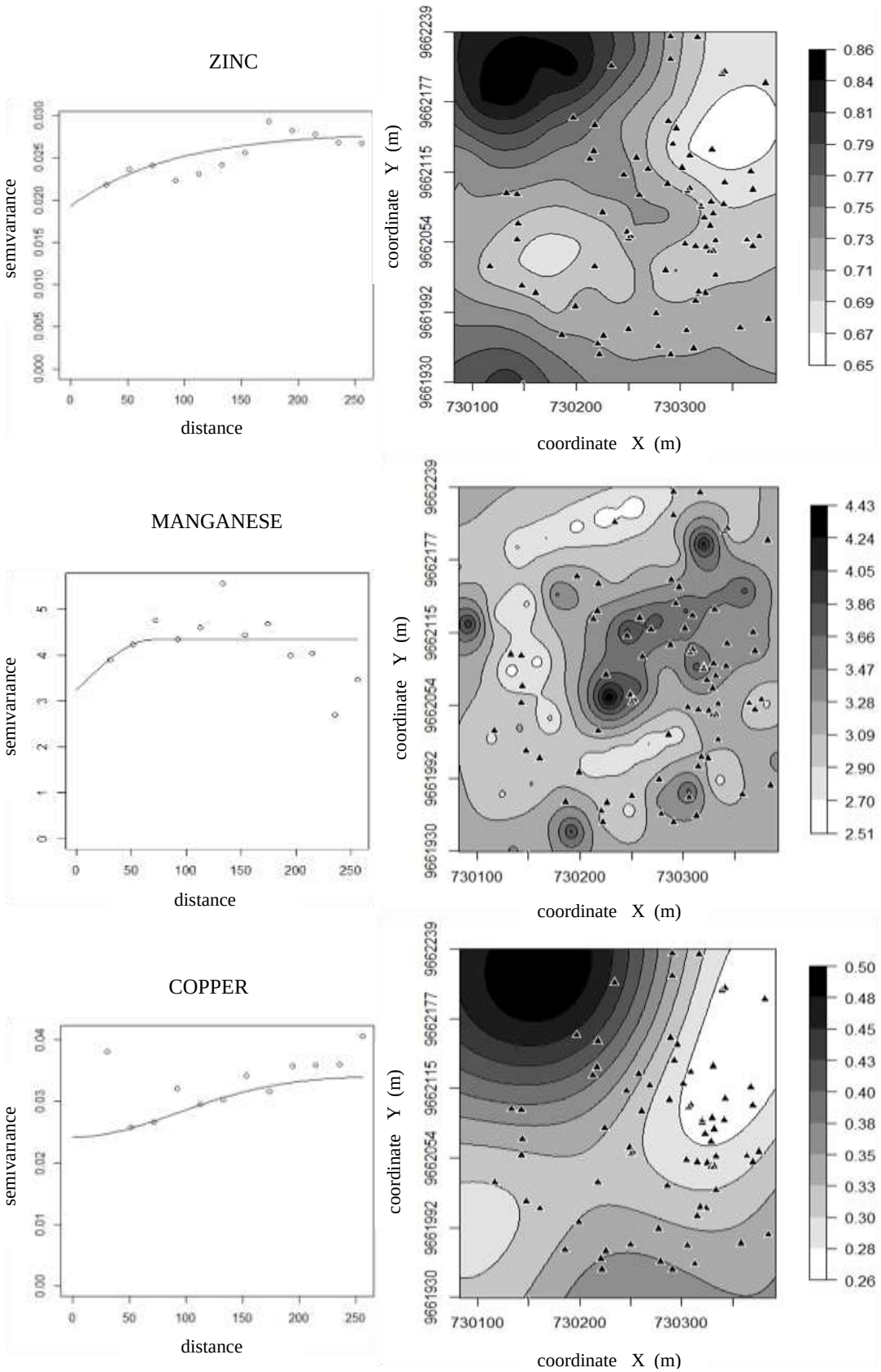

Figure 7. Semivariograms adjusted for the exponential (zinc), spherical (manganese) and gaussian (copper) models and their related maps obtained through the Simple Kriging process. The symbol in the shape of a triangle represents the georeferenced chestnut trees in the area of study.

Brazil nut tree plantation, and identified a good development in height and diameter of individuals on soil with low values of pH, cation exchange capacity and high values of aluminium saturation.

Considering that the quantity of nutrients in the soil also is an important factor in the production of fruits of the Brazil nut tree (Zuidema, 2003), highlights the importance of the products (simple kriging maps generated in this research) of the above variables, because they can direct management decisions aiming to foment the production of fruits in the area. Another study that reinforces this idea is the study by Kainer et al. (2007), that identified that the nutritional variable of the soil that best explained the variation of the yearly production of the fruits of the Brazil nut tree, in the State of Acre, was the cation exchange capacity (positive correlation) and the phosphorus concentration (negative correlation).

The kriging maps presented in this study provided information that can help to generate better practices for forest management that aim to maintain and/or enlarge the production of these Brazil nut trees in the FLONA Tapajós. The remaining variables, that do not present a more defined spatial behavior in relation to distribution of the Brazil nut trees, require more detailed studies to determine their correlation with this species.

The results for each variable presented in the kriging maps, in relation to the distribution of the Brazil nut trees, provide basic information that can be used to compare to other studies done in areas of Brazil nut tree production, and using these correlations it is possible to meet several of the objectives of the MapCast project, especially those whose goal is to understand whether or not there exists a pattern between the distribution of Brazil nut trees and soil physical and chemical properties in different regions of the Amazon basin, and also to identify environmental characteristics that should be taken into consideration in defining Brazil nut tree management practices.

The kriging maps can also be used to elucidate forest management practices that have the goal of maintaining and increasing the production of this area in the FLONA Tapajós since the results show the areas that have lower nutrient concentration in relation to the occurrence of Brazil nut trees.

Conclusions

Geostatistical analysis helped to elucidate to discern the spatial distribution of soil physical-chemical characteristics in the study area which will serve as a base for comparison for future studies in the same area to help to understand environmental aspects of other Brazil nut tree stands. The reach values obtained demonstrate lower variability for zinc, copper, and pH, and greater variability for microporosity, potassium, and silt. The simple krieging maps can help to more effectively choose areas in which future management actions should be focussed in order to optimize costs for fertilizers and soil sampling for variables that present reach values higher than those established by the 30 × 50 grid. For the variables that presented a pure nugget effect (carbon, nitrogen, sodium, calcium, magnesium, aluminum, iron) future spatial analyses should be done on a reduced grid of 15 × 50 m in size. This result is a reflection of the 120 sampling points used in the study area, and the viability of the use of geoestatistics and krieging in the elaboration of thematic maps that can be used in management of agricultural production systems. The variables that show a stronger spatial relationship with Brazil nut trees were silt, clay, macroporosity, pH, phosphorus, zinc, and copper. These are pioneering results that will be generated by the MapCast project, and will help to characterize and understand the environmental factors that influence production in Brazil nut tree stands in the Brazilian Amazon through geospatial data in order to contribute to adoption of good management practices.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Phytosociology and weed interference in okra under organic cropping system

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A research was carried out to study phytosociology and to determine the periods of weed interference in the okra crop in organic cropping system. The experiment was laid out in a randomized complete block design with 20 treatments and four replications. The treatments were 10 periods of weed coexistence with the crop during 0-7, 0-14, 0-21, 0-28, 0-35, 0-42, 0-49, 0-56, 0-63 and 0-74 days after crop emergence (DAE) and 10 periods weed free during 0-7, 0-14, 0-21, 0-28, 0-35, 0-42, 0-49, 0-56, 0-63 and 0-74 DAE. The weeds were evaluated by the number of individuals and accumulated dry biomass of each population in the treatments. The relative frequency, relative density, relative dominance and importance value index of each species were used to determine the effect the weeds. The most important weeds based on the Importance value index were *Commelina benghalensis* L., *Cynodon dactylon* (L.) Pers., *Eleusine indica* L., *Phyllanthus niruri* L. and *Alternanthera tenella* Colla. The period prior to interference and the total period of interference prevention were respectively of 12 and 36 days after emergence. The weed community caused yield losses of around 51%. In okra organic cropping system, weed control should be done early to boost okra plant growth in order to provide shade on the weeds to reduce the need for long control period.

Key words: *Abelmoschus esculentus* (L.) Moench, competition, weed community.

INTRODUCTION

Okra [*Abelmoschus esculentus* (L.) Moench], is a well adapted species to tropical and subtropical climates. It is widely grown in Brazil, particularly by smallholders (Purquerio et al., 2010) in conventional cropping systems, however it can be grown in organic cropping systems as well, since it generally demands high amount of organic fertilizer which is essential for proper plant nutrition, fruit quality and yield increase with less or no use of synthetic fertilizers (Sediyama et al., 2009).

Accordingly, Premsekhar and Rajashree (2009) observed that the application of organic fertilizers resulted in various positive effects on okra growth and yield. However, a limiting factor in okra production in organic system is weed interference.

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Ibrahim and Hamma (2012) found that treatments with no fertilizer and no weed control resulted in okra growth parameter values significantly lower, due to weed competition for soil nutrients, giving to weeds competitive advantages over the crop. Thus, it becomes necessary to know the weed species occurring in okra crop in order to decrease yield losses and production costs.

Weed community composition studies in conventional okra cropping system were carried out by Bachega et al. (2013). They identified the species *Portulaca oleracea, Eleusine indica* and *Nicandra physaloides* among the main weeds. Santos et al. (2010) found that the okra dry matter was affected by *Alternanthera tenella*, *Arachis pintoi*, *Bidens pilosa*, *Commelina benghalensis*, *Cyperus rotundus*, *Ipomoea nil* and, especially, *Eleusine indica* which reduced the number okra leaves. Conversely, Dada and Fayinminnu (2010), studying weed control in okra in Nigeria, reported that *Euphorbia heterophylla, Talinum triangulare, Eleusine indica, Cynodon dactylon, Commelina benghalenis, Cyperus iria, Cyperus difformis*, were the predominant species among the weed community.

Knowledge of the weed species growing in association with okra in organic cropping system is scarce, as well as the determination of the periods in which they interfere with crop yield. According to Pitelli (2014), knowledge of factors affecting the coexistence and interference relationships between crops and weeds is critical to establish cultural practices to direct resources to crop grow at the expense of weeds.

Studies on the determination of weed community interference periods in okra conventional cropping system carried out by Bachega et al. (2013), showed the Period Before Interference (PBI) of 57 days after emergence (DAE) that is, the crop can coexist with the weed community for this period without yield decrease, and the Total Period of Interference Prevention (TPIP) of 14 days, suggesting a short weed control period to ensure crop yield. However, Santos et al. (2010) also studying okra in conventional cropping system, observed lower PBI (25 DAE) and higher TPIP (100 DAE).

Carvalho et al. (2008) emphasized that different critical periods reflect the conditions of crop establishment and management in different times and locations, particularly with respect to soil and climate conditions, the weed community composition and the degree of weed infestation in the study area. Therefore, studies of periods of weed interference in okra should be performed in different cropping systems.

Taking into account that knowledge of the weed species growing in association with okra organic cropping system is of great importance for assessing the degree of interference on crop growth and yield, as well as to subsidize adaptations on weed management, this research aimed to study phytosociology and to determine the periods of weed interference in okra organic cropping system.

MATERIALS AND METHODS

The experiment was conducted between January and April 2014 in a certified organic production area in the municipality of São Luís, state of Maranhão (2°37'39.69'' S and 44°11'15.7'' W), northeastern Brazil. Local climate is of the Aw' type, according to Köppen classification, equatorial, hot and humid with a rainy season from January to June (average 2,010 mm) and a dry season from July to December (average 180 mm), average annual temperature is 26.1°C and average relative humidity is 88% (Instituto Nacional de Meteorologia, 2009).

The soil is classified as Paleudalf (Embrapa, 2013). Chemical analysis indicated the soil pH ($CaCl_2$) = 5.4; organic matter = 26 g dm^{-3}; P = 111 mg dm^{-3}; K = 2.3 mmolc dm^{-3}; Ca = 33 mmolc dm^{-3}; Mg = 14 mmolc dm^{-3}; SB = 49.3 mmolc dm^{-3}; H + Al = 26 mmolc dm^{-3}; CEC = 75.3 mmolc dm^{-3}.

Land preparation consisted of cleaning the area through mowing and opening of holes, with subsequent plot demarcation. Basal fertilization was done as 27.8 t ha^{-1} of chicken manure, 0.46 t ha^{-1} of natural phosphate, 0.023 t ha^{-1} potassium sulfate and 0.20 t ha^{-1} ash. Okra seeds were previously soaked in water for 24 h in order to break dormancy. Planting was done manually with four seeds per hole.

The experiment was laid out in randomized complete block design, with 20 treatments and four replications. Plots consisted of four rows of 3.20 m length spaced 1.20 m between rows and 0.40 m between plants within rows. The useful plot size for harvesting and non-destructive evaluation was composed of the two central rows, excluding two plants from each end, totaling 20 okra plants.

The treatments were divided into two groups: Weed control periods (weeded) and coexistence periods of the weeds with the crop (unweeded). In the first group, the crop was free from weed interference, by hoeing in the following periods: 0-7, 0-14, 0-21, 0-28, 0-35, 0-42, 0-49, 0-56, 0-63 and 0-74 DAE (harvest). After these periods, the weeds that emerged were left to grow freely, while in the second group, the crop was left in coexistence with weeds from emergence to the periods described above for the first treatment group. After each period of coexistence, the okra plants were kept weed free by hand weeding. The early application of treatments was considered from 80% of the crop emergence.

Okra plant thinning was performed at 10 DAE when the plants were 10-15 cm in height, leaving two plants per hole. The experiment was sprinkler irrigated during January and February in order to meet the crop water demands. Foliar fertilization was performed at 33 DAE with biofertilizers in the amount of 180 L ha^{-1} at the rate of 0.5 to 20 L of water.

Weed sampling was carried out using a 0.50 m × 0.50 m open metal rectangle which was placed at random three times in the plots. The aerial portions of the weeds were harvested, counted and identified by family, genus and species. Thereafter, the plants were placed in an oven with forced air ventilation at 65-70°C for 72 h until constant weight, and then weighed on a 0.01 g precision scale. The data obtained from each sample was used to perform the weed community phytosociological study, by computing the relative density, absolute and relative frequency and importance value index for each weed species (Mueller-Dombois and Ellemberg, 1974).

Plant height was taken at 32 DAE by measuring 10 okra plants from the soil to the apex from the useful area from each treatment plots. Crop harvest started at 46 DAE. Harvest was done every two days when the fruits showed intense green color, finishing at 74 DAE with a total of 13 harvests. Only fruits that had commercial grade "12" (fruits with a length between 12 and 15 cm) were harvested. Data on plant height and marketable yield were processed by treatment (initial periods of control or coexistence of weeds with the crop) and were subjected to analysis of variance by F test at 5% probability and then applied the Student t test through SAEG 9.1 software (SAEG, 2007). The yield data were studied by

nonlinear regression and adjusted according to the Boltzmann sigmoidal model (Kuva et al., 2000) with the aid of the software ORIGIN 8.0 (OriginLab Corporation, 2002).

RESULTS AND DISCUSSION

The weed flora in the okra organic cropping system was represented by 44 species from 17 families, of which 38.46% were from the monocot group and 61.54% from the dicot group (Table 1).

In the okra conventional cropping system, Bachega et al. (2013) found 19 species arranged in 12 families with 63% of species belonging to the dicot group and 37% to the monocot group. These results show that the weed species diversity in the okra organic cropping system was higher when compared to the conventional cropping system, indicating that no soil disturbance, use of manure and favorable weather conditions contributed to greater weed community diversity.

The highest weed species richness occurred in the period of coexistence, with 41 weed species compared to control, with 30 species. The most important families also occurred during the coexistence periods, these were Poaceae, Amaranthaceae and Cyperaceae with nine, six and five species each, respectively, whereas Poaceae and Cyperaceae with five species each and Amaranthaceae with four species were the most important families in the weed control periods (Table 1). This result suggests that weed control performed since the beginning of the crop growth served as a selection factor for various weed species. Due and Fayinminnu (2010) noted the predominance of Poaceae and Cyperaceae with six and four species each, respectively, in Okra cultivation with fertilizer. However, Smith and Ojo (2007) found that species of these families were rare in okra conventional cropping system.

The population density of the weed community sharply decreased with periods of coexistence in the okra organic cropping system. The highest density occurred at seven DAE with 3,175 plants m^{-2} and the lowest was observed after 74 DAE (crop), with 313 plants m^{-2} (Figure 1a). The initial high weed density can be explained by mowing used in the organic system which favored the spread of some species by their fragmentation influenced by good climate and soil conditions which favored their sprout (Figure 1a). With crop development, interspecific associated to intraspecific competition were intensified which suppressed several species, reducing weed density.

The weed dry matter accumulation increased significantly from seven up to 49 DAE, with values between 51.51 and 1,271.35 g m^{-2}, respectively. Thereafter there was a tendency to stabilize the weed dry matter accumulation to harvest (Figure 1b). This behavior suggests that with the decrease in the weed density, the development potential of some weed species was expressed by greater soil nutrients recruitment, resulting in higher dry matter accumulation in the weed community. Therefore, in the final period of coexistence, the biomass accumulation prevailed on the weed community density.

In the weed control periods, the weed community density increased up to 21 DAE when it reached 758 plants m^{-2}. Thereafter, there was a rapid decrease until the last evaluation (63 DAE) when it reached the value of 538 plants m^{-2} (Figure 2a). The sharp weed density decrease can be explained by the initial control efficiency that contributed to enhance the intra and interspecific competition resulting in weed death and showed that the crop required an initial period with no weeds to enhance its development. According to Coelho et al. (2009), the evaluation of the weed community in weed control periods does not allow portray the entire crop cycle, since it occurs only at harvest, but it assists in verifying the competitive crop potential.

A sharp decrease in the weed dry biomass in the weed control periods was observed until 35 DAE when they reached 123.13 g m^{-2}. Subsequently, the dry matter accumulation was constantly lower until the last evaluation with 100.47 g m^{-2} (Figure 2b). The weed community dry biomass decrease is associated with the control efficacy of their density which also enhances crop growth and development potential. Ibrahim and Hamma (2012) studying okra cultivation with organic fertilizer found that the supply of manure and the three weeding regime promoted crop development suppressing weeds that were competing mainly for soil nutrients.

The weed species that were common to both periods and had higher relative importance in the okra crop organic cropping system were *A. tenella*, *C. benghalensis* and *C. dactylon*. Other important species were *P. niruri* in periods of coexistence and *E. indica* in the weed control periods (Figure 3a and b). These species are well adapted to growing conditions in organic cropping system and should have special attention on the adequacy of weed management in this system to not reduce crop yield.

The species of greater relative importance in the periods of coexistence was *C. benghalensis* except at 14 and 21 DAE when it was surpassed by *A. tenella*. The larger *C. benghalensis* IVI values were obtained at 35 and 63 DAE with 93.4 and 94.8%, respectively (Figure 3a). This was the result of its high density in the area, coming from the land preparation with mowing that fragmented the plant facilitating its spreading and the use of organic fertilizer which favored its growth resulting in increased dry biomass accumulation. Pitelli (2014) emphasized that soil fertilization influences not only the crop but also weed growth, and some weed species grow faster than crops because they are able to recruit more resources, including those not added by fertilization thereby exerting greater competitive pressure on the crop.

The *A. tenella* population was relevant in the organic

Table 1. Family and weed species identified in different periods of coexistence with the crop and of weed control in okra organic cropping system (São Luís, state of Maranhão, northeastern Brazil, 2014).

Family/species	Okra organic cropping system	
	Coexistence with weeds	Weed control
Amaranthaceae		
Alternanthera tenella Colla	P	P
Amaranthus deflexus L.	P	P
Amaranthus retroflexus L.	P	P
Amaranthus spinosus L.	P	A
Amaranthus viridis L.	P	A
Amaranthus sp.	P	P
Asteraceae		
Eclipta alba (L.) Hassk.	P	A
Emilia sonchifolia (L.) DC	P	A
Synedrella nodiflora (L.) Gaertn.	P	P
Boraginaceae		
Heliotropium indicum L.	P	A
Brassicaceae		
Cleome affinis DC.	P	P
Cyperaceae		
Cyperus sp.	P	P
Cyperus flavus (Vahl) Nees	A	P
Cyperus distans L.f.	P	P
Cyperus brevifolius (Rottb.) Hassk.	P	P
Bulbostylis capillaris (L.) C.B. Clarke.	P	P
Kyllinga odorata Vahl	P	A
Commelinaceae		
Commellina benghalensis L.	P	P
Euphorbiaceae		
Chamaesyce hirta (L.) Millsp	P	P
Euphorbia heterophylla L.	P	A
Linderniaceae		
Lindernia crustacea (L.) F. Muell.	P	P
Loganiaceae		
Spigelia anthelmia L.	P	P
Malvaceae		
Sida sp.	P	A
Gaya pilosa K. Schum.	P	A
Corchorus argutus Kunth	P	P
Molluginaceae		
Mollugo verticillata L.	P	P
Nyctaginaceae		
Boerhavia difusa L.	P	A
Onagraceae		
Ludwigia octovalvis (Jacq.) PH. Raven	P	P
Poaceae		
Cynodon dactylon (L.) Pers.	P	P
Eleusine indica (L.) Gaertn.	P	P
Digitaria sp.	P	P
Cenchrus echinatus L.	P	P
Paspalum maritimun Trin.	P	P
Panicum maximum Jacq.	P	A
Panicum sp.	P	A

Table 1. Contd.

Brachiaria sp.	P	A
Brachiaria mutica (Forssk.) Stapf	P	A
Eragrostis ciliares (L.) R. Br.	A	P
Portulacaceae		
Portulaca oleracea L.	P	P
Talinum triangulare (Jacq.) Willd.	P	P
Phyllanthaceae		
Phyllanthus niruri L.	P	P
Rubiaceae		
*Hedyotis corymbosa (*L.) F. Muell	P	P
Spermacoce latifolia Aubl.	P	A
Spermacoce verticillata L.	A	P

P = Present; A = Absent.

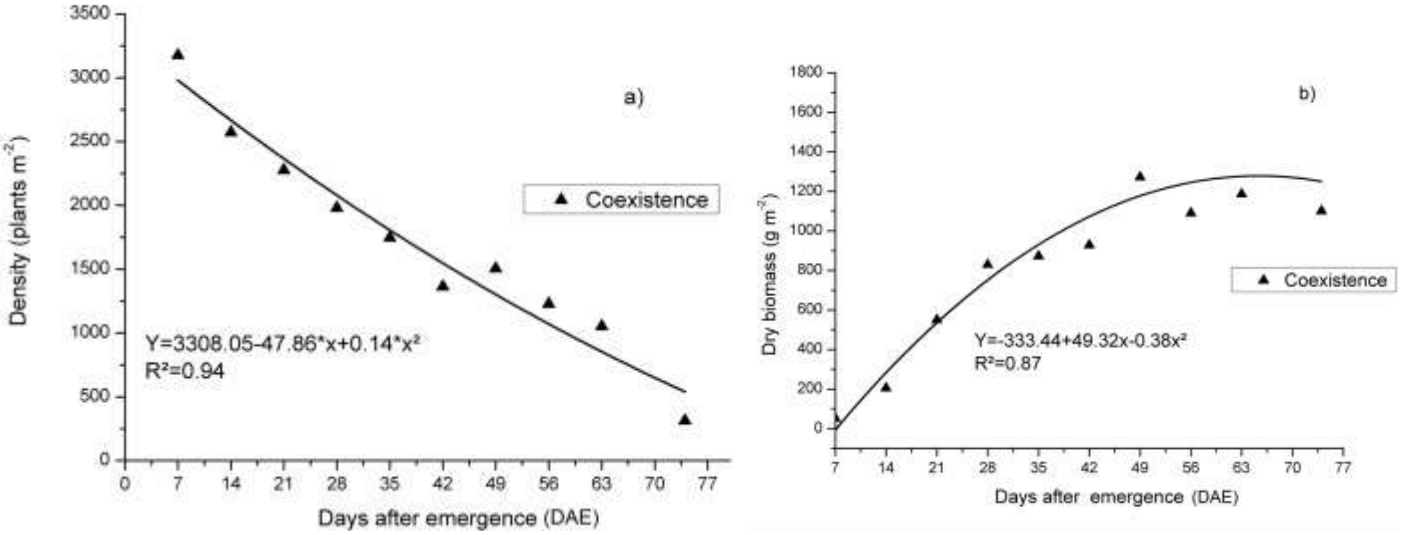

Figure 1. Density (a) and dry biomass (b) of the weed community in different periods of coexistence with weeds in okra organic cropping system in São Luís, state of Maranhão, northeastern Brazil, 2014.

cropping system particularly at the beginning of the coexistence period, at 14 DAE with IVI 85.2% and at 21 DAE with IVI 58.8% (Figure 3a). The practice of mowing in the organic cropping system also spread this species facilitating its regrowth. Santos et al. (2010) reported *C. benghalensis* and *A. tenella* as important species in the okra crop in conventional tillage.

Another important weed species noted in periods of coexistence was *C. dactylon*, particularly at seven and 56 DAE with IVI values above 60% (Figure 3a). This species is difficult to control when well established and it is also a potential host of root-knot nematode (*Meloidogyne* spp.) that causes severe reduction in okra yield, sometimes invalidating its cultivation. Therefore, it should be kept at low density to not harm the okra crop. Law-Ogbomo et al. (2013) noted that *C. dactylon* was one of the most important weeds found in okra conventional cropping systems.

In the weed control periods, *C. dactylon* population predominated in the weed community throughout the crop cycle except at 21 and 49 DAE, when the main species was *A. tenella* (Figure 3b). The highest incidence of light on the soil as a result of hoeing in the initial weed control periods, the wide spacing used and the okra plants slow growth favored the spread of *C. dactylon*. Ibrahim and Hamma (2012), studying okra cultivation with organic fertilizer also identified this species as one of the most important in the weed community.

It was noted that *C. benghalensis, A. tenella* and *E. indica* populations had IVI values below 35% for the whole control period, except at seven and 28 DAE for *E. indica* and at 21 DAE for *A. tenella* (Figure 3b). The initial

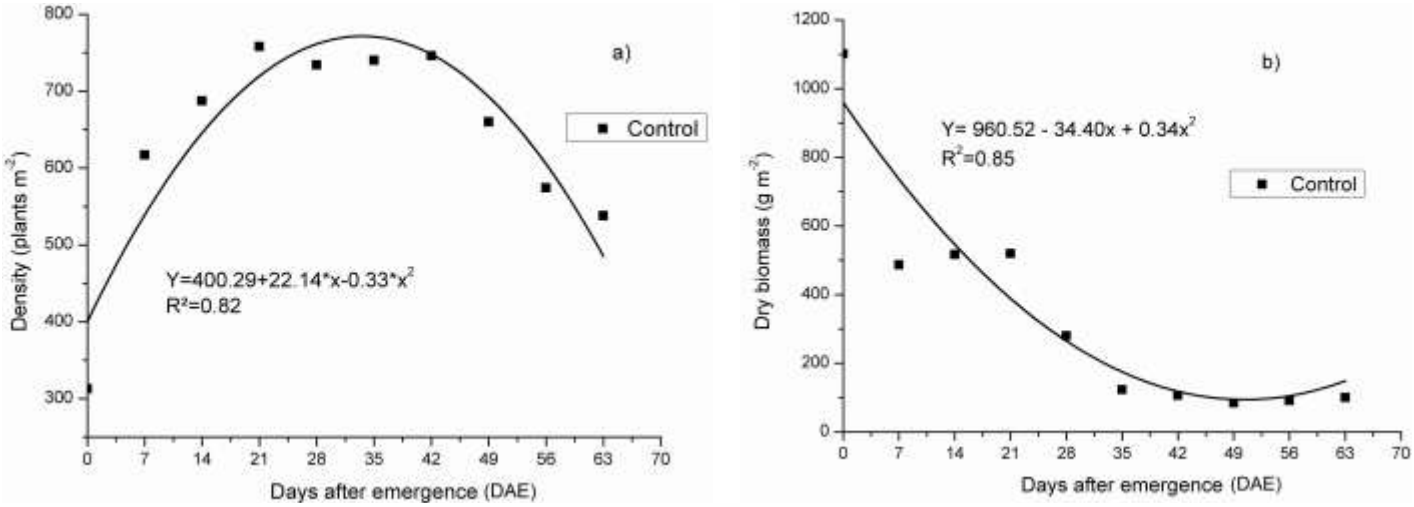

Figure 2. Density (a) and dry biomass (b) of the weed community in different periods of weed control in okra organic cropping system in São Luís, state of Maranhão, northeastern Brazil, 2014.

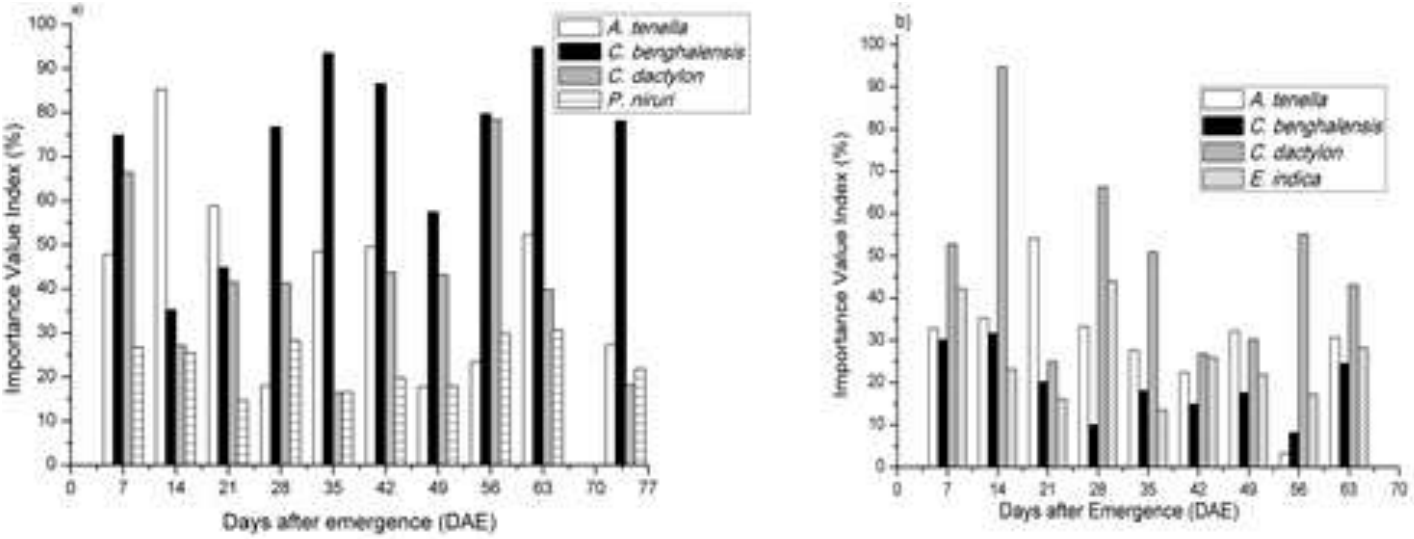

Figure 3. Importance Value Index of the most important weed species identified in different periods of coexistence with the okra crop (a) and of weed control (b) in okra organic cropping system in São Luís, state of Maranhão, northeastern Brazil, 2014.

control was efficient to intensify intra- and interspecific competition, especially the last, because, when the weeds emerged, okra plants were well established and therefore reduced their density.

The greatest okra plant height values were observed from treatments of period of coexistence with the weed community at 28 DAE (Figure 4). Similar results were obtained by Dada and Fayinminnu (2010) with okra grown with organic fertilizer whose plots with weeds had the highest okra plants as a result of competition with weeds for light. Law-Ogbomo et al. (2013) studying okra conventional cropping system observed that after 32 DAS, the okra plants had the lowest heights in treatments of periods of coexistence with weeds. This suggests that competition for light exerted higher effect on okra plant growth and development in an organic cropping system than in the conventional system.

In the weed control periods, the okra plant had the lowest height at 32 DAE compared to the coexistence periods, except in plots with weed control at seven and 14 DAE (Figure 4). Okra crop is slower in its early development, while the weeds are faster in capturing resources to survive and develop; therefore initial weed control enhances the crop growth and development.

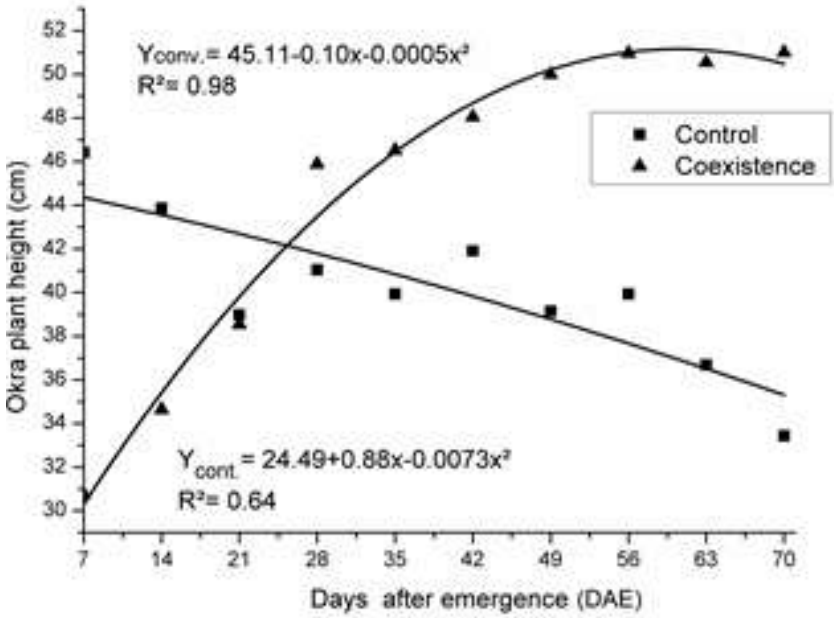

Figure 4. Okra plant height in organic cropping system in different periods of coexistence with the weeds and of weed control in São Luís, state of Maranhão, northeastern Brazil, 2014.

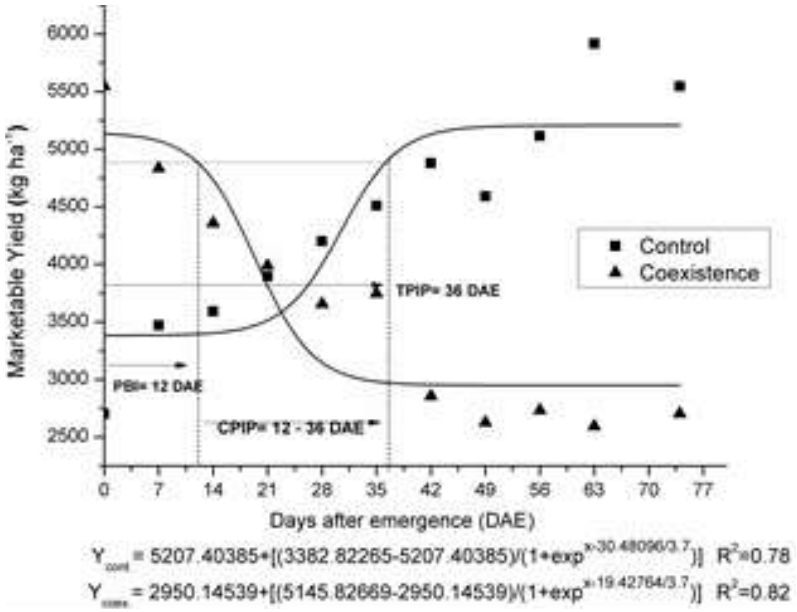

Figure 5. Okra marketable yield and yield data adjustment by the Boltzmann sigmoidal model in function of different periods of coexistence with the weeds and of weed control taking into consideration 5% yield loss. São Luís, state of Maranhão, northeastern Brazil, 2014.

The okra crop grown in organic system was affected by the different periods of coexistence with weeds. The interference was more intense at 12 DAE (Figure 5). The short period of coexistence indicates a crop disadvantage with respect to the weed community when growing in organic cropping system when compared to the results obtained by Santos et al. (2010) and Bachega et al. (2013) in okra conventional cropping system, whose coexistence periods were 25 and 57 DAE, respectively.

Law-Ogbomo et al. (2013) emphasized that the

frequency and cost of weed removal depends on the weed species, the crop grown, cultural practices, the cropping system and the growing season. Therefore, differences in periods of coexistence between okra organic and conventional cropping systems are related to the weed community management practices in these systems that select different species, the cultivar used and soil and climate conditions.

With regards to weed control it was found that up to 36 DAE, the control was enough since the weeds would not interfere in the crop yield, that is, control beyond this period did not increase the crop yield (Figure 5). This result confirms the good control complementation premise carried out by okra plants, which after this period, formed a canopy that shaded the soil and hindered weed growth and development.

In okra cultivation with organic fertilization, Ibrahim and Hamma (2012) found that weeding for three weeks significantly reduced the weed population that could compete with the crop for soil nutrients. However, in the okra conventional cropping system, Santos et al. (2010) found the need for weed control for 100 DAE. This suggests that in an organic cropping system, the weed control period is lower when compared to the conventional cropping system.

The CPIP was between 12 and 36 DAE covering 24 days of the crop cycle, that is, the period by which the weed control must be concentrated in order to reduce financial costs with unnecessary hoeing and to avoid yield losses. Dada and Fayinminnu (2010) found a CPIP between 21 and 42 days after sowing, covering 21 days of the okra crop cycle when grown with organic fertilizer as the most appropriate for optimal growth, development and fruit production. Bachega et al. (2013) studying okra conventional cropping system noted the non-occurrence of the CPIP since the PBI was more extensive than the TPIP. However, Santos et al. (2010) also found in okra conventional cropping system, values between 25 and 100 DAE for the CPIP, covering 79 days of okra crop cycle. These results indicate that in an okra organic cropping system, weed control should be carried out early to boost okra plant growth to provide shading on the weeds, because the time needed for control was not extensive.

Okra yield with in total absence of weed interference was 5,546.87 kg ha^{-1} and in coexistence with weeds throughout crop cycle was 2,703.12 kg ha^{-1} with yield losses of 51.3%. Ibrahim and Hamma (2012) reported yield losses around 40% in okra grown with organic fertilizer and three weeks of weeding. In okra grown in conventional cropping system, yield losses due to weed interference ranged from 78.59 to 95% (Bachega et al., 2013; Law-Ogbomo et al., 2013; Santos et al., 2010). This is an indication that organic fertilizer supplied the crop with the required amount of nutrients, and this resulted in a decrease in weed interference compared to the conventional cropping system whose crop yield losses were higher when in full coexistence with weeds.

Conclusions

The more important weeds in okra crop grown in organic cropping system were *A. tenella*, *C. benghalensis*, *C. dactylon*, *E.indica*, *P. niruri* and *A. tenella*. Weed control in okra crop under organic cropping system must be carried out earlier, between 12 and 36 days after crop emergence to boost growth in order to provide shade to reduce the need for long control period.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Stalk productivity and quality of three sugarcane varieties at the beginning, in the middle, and at the end of the harvest

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This paper evaluated the stalk productivity and quality of the RB961552 and RB98710 sugarcane varieties, compared with RB92579, during the cane-plant and regrowth cycles. The study was conducted in a random block experimental design with five repetitions. The plots composed of 7 furrows measuring 8 m in length with 1.0 m spacing. Juice quality from the lower, middle, and upper thirds of the industrialized stalks was evaluated at the beginning, in the middle, and at the end of the harvest. At the end of the harvest, industrialized stalk and sugar productivity was also quantified. In all the evaluation periods, RB961552 had a lower apparent sucrose level than the other two varieties. RB92579 and RB98710 only differed in the collections at the beginning of the harvest and in the first regrowth cycle, at which time RB98710 presented a higher sucrose level. RB92579 sugar production in the cane-plant and first regrowth cycles was 14.37 and 18.44%, respectively, and greater than the average for the RB961552 and RB98710 varieties.

Key words: Phosphorus in sugarcane juice, sucrose, production systalk, industrial quality.

INTRODUCTION

The sugar-alcohol sector in Brazil is one of the most technology intensive in the world. This is the result of research, with technological improvement programs developed in the country over decades making new varieties available with high productive potential. There have been great advances in knowledge of soils and plant nutrition, in cultivation practices, production management, and cane payment for juice quality

(Oliveira et al., 2014a; Simões et al., 2015; Rhein et al., 2016). The adoption of improved varieties has also contributed to an increase in cane productivity and crop profitability (Souza et al., 2012; Silva, 2013). Among these new varieties, RB92579, RB98710, and RB961552 stand out. RB92579 is characterized by its high productivity, hydric efficiency, optimal sucrose content, and short flowering period. RB98710 has a high productivity and sucrose content, low fiber content, early maturity, and is recommended for restricted environments, with it rarely flourishing or toppling. RB961552 is highly productive and responds excellently to irrigation (RIDESA, 2010).

Soil and climate conditions have a great impact on sugarcane juice production and quality. For this reason, it is important to carry out studies to determine the productivity and industrial quality of juice, in various production environments, in order to establish cultivation practices that make it possible to exploit sugarcane productive potential to the maximum (Calheiros et al., 2012; Silva et al., 2014).

In evaluations of the productive potential of particular sugarcane varieties, industrialized stalk productivity per hectare (TSH) and juice quality should be evaluated, especially apparent sucrose (AS) and inorganic phosphorus (Pi) levels (Tasso Júnior et al., 2014; Rhein et al., 2016). Sucrose is the raw material in sugar and alcohol production and the Pi concentration in juice is important both for yeast metabolism in alcoholic fermentation and in sugar production (Oliveira et al., 2011; Tasso Júnior et al., 2014; Mohammed et al., 2016). Pi is important in the sugarcane juice clarification process during sugar production. On reacting with the slaked lime ($Ca[OH]_2$), tricalcium phosphate is formed, which through flocculation and sedimentation draws the impurities to the bottom of the decanter (Calheiros et al., 2012; Oliveira et al., 2014b; Mohammed et al., 2016). Pi levels greater than 50 mg L^{-1} have been prescribed for good alcoholic fermentation to occur and Pi levels greater than 100 mg L^{-1} for efficient juice clarification (Martins, 2004; Tasso Júnior et al., 2014).

Juice quality varies according to stalk position and sugarcane maturity, with it therefore being important to evaluate juice quality in different parts of the stalk and different harvesting periods in order to identify the best period for each variety. In light of this, the aim of this paper was to evaluate the productive potential of the recently launched RB961552 and RB98710 sugarcane genotype varieties, compared to RB92579, in the cane-plant and first regrowth cycles, in cultivations in the Zona da Mata (scrub region) in Alagoas State, as well as juice quality in three harvesting periods in different parts of the stalk.

MATERIALS AND METHODS

The study was conducted at the Jequiá Plantation, located in the municipality of Anadia, AL, at 09°41'04"S latitude and 36°18'15"W longitude, and belonging to the Triunfo Mill, over the period from August 2011 to January 2014. The evaluations were carried out in two cycles: cane-plant and first regrowth. The region's climate is rainy tropical with dry summers, according to the Koppen classification. The average annual precipitation is 1200 mm, with an average annual temperature of 29°C. In 2012, accumulated rainfall in the months of November and December was only 1.4 mm. In 2013, the volume of rain in the months from January to March was lower than in 2012 (Figure 1).

The study was set up in Yellow Dystrophic Latosol (Embrapa, 2013) with an average texture. Prior to setting up the study, a chemical analysis of the soil was carried out at depths of 0 to 20 and 20 to 40 cm. With the results, dolomitic limestone and plaster were applied in a proportion of 3:1, and in sufficient quantity to raise base saturation to 60% in the topsoil layer and reduce aluminum saturation on the subsurface, as proposed by Oliveira et al. (2007) and Raij (2011). After 60 days, the soil was plowed, harrowed, and subsequently furrowed.

Planting was carried out in August 2011. 500 Mg ha^{-1} of 09-14-22 chemical fertilizer was applied at the bottom of the planting furrows. Table 1 shows the results of the soil analysis after soil fertilization. Three sugarcane varieties were planted: RB92579, RB961552, and RB98710, with treatments placed in random blocks with five repetitions. RB92579 was chosen as a reference due to it currently being the most widely-planted cane variety in Alagoas (Ridesa, 2012). RB961552 and RB98710 are promising varieties, but there is little information available in the literature.

The study was carried out in an random block experimental design with five repititions. The plots consisted of 7 furrows, measuring 8 m in length, with 1.0 m spacing and a total area of 56 m^2. The useful area was 30 m^2, composed of 5 central rows, excluding one meter borders. Plant density fluctuated between 15 and 18 seedlings per meter of furrow, which were collected from a nursery at the Triunfo Mill. The seedlings were manually covered with soil, with an approximately 5 cm layer of earth placed over them and Tebutiuron herbicide applied straight afterwards in doses of 1.0 kg of active ingredient per hectare. Fipronil was also used to control leafcutter ants. Leafhopper (*Mahanarva* species) and sugarcane borer *(Diatraea* species) controls were carried out via biological control, with the use of *Metarhizium anisopliae* and *Cotesia falvipes*, respectively (Benedini, 2006). Fertilization of the cane from first regrowth was carried out after cane-plant harvesting. 500 kg ha^{-1} of 20-05-25 fertilizer was applied by manually spreading the fertilizer. The weed and pest controls adopted in first cane regrowth were the same as those for the cane-plant stage.

The quality of the lower, middle, and upper thirds of the industrialized stalks were evaluated at 14, 15, and 17 months of sugarcane age, and at 9, 10, and 12 months in first regrowth, corresponding to the beginning, middle, and end of the harvest, respectively. In the Northeast of Brazil, the beginning of the harvest corresponds to the months of September and October, the middle of the harvest to November and December, and the end of the harvest to January and February (Souza et al., 2012).

In each of the evaluations, five stalks from the second row from

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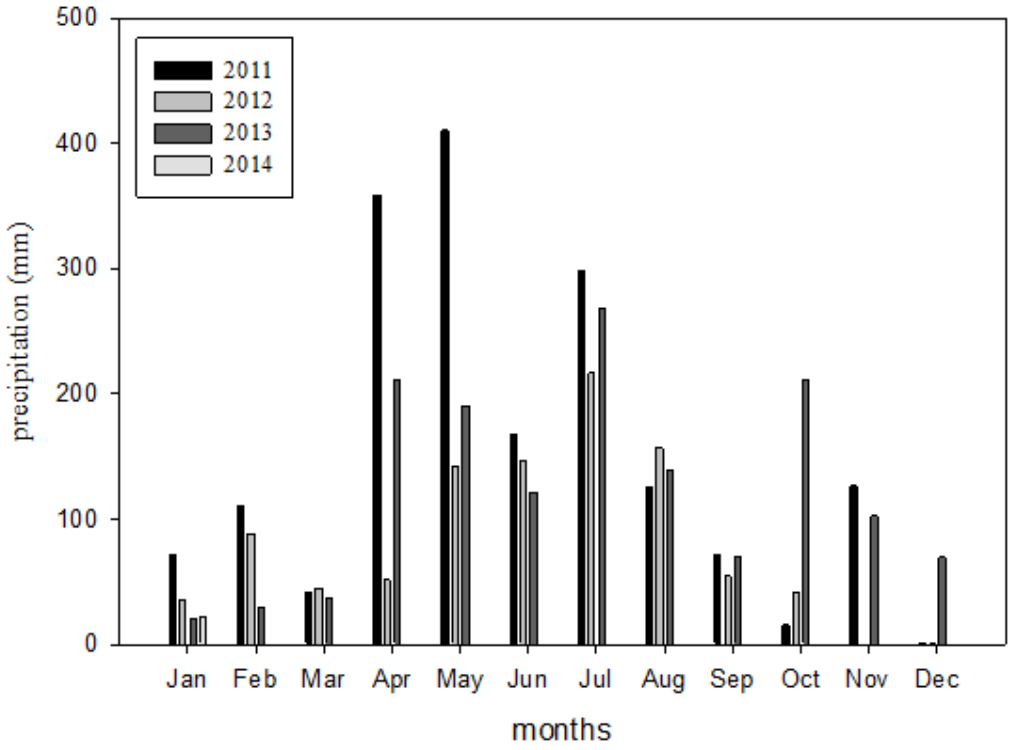

Figure 1. Monthly precipitation during the period studied.

Table 1. Chemical analysis of the soil from the experimental area after fertilization.

Determinations	Depth	
	0-20 cm	20-40 cm
pH (H_2O)	5.9	5.7
Na (mg dm^{-3})	21	21
P (mg dm^{-3})	6	4
K (mg dm^{-3})	48	28
Ca ($cmol_c$ dm^{-3})	2.8	1.3
Mg ($cmol_c$ dm^{-3})	0.9	1.2
Al ($cmol_c$ dm^{-3})	0.05	0.11
H + Al ($cmol_c$ dm^{-3})	3.0	2.2

left to right were collected, separated, clipped, divided into lower (LT), middle (MT), and upper (UT) thirds, and then weighed. The samples for each third were passed through a forage cutter and homogenized. A subsample of 500 ± 1.0 g of chopped stalks was pressed at 250 kgf cm^{-2} for 60 s to separate the juice from the pulp (CONSECANA, 2006). The juice obtained was analyzed for apparent sucrose levels in the juice (AS), juice purity (PUR), total recoverable sugars in the juice (TRS), and inorganic phosphorus (Pi). From the wet pulp, the stalk fiber level (fiber) was determined. The Pi calculation was carried out at the Center for Agricultural Sciences Agricultural Chemistry Laboratory (ASAC) of Alagoas Federal University (UFAL), in accordance with the methods described by Delgado et al. (1984). The other analyses were carried out at the Triunfo Mill Juice Quality Analyses Laboratory, following the methods described by Fernandes (2000) and Lavanholi (2008).

In the last evaluation, at 17 months after planting and at 12 months after cane-plant collection, industrialized stalk and sugar productivity were determined for each variety. The evaluation was carried out in plot rows 3, 4, and 5, from left to right. The plants were cut close to the soil, separated, clipped, and weighed, to determine industrialized stalk productivity in tons of sugarcane per hectare (TSH). In a subsample of these stalks, the juice was extracted and the TRS was determined (Fernandes, 2000). Gross income per hectare of sugar (GIS) was obtained by multiplying stalk productivity per hectare by TRS.

The data were submitted for variance analysis using the F test and the averages compared using the Scott-Knott test with a 5% probability. These analyses were carried out with the help of the Sisvar software (Ferreira, 2011).

RESULTS AND DISCUSSION

In the cane-plant cycle, the AS and TRS levels were significantly influenced by the varieties, by the harvesting period, and by the stalk third (Tabel 2). There was a significant difference in fiber between the stalk thirds and the harvesting periods, while PUR was only affected by the stalk third (Table 2). Pi was influenced by the variety and by the stalk third. In the first regrowth cycle, all of the variables were influenced by the varieties, by the harvesting period, and by the stalk third (Table 2). There was interaction between the harvesting period and the stalk third for all the juice quality variables analyzed in the first regrowth cycle (Table 2) and for AS, PUR, and TRS in the cane-plant cycle. The interaction between the varieties and harvesting period was significant for Pi in the cane-plant cycle and for AS, PUR, and TRS in the first regrowth cycle.

RB961552 presented AS and TRS levels that were 10 and 9% lower than the averages for the RB92579 and RB98710 varieties, in the cane-plant cycle, and 10 and 8% lower in the first regrowth cycle, respectively (Table 3). The R92579 and RB98710 varieties presented statistically similar averages in the two cycles, for AS and TRS, except for AS in the first regrowth cycle, when RB98710 presented a 2.4% higher average. TRS and AS behaviors are similar, since the TRS variable depends on the sucrose level present in the cane juice (Oliveira et al., 2014b).

In a study conducted by Calheiros et al. (2012), regarding RB867515 and RB92579 cultivated in Rio Largo, in the Zona da Mata in Alagoas, in the cane-plant cycle, AS values were obtained for RB92579 that are similar to those observed in this study. Moreover, Oliveira et al. (2011), in a study conducted in Boca da Mata, AL, observed AS levels fluctuating around 18.0% for RB98710, RB867515, and RB92579, collected at the end of the Alagoan harvest.

AS and TRS levels were always lower at the beginning of the harvest (Table 3). The increase in sucrose in the stalk is associated with the reduction in available soil water and the action of invertase enzymes and sucrose phosphate, as cited by Vieira (1988) and Casagrande and Vasconcelo (2008). In these studies, it was verified that acid invertase activity is high in internodes in elongation, but absent in mature internodes. In the mature stalk internodes, there is an increase in alkaline invertase and sucrose phosphate synthesis. In the cane-plant cycle, there was no statistical difference for AS and TRS levels in the middle and at the end of the harvest. However, in the second year of cultivation, the sugarcane collected at the end of the harvest presented a higher AS level. This occurs due to the reduciton in available soil water and consequent hydric restriction in the plant, which influences sugarcane maturation (Toppa et al., 2010). In Brazil, the low temperatures associated with hydric deficit are the main climatic factors responsible for sugarcane maturation. In Alagoas, it is the main climatic factor related to sugarcane maturation (Calheiros et al., 2012).

In the MT and LT, AS and TRS levels did not differ between any crop cycle (Table 3), with an average of 18.93% and 149.70 kg Mg^{-1} in the cane-plant cycle and 19.47% and 156.75 kg Mg^{-1} in first regrowth, respectively. The difference between the UT and MT and LT averages was 25.94 and 17.15%, respectively. This difference was lower than that observed by Martins (2004), who while working with the SP823530, SP835073, and RB835486 varieties observed an average difference of 71.43%. This greater difference in sucrose levels between stalks is due to a shorter sugarcane maturation phase, as excess rainfall in the study delayed cane dehydration.

In the interaction between the harvest period and stalk thirds (Tables 4 and 5), it was observed that the UT always presented lower AS and TRS levels. For cane-plant, there was no statistical difference for AS and TRS levels in the MT and LT in any harvest period. On the other hand, for first regrowth, the AS and TRS levels increased in the UT, MT, and LT at the beginning of the harvest. As the harvest advanced, the difference in sucrose concentrations between the thirds decreased, showing advanced maturity (Leite et al., 2010; Toppa et al., 2010).

From the analysis of the interaction between variety and harvest period, in the first regrowth cycle (Table 6), it is observed that AS and TRS levels increase progressively as the harvest advances. The RB92579 and RB98710 varieties did not differ between each other in the three harvest periods, and were always greater than RB961552. The percentage difference between the AS level in RB961552 and the average for the other two varieties was 10.07, 15.29, and 14.23%, at the beginning, in the middle, and at the end of the harvest, respectively. On the other hand, RB961552 presented a lower TRS at the beginning and in the middle of the harvest, however was similar to RB92579 and RB98710 at the end of the harvest.

In the cane-plant cycle, there was no statistical difference between the varieties studied, with the average PUR value being 87.15% (Tables 2 and 3). In the first regrowth cycle, RB92579 was the variety that presented the greatest PUR, with 88.75%, this being 1.6% greater than the average for the other varieties. The harvest period only influenced PUR in the first regrowth cycle, when the juice collected at the end of the harvest presented greater purity. In both cycles, the UT presented lower purity than the MT and LT. This lower purity in the UT is the result of the sugarcane maturation process, which occurs from the base to the apex (Segato et al., 2006; Leite et al., 2010; Toppa et al., 2010).

On analyzing the interaction between the stalk thirds and harvest period, it was observed that, in the two cultivation cycles (Tables 4 and 5), the UT had less juice purity, however, at the end of the harvest, there was no

Table 2. Values and significance of average squares from variance analyses and variation coefficients of soluble solid percentages (SS), apparent sucrose in juice (AS), purity (PUR), total recoverable sugars (TRS), and inorganic phosphorus (Pi), for three sugarcane varieties, in three harvesting periods (P), in three parts of the stalk (third), in the cane-plant and first regrowth cycles.

Source of variation	G.L	Average squares				
		AS (%)	PUR (%)	Fiber (%)	TRS (Mg ha^{-1})	Pi (mg L^{-1})
Cane plant						
Variety (V)	2	43.55**	11.36^{ns}	1.31^{ns}	2,419.14**	1,331.58**
Third (T)	2	361.79**	822.42**	10.88**	20,792.27**	1,290.13**
Period (P)	2	64.62**	14.59^{ns}	30.86**	2,142.24**	119.08^{ns}
Block	4	1.35	11.99	0.39	59.99	313.35
V × T	4	2.42^{ns}	6.33^{ns}	0.43^{ns}	61.60^{ns}	50.556^{ns}
V × P	4	2.43^{ns}	10.67^{ns}	0.99^{ns}	61.01^{ns}	417.58**
T × P	4	30.76**	223.26**	0.44^{ns}	1,664.56**	19.21^{ns}
V × T × P	8	1.13^{ns}	12.90^{ns}	0.63^{ns}	17.95^{ns}	121.38^{ns}
Residual	104	2.56	6.52	0.66	96.45	93.14
General average		17.29	87.15	14.34	137.32	49.03
C.V (%)		9.27	2.93	5.66	7.15	19.68
First regrowth						
Variety (V)	2	46.82**	30.35**	1.51*	2,243.32**	1,343.41**
Third (T)	2	199.56**	201.16**	110.27**	16,524.14**	333.12*
Period (P)	2	114.90**	35.44**	28.48**	4,108.60**	2,084.41**
Block	4	0.94	4.71	0.29	93.14	348.37
V × T	4	2.62^{ns}	10.03^{ns}	2.59^{ns}	108.96^{ns}	40.69^{ns}
V × P	4	3.51*	16.03*	0.38^{ns}	191.40*	114.01^{ns}
T × P	4	10.91**	29.73**	5.55**	358.45**	556.57**
V × T × P	8	1.30^{ns}	4.96^{ns}	0.52^{ns}	45.54^{ns}	353.21**
Residual	104	1.15	6.16	0.35	74.26	96.53
General average		18.56	87.81	14.90	145.73	105.67
C.V (%)		5.78	2.83	3.99	5.91	9.30

^{ns},*,** represent, respectively, not significant and significant to 5.0% and 1.0% probability using the F test.

Table 3. Average levels of Apparent Sucrose in Juice (AS) in %, Total Recoverable Sugars (TRS) in kg Mg^{-1}, Purity (PUR) in %, Fiber (F) in %, and inorganic Phosphorus (Pi) in mg L^{-1}, for the RB92579, RB961552, and RB98710 sugarcane varieties, cultivated in Anadia, in the Alagoan *agreste* region, and collected at the beginning, in the middle, and at the end of harvest, in the upper, middle, and lower thirds.

Parameter	AS	PUR	F	TRS	Pi	AS	PUR	F	TRS	Pi
		%		kg Mg^{-1}	mg L^{-1}		%		kg Mg^{-1}	mg L^{-1}
Variety			Cane-plant					First regrowth		
RB92579	17.99^{a}	87.7^{a}	14.30^{a}	141.64^{a}	45.80^{b}	18.90^{b}	88.7^{a}	14.74^{a}	147.98^{a}	99.4^{b}
RB98710	17.72^{a}	86.7^{a}	14.20^{a}	141.48^{a}	55.31^{a}	19.97^{a}	87.4^{b}	15.10^{a}	151.39^{a}	108.7^{a}
RB961552	16.17b	87.0^{a}	14.53^{a}	128.86^{b}	45.98^{b}	17.41^{c}	87.3^{b}	14.87^{b}	137.81^{b}	108.9^{a}
Harvesting periods										
Beginning	15.91^{b}	86.5^{a}	13.42^{c}	129.64^{b}	47.71^{a}	17.08^{c}	87.1^{b}	14.10^{c}	137.01^{b}	101.8^{b}
Middle	18.06^{a}	87.5^{a}	14.59^{b}	142.98^{a}	48.54^{a}	18.34^{b}	87.5^{b}	14.93^{b}	144.23^{a}	113.5^{a}
End	17.92^{a}	87.5^{a}	15.02^{a}	139.36^{a}	50.85^{a}	20.25^{a}	88.8^{a}	15.69^{a}	155.94^{a}	101.6^{b}
Stalk thirds										
Upper	14.02^{b}	82.2^{b}	14.89^{a}	112.57^{b}	43.65^{c}	16.13^{b}	85.4^{b}	16.71^{a}	123.67^{b}	108.8^{a}
Middle	18.87^{a}	89.4^{a}	14.21^{b}	148.16^{a}	49.08^{b}	19.61^{a}	88.8^{a}	14.07^{b}	155.24^{a}	103.8^{b}
Lower	29.99^{a}	89.6^{a}	13.94^{b}	151.25^{a}	54.36^{a}	19.33^{a}	89.3^{a}	13.93^{b}	158.27^{a}	104.4^{b}

[1]Averages followed by the same letter in the column do not differ statistically between each other using the Scott-Knott test with a 5% probability.

Table 4. Average values of Apparent Sucrose in Juice (AS) in %, Total Recoverable Sugars (TRS) in kg Mg^{-1}, Purity (PUR) in %, in the upper, middle, and lower thirds, collected at the beginning, in the middle, and at the end of harvest, in the cane-plant cycles.

Stalk thirds	Collection periods		
	Beginning of harvest	Middle of harvest	End of harvest
AS (%)			
Upper	10.79^{B}	15.47^{B}	15.79^{B}
Middle	18.06^{A}	19.23^{A}	18.86^{A}
Lower	18.88^{A}	19.45^{A}	19.09^{A}
TRS (kg Mg^{-1})			
Upper	91.36^{B}	122.61^{B}	123.73^{B}
Middle	145.55^{A}	153.77^{A}	145.15^{A}
Lower	152.01^{A}	152.56^{A}	149.18^{A}
PUR (%)			
Upper	76.78^{B}	83.35^{B}	86.53^{A}
Middle	90.73^{A}	89.44^{A}	88.05^{A}
Lower	91.98^{A}	89.63^{A}	87.88^{A}

Averages followed by the same letter in the column do not differ statistically between each other using the Scott-Knott test with a 5% probability.

Table 5. Average values of Apparent Sucrose in Juice (AS) in %, Total Recoverable Sugars (TRS) in kg Mg^{-1}, Purity (PUR) in %, Fiber (F) in %, and inorganic Phosphorus (Pi) in mg L^{-1}, in the upper, middle, and lower thirds, collected at the beginning, in the middle, and at the end of harvest, in the first regrowth cycles

Stalk thirds	Collection periods		
	Beginning of harvest	Middle of harvest	End of harvest
AS (%)			
Upper	13.81^{c}	15.86^{b}	18.73^{b}
Middle	18.10^{b}	19.75^{a}	20.97^{a}
Lower	19.32^{a}	19.43^{a}	21.05^{a}
TRS (kg Mg^{-1})			
Upper	110.48^{b}	121.86^{b}	138.67^{b}
Middle	145.77^{b}	156.41^{a}	163.55^{a}
Lower	154.79^{a}	154.41^{a}	165.62^{a}
PUR (%)			
Upper	83.55^{b}	80.16^{b}	87.88^{a}
Middle	88.15^{a}	88.97^{a}	88.56^{a}
Lower	89.57^{a}	90.35^{a}	89.95^{a}
Fiber (%)			
Upper	15.18^{a}	13.86^{a}	18.16^{a}
Middle	13.54^{b}	13.17^{b}	14.49^{b}
Lower	13.56b	13.23^{b}	14.40^{b}
Pi (mg L^{-1})			
Upper	109.98^{a}	115.90^{a}	85.06^{b}
Middle	87.77^{b}	108.23^{a}	92.22^{b}
Lower	86.15^{b}	101.00^{a}	107.92^{a}

Averages followed by the same letter in the column do not differ statistically between each other using the Scott-Knott test with a 5% probability.

Table 6. Average values of Apparent Sucrose in Juice (AS) in %, Total Recoverable Sugars (TRS) in kg Mg^{-1}, and Purity (PUR) in %, in the RB92579, RB961552, and RB98710 varieties, collected at the beginning, in the middle, and at the end of harvest, in the first regrowth cycle.

Variety	Collection periods		
	Beginning of harvest	Middle of harvest	End of harvest
AS (%)			
RB92579	17.35[a]	18.92[a]	20.44[a]
RB961552	15.89[b]	16.67[b]	19.67[b]
RB98710	17.99[a]	19.44[a]	20.65[a]
TRS (kg Mg^{-1})			
RB92579	138.60[a]	148.54[a]	156.81[a]
RB961552	128.50[b]	132.65[b]	152.30[a]
RB98710	143.93[a]	151.49[a]	158.73[a]
PUR (%)			
RB92579	87.48[a]	88.03[a]	89.179[a]
RB961552	86.32[a]	86.01[b]	89.203[a]
RB98710	87.46[a]	85.45[b]	88.020[a]

Averages followed by the same letter in the column do not differ statistically between each other using the Scott-Knott test with a 5% probability.

statistical difference between the thirds. Uniformity of purity in the stalk is expected when sugarcane reaches maximum maturity, presenting a maturity index between 0.85 and 1.0 (Toppa et al., 2010). The maturation index is the proportion of apparent sucrose content, determined using polarimetry, from the base to the industrially useable stalk. It is an index used to evaluate sugarcane maturation (Liz et al., 2016).

Analyzing the interaction between the varieties and the harvest period (Table 6), in the first regrowth cycle, it is found that in the middle of the harvest, RB92579 presented greater purity, however it did not differ from the other varieties at the beginning and end of the harvest.

In all of the harvesting periods, all of the varieties presented over 80% purity, which is considered adequate for sugarcane industrialization (Rhein et al., 2016; Rodolfo Junior et al., 2016). In this paper, average purity in the cane-plant and first regrowth cycles was greater than that reported by Oliveira et al. (2011) and Silva (2013). High PUR in sugarcane juice is desired at the time of harvesting, since it implies a higher concentration of sucrose and reduced amino acids, organic acids, starch, reducing sugars, and other color precursor compounds (Rhein et al., 2016; Rodolfo Junior et al., 2016).

The average fiber level in the three varieties for cane-plant was 14.34% (Table 3), approximately 11.08% higher than that observed by Oliveira et al. (2011) and Silva (2013). In the first regrowth cycle, RB98710 presented a higher level of fiber, with 15.10%, while the RB92579 and RB961552 varieties did not differ between each other and present an average of 14.80%. In both cycles, the level of fiber increased as the harvest advanced and the UT presented a higher level of fiber than the MT and LT, which did not differ between each other. The difference between the UT and the average for the MT and LT was 5.51 and 16.21% in the cane-plant cycle and first regrowth, respectively. The higher level of fiber in the UT is probably due to the lower accumulation of sucrose compared to the other thirds.

Fiber is important when it comes to industries' energy balance, as pulp is used for obtaining electrical energy; however, a high level of fiber causes resistance to juice extraction (Simões et al., 2015; Rodolfo Junior et al., 2016). To maintain energy balance, a percentage of fiber between 10 and 12.5% has been recommended. However, the Northeast region of Brazil presents greater evapotranspiration than the Center-South region, for which reason sugarcane cultivated in the Northeast has a higher level of fiber at the time of harvesting (Oliveira et al., 2011, 2014b).

RB92579 presented a lower level of Pi in the two cultivation cycles, although it did not differ from RB961552 in the cane-plant cycle (Table 3). On the other hand, RB98710 was the variety that presented the highest level of Pi in the juice. In the cane-plant cycle, the level of Pi in RB92579 juice was lower at the beginning of the harvest, but the concentration rose during the harvest, reaching the same values as RB98710 at the end of the harvest (Table 7). The Pi concentration in the sugarcane cycle was lower than in the first regrowth cycle, probably due to the greater production of biomass in the cane-plant cycle, resulting in dilution of the absorbed phosphorus (Oliveira et al., 2007). The average

Table 7. Average values for inorganic phosphorus (Pi) in mg L^{-1} in the RB92579, RB961552, and RB98710 varieties, collected at the beginning, in the middle, and at the end of harvest, in the cane plant cycle.

Variety	Collection periods		
	Beginning of harvest	Middle of harvest	End of harvest
Pi (mg L^{-1})			
RB92579	38.99[c]	44.38[b]	54.04[a]
RB961552	47.11[b]	46.93[b]	46.90[b]
RB98710	57.03[a]	54.30[a]	54.61[a]

Averages followed by the same letter in the column do not differ statistically between each other using the Scott-Knott test with a 5% probability.

Pi levels in RB92579, RB961552, and RB98710 juice for the two cycles were 73, 77, and 82 mg L^{-1} of P, respectively. High Pi levels in sugarcane juice during industrialization are desirable for reducing the cost of clarifying the juice, since the addition of exogenous Pi is necessary when juice levels are not adequate for good clarification (below 100 mg L^{-1}) (Mohammed et al., 2016). Pi levels in juice of around 180 mg L^{-1} were obtained by Oliveira et al. (2011) in studies conducted in the Alagoan Agreste region involving the RB867515 variety. Tasso Júnior et al. (2014) evaluated Pi levels in the CTC9, CT15, and CTC16 cane varieties and did not find any difference between the varieties with regards to Pi in the juice, finding an average juice value of 147 mg L^{-1}. In the study conducted by Martins (2004), the Pi level was influenced by the variety, with Pi values of 151, 236, and 388 mg L^{-1} for the SP823530, SP835073, and RB835486 varieties, respectively.

The Pi levels only differed between the harvesting periods in the first regrowth cycle, when the sample corresponding to the middle of the harvest presented a higher Pi level than in the other samples (Table 3). Pi level behavior in the thirds differs between the cycles studied. In the cane-plant cycle, the highest Pi content was observed in LT, however in first regrowth, the UT presented the highest Pi level. By analyzing the interaction between the thirds and the harvesting periods in the first regrowth cycle (Table 5), it is observed that at the beginning of the harvest, the UT presented the lowest Pi level, in the middle of the harvest there was no difference between the thirds, and at the end of the harvest the LT has the highest Pi level. When sugarcane is not yet completely mature, the UT is the most biochemically active part, demanding greater quantities of Pi (Oliveira et al., 2014b; Tasso Júnior et al., 2014). With sugarcane maturity, Pi comes to be required in greater quantities in the LT and MT, where it acts as an energy source in the sucrose accumulation process in the cell vacuoles (Casagrande and Vasconcelos, 2008). Thus, when sugarcane starts the maturation process, Pi migrates from the UT to the MT and LT.

Table 8 presents the average square results from the variance analysis for TSH, TRS, and GIS, for the RB92579, RB98710, and RB961552 varieties, in the cane-plant and first regrowth cycles. It is observed that there was a varietal effect for all the variables only in the cane-plant cycle. The averages for TSH, TRS, and TRS_{ha} in the cane-plant cycle are shown in Table 9.

RB92579 was the variety that presented the highest TSH, at around 15% more than the other varieties, and consequently the highest GIS. RB98710, despite having similar TRS to RB92579, produced fewer stalks and therefore its GIS was lower. RB961552 was less productive for all the varieties analyzed. The RB92579 variety presented a higher GIS than those observed by Aquino et al. (2016). Ferreira Junior et al. (2014) indicated that RB98710 has a high sugar level and high productivity. When cultivated using drip irrigation, they observed that RB98710 sugar productivity was 17.8 Mg ha^{-1} (Ferreira Junior et al., 2014), similar to the RB92579 productivity observed in this study. The stalk productivity obtained in this study (118.52 Mg ha^{-1}) is considered as average to high for the state. In Alagoas, the maximum sugarcane growth phase occurs on short days, and therefore under low luminosity, unlike in the Center-South of Brazil, where increased luminosity coincides with greater hydric availability. The non-coincidence of maximum hydric availability with luminosity negatively influences photosynthetic rates, resulting in lower cane productivity in Alagoas compared to the Center-South (Oliveira et al., 2011; Calheiros et al., 2012).

Studies carried out in Brazil (Calheiros et al., 2012; Oliveira et al., 2014b) indicate RB92579 as one of the most productive varieties, and this is one of the reasons for which, together with RB867515, it is in expansion. However, the juice from this variety presents high phenolic and flavonoid levels (Oliveira et al., 2011), characteristics that are not contemplated in sugarcane payment for recoverable sugar (TRS), but which negatively contribute to juice color and makes industrialization difficult. Phenolic compounds are substances that negatively influence juice color and consequently that of the sugar, reducing the quality and acceptability of the product (Qudsieh et al., 2002). They

Table 8. Average values from variance analysis for Industrialized Stalk Production (ISP) in Mg ha^{-1}, Total Recoverable Sugars in Juice (TRS) in kg Mg^{-1} and Gross Income of Sugar (GIS) in Mg ha^{-1}, for the RB92579, RB961552, and RB98710 varieties, in the cane plant and first regrowth cycles, collected at the end of harvest.

Source of variation	GL	Average squares					
		Cane-plant			First regrowth		
		ISP (Mg ha^{-1})	TRS (kg Mg^{-1})	GIS (Mg ha^{-1})	ISP (Mg ha^{-1})	TRS (kg Mg^{-1})	GIS (Mg ha^{-1})
Variety	2	800.62**	162.60*	19.17**	226.301^{ns}	89.10^{ns}	4.51^{ns}
Blocks	4	242.08	28.47	4.65	170.61	29.19	3.40
Residual	8	42.19	35.45	0.57	45.67	19.98	1.06
General average		118.52	132.70	15.73	66.98	156.61	10.47
C.V. (%)		5.48	4.49	4.83	10.09	2.85	9.86

**Significant to 1% probability using the F test; ^{ns}Not significant to 1% probability.

Table 9. Average values of Industrialized Stalk Productivity (ISP) in Mg ha^{-1}, Total Recoverable Sugars (TRS) in kg Mg-^{1}, and Gross Income of Sugar (GIS) in Mg ha^{-1}, for the RB92579, RB961552, and RB98710 varieties, in the cane plant cycle, collected at the end of harvest.

Variety	ISP (Mg ha^{-1})	TRS (kg Mg-^{1})	GIS (Mg ha^{-1})
RB92579	133.10^{A}	135.15^{A}	17.92^{A}
RB961552	112.00^{B}	126.18^{B}	14.14^{B}
RB98710	110.46^{B}	136.77^{A}	15.13^{B}
General Average	118.52	132.70	15.73

Averages followed by the same letter in the column do not differ statistically between each other using the Scott-Knott test with a 5% probability.

also have a negative effect on fermentation, especially by reducing the action of invertase excreted by the yeast.

The productive superiority of RB92579 was not proven in the first regrowth cycle, as there was no significant difference for any of the variables in this cycle. The averages for TSH, TRS, and GIS in the first regrowth cycle were 66.98 Mg ha^{-1}, 156.61 kg Mg^{-1}, and 10.47 Mg ha^{-1}, respectively (Table 8). The TRS was similar to that found by Silva (2013), however the TSH and GIS were lower. The decrease in productivity in the first regrowth cycle was high and probably influenced due by the hydric stress in the growth phase in the second cycle of the study.

Hydric deficit in the growth phase is one of the main causes of reduced sugarcane productivity (Rhein et al., 2016; Rodolfo Junior et al., 2016), since it causes morphophysiological defense alterations such as reductions in leaf area and gas exchange. Bueno et al. (2012) studied 10 sugarcane genotypes in the first regrowth cycle collected in different periods in the state of Paraná, where it was observed that hydric deficit in the cane growth phase reduced agricultural production and the accumulation of sugar collected in April, the beginning of the harvest in the region.

Conclusions

RB961552 has lower levels of apparent sucrose, total recoverable sugars, and purity, than RB92579 and RB98710. However, all the varieties have ideal apparent sucrose and purity levels for the samples in the three harvest periods, with these values increasing as the harvest advanced.

RB98710 has higher Pi levels in the juice than RB92579 and RB921552. In the cane-plant cycle, all the varieties have lower than ideal Pi levels for juice clarification, while in the first regrowth cycle, RB98710 and RB961552 have Pi levels within the ideal range.

In the cane-plant cycle, the RB92579 variety has higher stalk and sugar productivity than the other varieties.

Conflict of Interests

The authors have not declared any conflict of interests.

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Physiological aspects in cotton cultivars in response to application leaf gibberellic acid

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The cotton plant is a species of the *Malvaceae* family and is relevant to the Brazilian and world economy, mainly because of the textile fiber. However, there was an increase in cotton production because it is necessary for the use of inputs or stimulants, such as the use of gibberellic acid, which has contributed in improving the physiological processes of plants. The study aimed to evaluate the effects of gibberellic acid doses and foliar application on the physiological aspects of different cotton cultivars. The experiment was conducted under field conditions in 5 × 3 factorial scheme, corresponding to five doses of gibberellic acid (0, 0.01, 0.02, 0.04 and 0.06 mg L^{-1}) and three cultivars upland cotton (BRS 8H, BRS Rubi and BRS Safira) in the design of randomized blocks, three replications and 25 plants per plot. The photosynthetic pigments, which are represented by the contents of chlorophyll *a* and *b*, total, carotenoid and relative water content in the sheet were determined. In BRS 8H the chlorophyll levels were high, 287.914 µmol m^{-2} to 468.796 µmol m^{-2} being the treatments without and sprayed with 0.06 mg L^{-1} GA_3, with 62.82% increase. The application of 0.06 mg L^{-1} GA_3 generally promotes increased levels of photosynthetic pigments and relative water content in cotton leaves. The cotton BRS 8H was the culture that best meets the application of gibberellic acid.

Key words: Genetic material, *Gossypium hirsutum* L., photosynthetic pigments phytohormone.

INTRODUCTION

The cotton (*Gossypium hirsutum* L.) is a species of the *Malvaceae* family originating from the Mesoamerican region (D'eeckenbrugge and Lacape, 2014). The cotton crop has been cultivated for thousands of years and has great relevance in the Brazilian and world economy, mainly because of textile fiber (Carvalho et al., 2015). On the international scene, according to Carvalho et al. (2015), Brazil ranks fifth in world's cotton ranking, with

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Table 1. Soil chemical characterization as fertility before the experiment

pH	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	BS	$H^{+} + Al^{3+}$	CEC	V	P	O.M
1:2.5				$mmol_c\ dm^{-3}$				%	$mg\ dm^{-3}$	$g\ kg^{-1}$
7.5	51.6	6.9	0.9	2.9	62.3	0.0	62.3	100	126.2	15.3

BS = Base sums; O.M. = Organic Matter; CEC = Cation Exchange Capacity.

production of about 4.404,600 t in an area of 1.121,600 ha during the 2013 to 2014 season (Conab, 2015). Brazil comes after China, India, the United States and Pakistan in the world's ranking. However, that there is an increase in cotton production, it is necessary for the use of inputs or stimulants, such as the use of growth regulators which is an agronomic technique interfered for plant growth and increasing production in various cultures (Campos et al., 2009; Ferrari et al., 2008). Among the growth regulators, the gibberellic acid (GA_3) which is a plant growth hormone ($C_{19}H_{22}O_6$) widely used in the improvement of the regulation of physiological processes of plants, including cotton was used (Onanuga et al., 2012). It is known that the application of gibberellic acid is important in many metabolic processes of plants, works in stimulated seed germination, elongation and cell division, leaf expansion, flowering and fruit development, and stimulate the secondary metabolism species (Ahmad Dar et al., 2015). Recently, research has turned to elucidate the role of GA_3 in the preservation and stimulation process in the production of photosynthetic pigments in plants. The results have shown that the application of low concentrations phytoregulator has increased the carotenoid and chlorophyll content in leaves (Ali et al., 2012; Jaleel et al., 2009).

In addition, gibberellic acid is often employed when plants are under stress, especially those related to water and salt (Ali et al., 2012; El-Tohamy et al., 2015), since under such conditions, leaf water content is reduced and it in turn, severely affects the growth and development of the plant. Thus, the exogenous application of GA_3 as mitigating, has enabled the plants retain a larger amount of water in the leaves, favoring the growth process, mainly related to elongation and cell division, even under stress conditions (Kaya et al., 2006; Taiz and Zeiger, 2013). However, so that the plants can respond to the stimulus applying GA_3, studies are needed to elucidate the application form and the correct dose of phytohormone for each species. Since, according to Carvalho et al. (2016), the answer depends on several factors, such as plant species and variety within the same crop species. This is proven by Alia-Tejacal et al. (2011) and Onanuga et al. (2012) who found that there are differences in the requirement of gibberellic acid-flower native cultivars (*Euphorbia pulcherrima* Willd. Ex Klotz) and cotton varieties. Due to the economic importance of the cotton crop to the national scene and the lack of information on the effect of GA_3 on cotton cultivars in parameters related to the physiology of the species. The study aimed to evaluate the physiologically effects of gibberellic acid doses and foliar application on cotton cultivars.

MATERIALS AND METHODS

The experiment was conducted under field conditions between March and June 2012, at the National Center for Research on Cotton, Brazilian Agricultural Research Corporation - Embrapa Cotton, situated in Campina Grande city, Paraiba, Brazil. The municipality is geo-referenced by the coordinates: latitude 7°13'1 "South and 35°52'31" West and at an altitude of 551 m. The climate of the region is related with hot and humid climate with autumn-winter rain, according to Köppen climate classification. The rainy season is between the months of April and July, and the monthly rainfall in the experimental period was 12.1; 5.0; 58.3; 213.1 mm respectively in the months of March, April, May and June (AESA, 2016). The experiment was conducted in a 5 × 3 factorial design, the design of randomized blocks, with three replications and 25 plants per plot. The factors corresponded to five doses of gibberellic acid (0, 0.01, 0.02, 0.04 and 0.06 mg L^{-1}) and three varieties of herbaceous cotton (BRS 8H, BRS Rubi and BRS Safira). The plants were grown in 5 rows of 5 plants each, spaced at 0.80 m between rows and 0.50 m between plants, which corresponds to 25,000 plants per ha. However, for evaluation purposes, only the three central rows of block, totaling 15 plants per plot were considered. This choice was made in order to avoid border errors which are not controllable in this case. The soil of the experimental area was classified as Entisol, dystrophic, of sandy loam texture (Santos et al., 2013). Before the experiment, samples were collected from the soil at a depth of 0-20 cm, which were homogenized, transformed into a sample and put in a dry shade. After these procedures, the sample was taken to the Soil and Water Analysis Laboratory to perform analysis of the chemical, following the methodology contained in Donagema et al. (2011) as indicated in Table 1.

During the experiment, the driving period made daily collections of the maximum temperature, average, minimum and relative humidity of experimental area through a weather station located at Embrapa Cotton, as is verified the results in Figure 1. Fertilizing plants of cotton cultivars, followed the recommendations suggested by the Soil Analysis Laboratory at Embrapa Cotton, which indicated the application of 20 kg ha^{-1} of N and 30 kg ha^{-1} of P_2O_5, provided in form of urea (45% N) and superphosphate (18% P_2O_5), respectively. Fertilization in foundation and coverage was performed 15 days after emergence (DAE), applying the phosphate fertilizer directly into furrows in a half moon shape, the depth of 30 cm. As for nitrogen fertilization, this was partitioned into three equal applications, the first fertilization performed at 15 DAE, the second at 30 DAE and third at 45 DAE. Because of the irregularity in precipitation, the plants were irrigated daily in accordance with the water demand of the culture, i.e 6.5 mm day^{-1}, as determined for upland cotton (Azevedo et al., 1993). The water used for irrigation of the plants was analyzed for its chemical composition in the Soil

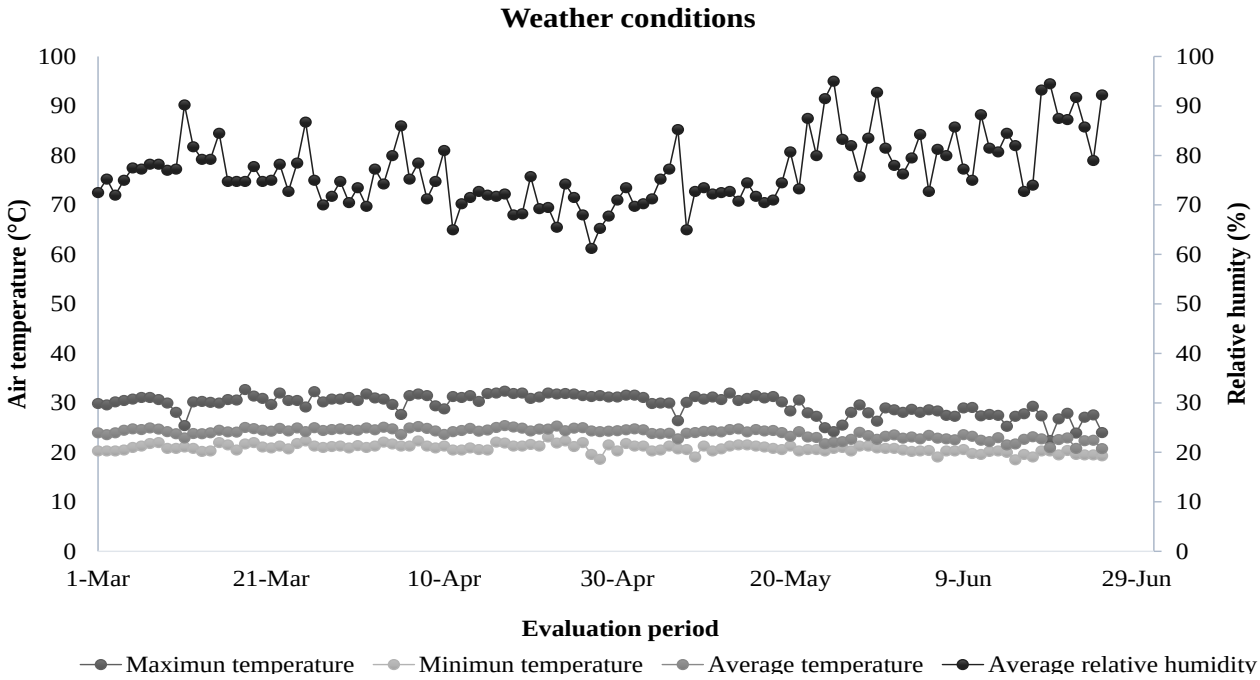

Figure 1. Values of maximum temperatures, average and minimum relative humidity recorded during the experiment period of driving.

Analysis Laboratory and Water Embrapa Cotton, following the methodology described by Richards (1954), with the following characteristics: pH = 7.7 (alkaline) , Cl = 266.25 mg L^{-1} (moderate); $CaCO_3$ = 92.50 mg L^{-1} (high level); Ca^{2+} = 29,00 mg L^{-1}; Mg^{2+} = 30.60 mg L^{-1}, Na^{+} = 98.90 mg L^{-1} (low level), ECiw (Electrical conductivity of irrigation water) = 730 µS cm^{-1} and SAR (Sodium adsorption ratio) = 3 mmol L^{-1}, classified as C_2S_1 (water with average risk of salinization and low risk of sodium), according to Ayres and Westcott (1999).

The gibberellic acid doses were applied at 20, 40 and 60 DAE, and the application through foliar sprays directed on abaxial faces and adaxial of cotton leaves. To better absorption efficiency of gibberellic acid in the leaf surface was used surfactant in the spray solution (Carvalho Júnior et al., 2016). Spraying the plant growth regulator, it was made with the aid of a prior compression manual spray with high molecular weight polyethylene tank with volume capacity of 3 L and pump type piston diameter of 34 mm nozzle. At 90 days after the application of GA_3 solutions, which corresponds reproductive cotton stage, the third pair of fully expanded leaves were collected, counting from the apex to the base of the plant, for the determination of the photosynthetic pigments, which are represented by contents of chlorophyll a (CL*a*), b (CL*b*), total (CL*t*), carotenoids (CAR) and the chlorophyll *a*/*b* (Cl*a*/CL*b*). For this, the leaves were collected and immediately placed in aluminum envelopes, stored in boxes with thermal insulation containing dry ice and transported to the laboratory. Then the middle part circular fractions were taken without the midrib of the leaf tissue with size of 113 mm^2. Fractions were macerated tissue and placed in test tubes coated aluminum foil, which was added 5 ml of dimethylsulfoxide (DMSO). The tubes were left in a dark environment at room temperature of 25°C for a period of 48 h. After this time the solution containing DMSO + fraction of the plant tissue was filtered through a "filter paper" during the 5 min period. With the solution extracted absorbance readings were performed in a spectrophotometer at respective wavelengths of 480, 649 and 665 nm (Wellburn, 1994).

For quantification of photosynthetic pigments, the following equations were used according to the proposed by Wellburn (1994):

Chlorophyll *a* (µmol m^{-2}) = 12.19 × A_{665} – 3.45 × A_{649}
Chlorophyll *b* (µmol m^{-2}) = 21.99 × A_{649} – 5.32 × A_{665}
Total chlorophyll (µmol m^{-2}) = chlorophyll *a* + chlorophyll *b*
Carotenoids (µmol m^{-2}) = (1000 × A_{480} – 2.14 × chlorophyll *a* – 70.16 × chlorophyll *b*)/220

In the same period of evaluation of photosynthetic pigments content, was measured relative water content in the leaf (RWC), using the methodology proposed by Weatherley (1950) and as is seen in the following equation:

RWC (%) = [(Mf - Ms)]/(Mt-Ms)] × 100

Where, Mf = Fresh pasta sheet; Ms = dry weight of leaf and Mt = Mass turgid leaf.
The data were submitted test by analysis of variance F to 5% probability to check the effects of the interaction doses of gibberellic acid × cotton cultivars on the variables analyzed. The average regarding cotton cultivars were compared by Tukey test at 5% probability and the average regarding the gibberellic acid doses by polynomial regression ($p<0.05$). For data analysis, we used the statistical software SISVAR 5.3 (Ferreira, 2014).

RESULTS AND DISCUSSION

As noted in the summary of the mean square values of variance analysis, interaction cotton cultivars × gibberellic acid doses led to significant effects on variables related to the content of photosynthetic pigments and relative water content in leaves of cotton cultivars, with coefficient

Table 2. Summary variance analysis for chlorophyll *a* (CL*a*), chlorophyll *b* (CL*b*), carotenoid (CAR), chlorophyll (CL*t*), relative water content (CRA) and chlorophyll *a*/*b* (CL*a*/CL*b*) in three cotton cultivars treated with gibberellic acid doses.

		Mean square					
S. V	**DF**	**CL*a***	**CL*b***	**CL*t***	**CL*a*/CL*b***	**CAR**	**RWC**
Blocks	2	50.86^{ns}	43.46^{ns}	75.35^{ns}	0.08^{ns}	40.15^{ns}	5.42^{ns}
Cultivars (C)	2	9064.06**	71.46^{ns}	8144.02**	4.62**	699.08**	0.82^{ns}
Gibberellic acid (G)	4	13696.36**	466.41**	17212.30**	2.14**	3669.55**	58.81^{ns}
C × G	8	8828.15**	607.32**	12600.80**	0.84**	1865.67**	121.87**
Residue	28	152.22	86.75	204.92	0.11	50.03	25.39
C.V (%)		3.86	17.64	3.86	5.45	3.94	7.30
Mean		319.60	50.80	371.24	6.15	191.55	69.04

S.V. = Source of variation; C.V = coefficient of variation; D.F. = degree of freedom; * = Significant at 5% probability by the F test; ** = Significant at 1% probability by the F test; ns = not significant.

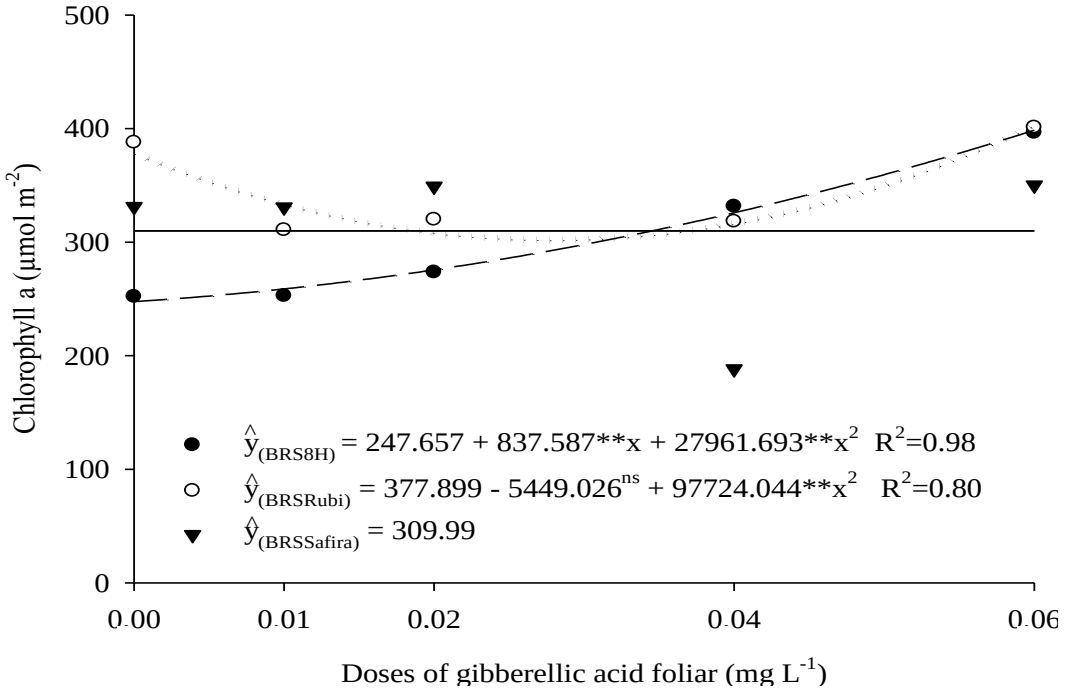

Figure 2. Leaf chlorophyll *a* content in the BRS-8H cotton cultivars (- -), BRS-Rubi (-) and BRS-Safira (•••) due to the application of increasing doses of gibberellic acid foliar.

relatively low variation, ranging from 17.64 to 3.86%, depending on the variable analyzed (Table 2).

The foliar concentration of chlorophyll *a* in cotton cultivars increased with the rise of gibberellic acid doses, except BRS Safira, not set to any regression model with increasing doses of GA_3 and was represented by the average level of 309.09 µmol m^{-2} (Figure 2). In cultivating cotton cv. BRS 8H, it was found that the application of 0.06 mg L^{-1} GA_3 provided increase in leaf chlorophyll *a*, it is found to confront the contents of 247.657 µmol m^{-2} (0 mg L^{-1}) and 398.574 µmol m^{-2} (0.06 mg L^{-1}) in which the highest dose of the plant growth regulator in increased 60.93% pigment content in the leaves. In cotton cv. BRS Rubi, there is a reduction in CL*a* content to the estimated dose of 0.0278 mg L^{-1} GA_3 above this dose increased the chlorophyll content of the cotton sheets, until the 402.763 µmol m^{-2} content when applied dose of 0.06 mg L^{-1}. In soybean (*Glycine max* L.), Campos et al. (2008) reported that leaf application of gibberellic acid inhibits degradation or even increases the chlorophyll content in the leaves. The gibberellic acid is often applied in harvest treatments and post-harvest in order to keep the fruits with greenish longer and hence promote the delay harvest, and increase the sale period of fruits (Modesto et al., 2006).

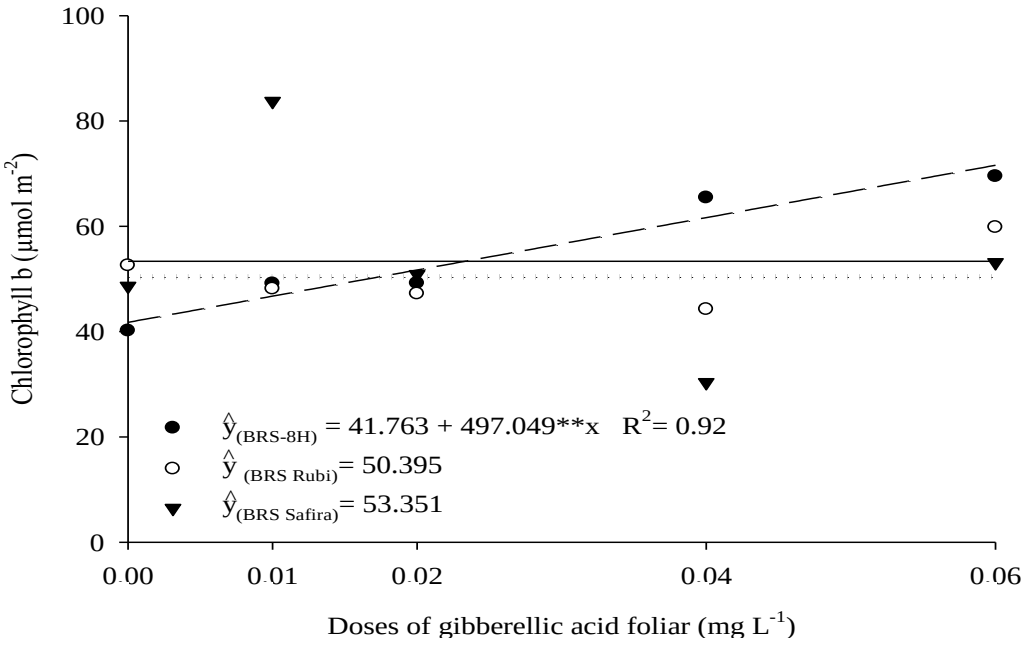

Figure 3. Foliar content of chlorophyll *b* in BRS-8H cotton cultivars (- -), BRS-Rubi (-) and BRS-Safira (•••) due to the application of increasing doses of gibberellic acid foliar.

These differences are also dependent on the genetic material as found Alia-Tejacal et al. (2011) evaluated five-flower native cultivars, plant for ornamental purposes originating in Mexico, found differences in chlorophyll content between cultivars. The foliar content of chlorophyll *b* in cultivars BRS Rubi and BRS Safira did not fit with any regression model with increased doses of gibberellic acid, with average levels of 50.395 and 53.351 µmol m^{-2}, respectively (Figure 3). In cv. BRS 8H, the CL*b* content increased linearly 497.049 µmol m^{-2} per unit increase in the dose of plant growth regulator applied. This is evidenced by comparing the plants without and sprayed with a dose of 0.06 mg L^{-1}, in which the highest dose promoted 71.4% gains over the plants was not applied GA_3.

Campos et al. (2009) found that the application of gibberellic acid in soybean, increased the chlorophyll content up to 105 days after sowing. This is due mainly because of plant growth regulators influence in maintaining the integrity of the photosynthetic apparatus, interfering with chlorophyll synthesis (Synková et al., 1997). Onanuga et al. (2012) found that the production of chlorophyll *b* in cotton produced in China are variable depending on the cultivar, this response being attributed to the interaction of gene expression of each material with the culture environment. The maintenance and increases chlorophyll *b* content in the plant species sheets is of great importance to all photosynthetic process, since chlorophyll *b* is considering an accessory pigment, aiding in the absorption of light and the radiant energy transfer to the centers reaction that are located on the membranes of the thylakoids (Taiz and Zieger, 2013).

The total chlorophyll content in cotton cultivars of leaves increased with the rise of gibberellic acid doses, except BRS Safira, not set to any regression model, with average content of chlorophyll 359.719 µmol m^{-2}, as seen in Figure 4. To BRS 8H in the absence of GA_3, CL*t* content was 287.914 µmol m^{-2}, and increased to 468.796 µmol m^{-2} to apply 0.06 mg L^{-1} plant growth regulator, this increase corresponds to an increase of 62.82% in the foliar chlorophyll. In cv. BRS Rubi, the total chlorophyll content decreased to the estimated dose of 0.0277 mg L^{-1} GA_3, doses above notes to increase the levels of total chlorophyll pigments and applied at the maximum dose (0.06 mg L^{-1}) there is CL*t* content of 461.745 µmol m^{-2}.

Onanuga et al. (2012) found significant differences in the production of chlorophyll pigments (total chlorophyll) between cotton varieties tested under application of a solution containing hormones, which contained 40 µg L^{-1}gibberellic acid. Similarly, the application of 5 µg L^{-1} GA_3 in leaves of the vinca-of-Madagascar (*Catharanthus roseus*) stimulated increased leaf chlorophyll concentration (Jaleel et al., 2009). Furthermore, the response is variable and depends on the interaction between the genotype and environmental conditions imposed cultivation (Ferrari et al., 2008), given that each of cotton cultivar responded differently.

It can be seen in Figure 5, the cotton cultivars BRS 8H and BRS Rubi showed reductions in chlorophyll *a*/*b* to respective gibberellic acid doses of 0.033 and 0.030 mg L^{-1}. Lifting these doses appears in the list of chlorophyll pigments with 5.72 values in cv. RBS 8H and 7.23 in cv.

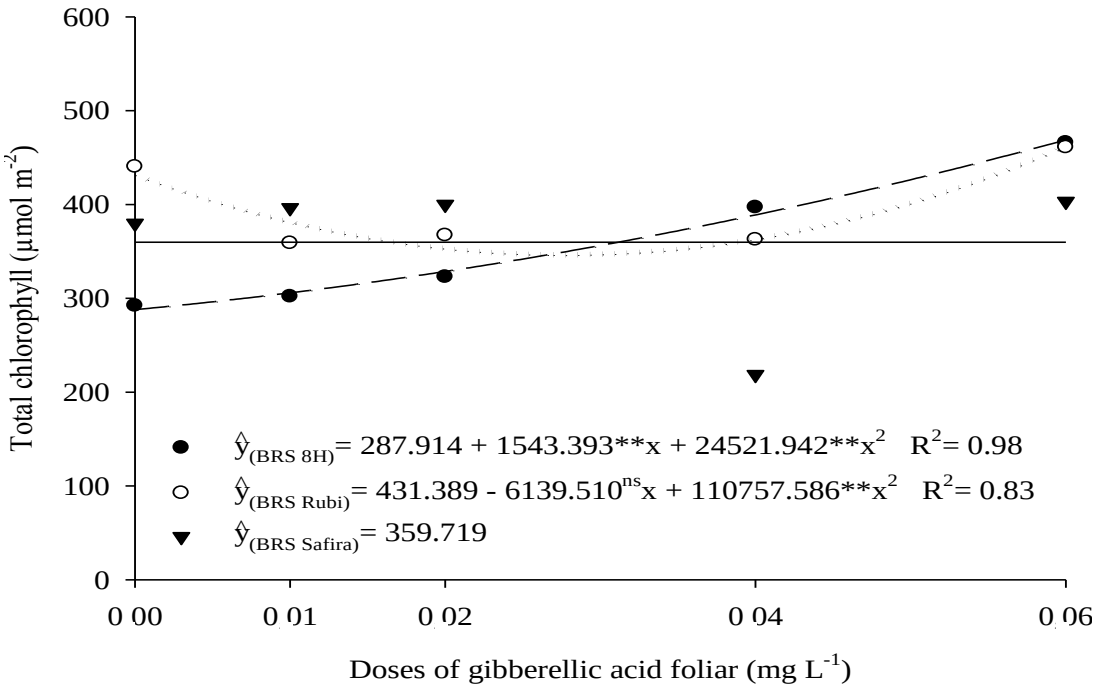

Figure 4. Foliar content of total chlorophyll in the BRS-8H cultivars (- -), BRS-Rubi (-) and BRS-Safira cotton (•••) due to the increasing doses application of gibberellic acid foliar.

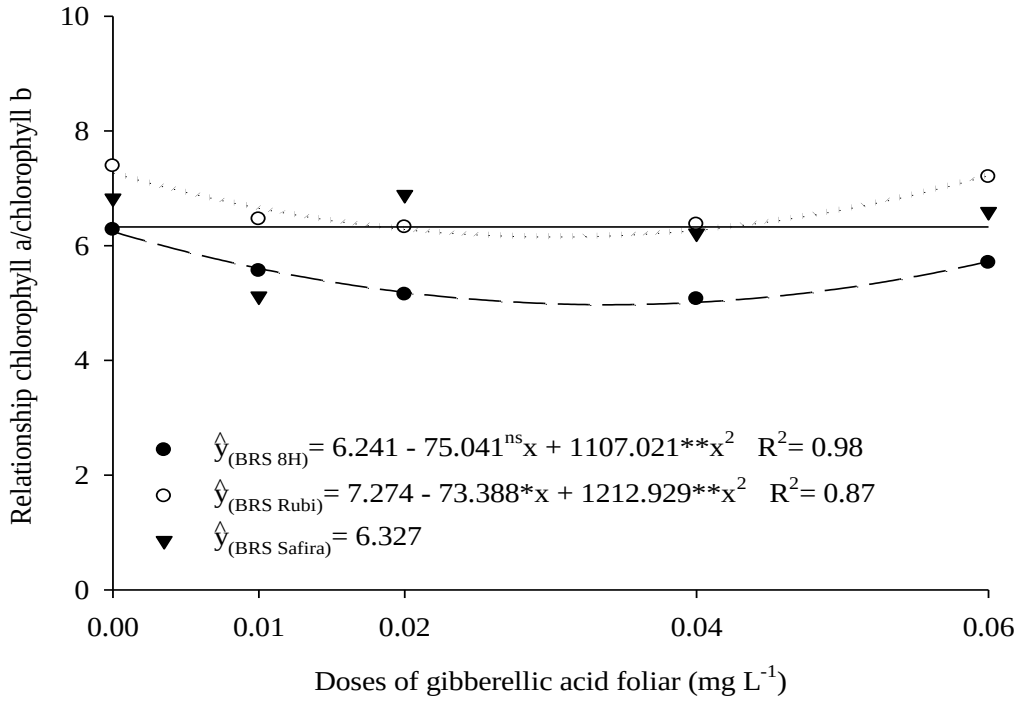

Figure 5. Relationship chlorophyll *a*/chlorophyll *b* in leaves of cotton cultivars BRS 8H (- -), BRS-Rubi (-) and BRS-Safira (•••) due to the application of increasing doses of gibberellic acid foliar.

BRS Rubi the maximum applied dose of 0.06 mg L^{-1}, however, these values are lower than the treatments without applying the plant growth regulator. As noted in the other variables related to chlorophyll levels, the

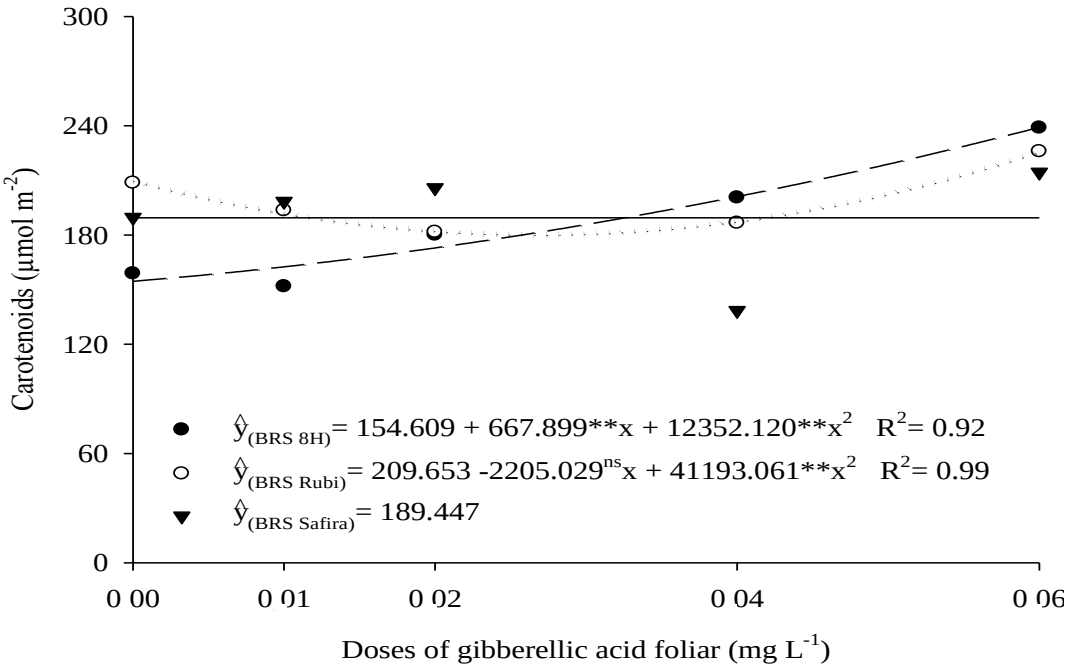

Figure 6. Foliar content of carotenoids in cotton cultivars BRS 8H (- -), BRS Rubi (-) and BRS Safira (•••) due to the foliar application of increasing doses of gibberellic acid foliar.

relationship CL*a*/*b* cv. BRS Safira, not set to no regression model with increased doses and GA_3, was represented by the average value of 6.32. Unlike the results observed in this study, Fioreze and Rodrigues (2012) evaluating the effect of applying foliar biostimulant auxin base + gibberellin + cytokinin in wheat plants (*Triticum* spp.), found no response in chlorophyll *a*/*b* flag leaf. The chlorophyll *a*/*b* is an important variable since the reduction ratio value is assigned a lot of times and it leads to increased leaf chlorophyll *b*; this response may be considered as an adaptive characteristic of the ambient conditions, since pigment that absorbs energy at a different wavelength of chlorophyll maximizes energy capture used in the photochemical step in photosynthesis and then transfer to the reaction centers - photosystems (Taiz and Zeiger, 2013). The leaf carotenoid levels responded differently for each cotton cultivar according to gibberellic acid levels (Figure 6). BRS 8H observed increase in levels of CAR 154.60 to 239.15 µmol m^{-2} between plants without spraying.

It was sprayed with 0.06 mg L^{-1} which corresponds to a larger percentage, 54.68%, in the sprayed treatment with the highest dose biostimulant. In BRS Rubi, the leaf levels of carotenoid decreased to 0.026 mg L^{-1} dose and from that dose the CAR levels became high, reaching a maximum value of 225.64 µmol m^{-2} at a dose of 0.06 mg L^{-1}. Meanwhile, the carotenoid content in cv. BRS Safira presented an average of 189.47 µmol m^{-2} with the increase of doses for plant growth regulator without adjusting any regression model. The increase in the dose of gibberellic acid promoted growth in the concentration of carotenoid per gibberellic acid and carotenoids to possess the same precursor, geranylgeranyl pyrophosphate (GGPP, C20). Therefore, the application of exogenous plant growth regulator may be applied to the need of the plant in relation to the concentration of gibberellic acid and the most of it diverted to the precursor for the synthesis of carotenoids (Castro et al., 2012). This trend of results was observed by Jaleel et al. (2009) in plant vinca-of-madagascar. After applying gibberellic acid foliar, they found that the leaf carotenoid content was high when applied to 5 µM L^{-1} GA_3. Carotenoids are extremely important for the plants, as they play a significant role in protecting the photosynthetic apparatus against photobleaching of photosystems (Taiz and Zeiger, 2013).

BRS 8H and BRS Safira cotton cultivars showed distinct trends cv. BRS Rubi relative water content in leaves in response to GA_3 doses (Figure 7), demonstrating that the response to plant growth regulator varies within the cultivars of the same species. In BRS 8H and Safira, the CRAs increased linearly from 65.90 to 78.90% and 62.74 to 77.82%, respectively, between treatments without application and application 0.06 mg L^{-1} biostimulant. Inverse response was observed in cv. BRS Rubi, where increasing doses GA_3 linearly reduced water on the leaves. From the results, it was found that when gibberellic acid was not applied, the sheets had an CRA 74.58%, reducing the value of 62.04% at the applied dose of 0.06 mg L^{-1} GA_3. Many authors have observed

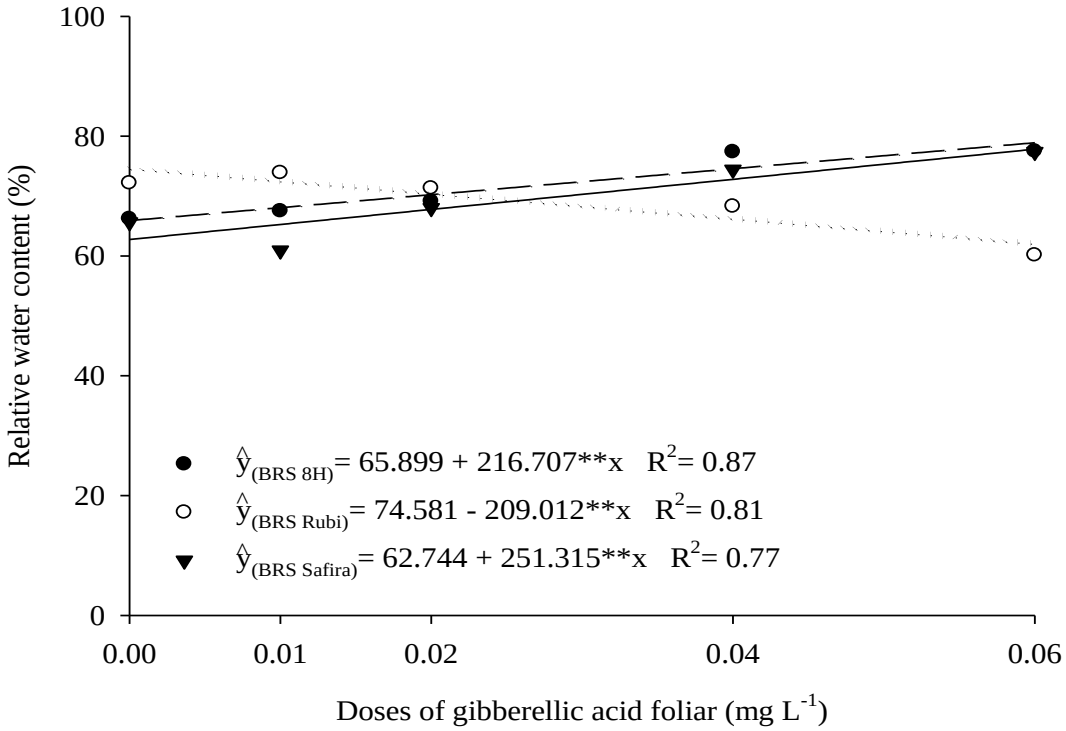

Figure 7. Relative water content in leaves of BRS 8H (- -), BRS-Rubi (-) and BRS-Safira cotton (•••) according to the foliar application of increasing doses of gibberellic acid foliar.

the benefits of gibberellic acid's foliar application in the relative water content of various crops, however, the correct dose to be applied depends on the plant species (Carvalho Júnior et al., 2016). This is verified by Ali et al. (2012) who found that the application of GA_3 increased the relative water content in the leaves of hibiscus (*Hibiscus sabdariffa* L.) under salt stress. In sweet potato plants (*Ipomoea batatus* L.), the exogenous application of gibberellic acid at a concentration of 10 ml L^{-1}, as well as improving the biometric and production parameters, promoted the increase in CRA in the leaves (El-Tohamy et al., 2015).

Conclusions

The application of 0.06 mg L^{-1}gibberellic acid, in general, promotes increased levels of photosynthetic pigments and relative water content in cotton leaves. However, cultivars respond differently to the application of plant growth regulator.

The BRS 8H cotton was among the evaluated materials with the plant variety that best meets the application foliar gibberellic acid. Despite the satisfactory results obtained in this work, although more studies are needed to clarify the effects of gibberellic acid on the physiological responses of cotton cultivars, especially those with colored fiber.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Quality of seeds from Leucaena species stored under ambient conditions

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The objective of this research was to know the performance of the seeds from different species of the Leucaena genus (*Leucaena leucocephala*, *Leucaena lanceolata*, *Leucaena diversifolia*, *Leucaena macrophylla* and *Leucaena esculenta*), stored under ambient conditions and subject to an evaluation of their physical, physiological and sanitary quality. The material came from the harvests of the collection planted at the Pastures and Forages Research Station Indio Hatuey (Matanzas province, Cuba). Samples were taken from two seed lots, with 6 and 18 months of storage, in which the moisture content, germination and viability were determined. In addition, the hard, fresh and dead seeds were counted. A completely randomized design was used, and the data were processed through a variance analysis (ANOVA). The results indicated the variation, in terms of quality, of the stored seeds at 6 months with regards to those that had been stored for 18 months; however, both lots are considered adequate. The rotting increased (75%) with the months of storage; while the viability decreased as the storage time and physiological age increased (95 vs. 65,7% at 6 and 18 months, respectively). Fungi were the main microorganisms that caused seed rot, with higher effect on the seeds that had been stored for 18 months.

Key words: Physical and physiological quality, *Leucaena spp.*, seeds, storage

INTRODUCTION

The need to produce food for a constantly growing population causes large areas of forests and rainforests to be degraded, with which the biodiversity of these ecosystems is lost. In such areas there are tree and shrub species with potential to contribute to the livelihood" instead of economy of farmers. In this sense, Ramos-Quirarte et al. (2009) stated that trees and shrubs, due to the many products and benefits they provide, represent an important patrimony for the dwellers. Tree and shrub species are valuable resources for animal feeding and the wild fauna. Their use represents an option in livestock production systems, to avoid dependence on concentrate feeds (Suárez et al., 2011). In the tropic and subtropical regions the *Leucaena leucocephala* species is one of the most used in such systems, as well as in cut-and-carry systems for small

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farmers (Ruiz and Febles, 2006). However, its initial growth is slow and has some disadvantages regarding its establishment, due to the dormancy of seeds, which is caused by the presence of a cuticle impermeable to water and oxygen (Sánchez and Ramírez-Villalobos, 2006; González et al., 2009). The initial propagation of the species of leucaena is by sexual seed (Hughes, 1998), which is a practical and economic process, highly used by farmers. The physiological quality of the seeds of this species is determined by the germination percentage, moisture content and vigor, if it is considered that they are very important factors at plantation level (Román-Miranda et al., 2013).

In this sense, seed conservation (storage) constitutes the basis of breeding works and allows the exchange of germplasm and the preservation of genetic viability, mainly. However, despite this, there are few studies on all species of the genus. The main reason for seed storage according to reports by Besnier Romero (1989), is their distribution in time and space, which from the point of view of their utilization, for which they are reproduced, should allow their longevity; that is, the preservation of their adequate viability and vigor in the most appropriate places for their germination and establishment during a reasonable time.

In this sense, according to Alves et al. (2002), seed storage acquires high importance in the production process because there is generally a time interval between the seed harvest and the later seeding, which can last a few days or be extended for several months, according to the species and crop, production site, prevailing environmental conditions and production technology. The fundamental reason for storage is related to the preservation of the physiological and sanitary quality of the seeds, due to the reduction of contamination by potential insect pests, the incidence of microorganisms and the minimization of the deterioration rate.

During storage, seed deterioration cannot be prevented, although the rate of the process can be minimized through adequate procedures of production, harvest, drying, processing, transportation and through biochemical and physiological disturbances started right after the physiological maturation, which causes vigor reduction, ending in the loss of germination capacity. Among the factors that affect the maintenance of seed quality along a certain period, the humidity degree, condition of the storage environment (mainly air temperature and relative humidity) as well as the type of packing used stand out (Gálvez, 2012).

Nevertheless, in spite of the studies conducted, specifically with the species *L. leucocephala*, as well as of the importance of seed quality for farmers, there are limit studies about the performance of stored seeds; therefore, the objective of this research was to determine the performance in different species of the *Leucaena spp*. genus, stored at ambient temperature.

MATERIALS AND METHODS

Provenance of the evaluated plant material

The seeds of the evaluated species of the *Leucaena spp*. Genus (*L. leucocephala*, *Leucaena lanceolata*, *Leucaena diversifolia*, *Leucaena macrophylla* and *Leucaena esculenta*), stored under ambient conditions with time difference of 6 and 18 months, came from the harvests of the collection planted at the Pastures and Forages Research Station Indio Hatuey –Matanzas province, Cuba.

Edaphoclimatic conditions

The mother plants from which the seeds were collected are planted on a humic nodular ferruginous hydrated lixiviated Ferralitic Red soil, of fast dessication, clayey and deep on limestone (Hernández et al., 2015). According to the above-mentioned author's reports, this soil is slightly acid. The results of the chemical composition of the soil are shown in Table 1.

Climate characteristics

The climate of the site is classified as of tropical savanna, characteristic of Cuba (Academia de Ciencias de Cuba, 1989), in which the tropical marine conditions prevail with marked seasonality of the rainfall, where the influence of the arctic and continental polar air masses is felt in the winter dry season which is extended from November to April. In the last 15 years before the research, the annual average temperature of the zone was 24.3°C, July was the warmest month, with 28.6°C, and January the coldest one, with 20.6°C. During the study the maximum temperatures reached 33.4°C in August and the minimum ones went down to 14.2°C in January. The average sum of the annual rainfall was 1 331.18 mm, with the highest value in June (235,8 mm) and the lowest one in February (only 27.4 mm). The rainfall during the rainy season (May-October) represented, as average, 79.8 % of the total annual volume. The evaporation in the zone increased since January, with maximum values in April (220 mm). The annual average relative humidity was 82.6%, with the highest value in July (89.0%) and the lowest one in April (75.5%).

Post-harvest seed management

The pods were manually harvested. Afterwards, the ginning and natural drying (72 h under sunlight) of the seeds was carried out, in order to reduce their moisture content between 10 and 13%, with which, according to Gálvez (2012), the growth of fungi and, thus, the destruction of embryos, is avoided. Then, they were processed and stored (6 and 18 months) under ambient conditions, during the two years of research, in a rectangular shed of 52 m^2 of basis and 3 m of height, with continuous adequate ventilation, according to the regulations for this type of facility (Harrigton, 1972; Gálvez, 2012). The seeds were selected according to ISTA rules (2005), and the physical and physiological quality of the seeds with 6 and 18 months of storage, through three of the recommended essays; in addition, the sanitary quality was evaluated. The seeds were not treated.

Determination of the physical and physiological quality of the seeds

To determine the humidity content (%), the gravimetric method in hot stove (130 ± 30°C) was used, according to the recommendation

Table 1. Chemical characteristics of the soil1 of the experimental area.

Indicator	Mean value	Analytical method
pH (H_2O)	6.34	Potentiometric
Organic matter (%)	5.42	Oniani
Total nitrogen (%)	0.22	Kjeldahl
P_2O_5 (mg/100 g of soil)	3.75	Walkley-Black
K^+ (meq/100 g of soil)	0.19	Maslova
Ca^{++} (meq/100 g of soil)	17.1	Maslova
Mg^{++} (meq/100 g of soil)	2.30	Maslova
Na^+ (meq/100 g of soil)	0.19	Maslova

[1]Results of the chemical analysis of the soil, conducted in the Soil Laboratory of the Soil Resarch Institute.

made by ISTA (2005); the calculation was made based on the fresh weight. Four replications of 10 g of seeds from each lot were used, and the formula proposed by ISTA (2005) was applied. The germination test (%) was performed according to ISTA (1999) standard for tropical trees using four replicates of 100 seeds for each species evaluated. The emission of the radicle from the seed coats was taken as germination criterion (ISTA, 2005). The seedlings were evaluated after planting and were classified from the criterion of the seedling evaluation handbook, according to ISTA rules (2005). The hard, fresh and dead seeds were also counted. The result was expressed in percentage of normal seedlings. To corroborate the accuracy of the results, the tolerance table 14.3 of chapter 14 of the Handbook of Seed Technology for Genebanks was used (Ellis et al., 1985). In the case of dead seeds, the ones damaged by fungi or other microorganisms and the rotten ones without visible signs or symptoms of affectation were included. The viability test was estimated through the biological method recommended by ISTA (1999), thus determining the viability percentage, which was expressed in viable and non-viable seeds.

Statistical analysis

The data expressed in percentages were transformed using the mathematical function sen-1√% (Steel and Torrie, 1992). They were processed by a one-way variance analysis (ANOVA), through the InfoStat program. The means were compared by Duncan's test (1955), at 5% level of significance, after verifying that they fulfilled the fit of normal distribution and variance homogeneity.

RESULTS AND DISCUSSION

Regarding the moisture content, there were no significant differences between both lots (Table 2). All the seeds did not respond equally to storage, independently from the fact that there were no significant differences among the species: the seeds of *L. esculenta* showed lower moisture content, followed by those of *L. lanceolata*, with regards to the ones of *L. leucocephala*, at 6 as well as at 18 months.

The storage of leucaena seeds in the tropic is carried out under environmental conditions of high relative humidity (85% of relative humidity) and temperature (> 26°C), which are not controlled; these associated variables significantly influence quality. Bustamante (2010) reports that humidity contents have direct influence on seed longevity, because they stimulate the metabolic activity of the embryo. This circumstance is relevant because the physiological quality of the seed is negatively affected, while what is required is high physiological potential represented in seed vigor. The concepts of 'vigor', attribute belonging to the seeds capable of germinating, and 'deterioration', are physiologically linked and are reciprocal aspects which have incidence on seed quality. Deterioration has a negative connotation, while vigor has an extremely positive meaning: vigor decreases as deterioration increases due to the rise of temperature and relative humidity (RH) in the storage time. Deterioration refers to the ageing process and death of the seeds, and thus, vigor is the main component of quality that is affected by the deterioration process (Teofilo et al., 2004).

Seed longevity is given by a balance between intrinsic and extrinsic factors that mainly affect the deleterious mechanisms of metabolism and the repair processes. Likewise, the period in which the seeds remain viable is very variable and is genetically determined, although the storage conditions and environmental factors have a determinant effect on the duration of a seed's life (Bustamante, 2010). *Leucaena* seeds are small; for such reason they can register differences in the absorption of water from the prevailing RH in the storage medium, which can originate variations in the moisture contents of the samples. The hygroscopic nature of the seeds and the environmental conditions under which they are kept influence the process of water uptake or loss; this causes damage which reduces the seed physiological quality. That is why, as the hydration and water loss cycles increase, germination decreases and the effects are more critical with the hydration periods (Aramendiz-Tatis et al., 2007). The increase of RH and temperature, associated with the storage time of the seed, leads to a progressive decrease of seed vigor due to the deterioration caused by the loss of membrane integrity (Delouche et al., 1973). The RH exerts influence on the moisture content of the seed and its effect on their

Table 2. Moisture content in the analyzed seeds.

Seeds	Moisture content (%)	SE (±)
6 months		
L. leucocephala	11.09^{NS}	
L. lanceolata	8.89^{NS}	
L. diversifolia	10.07^{NS}	0.01
L. macrophylla	9.07^{NS}	
L. esculenta	7.06^{NS}	
18 months		
L. leucocephala	9.97^{NS}	
L. lanceolata	7.89^{NS}	
L. diversifolia	9.87^{NS}	0.01
L. macrophylla	8.79^{NS}	
L. esculenta	6.96^{NS}	

*Average values expressed in %). Equal letters do not differ for p ≤ 0.05.

Table 3. Germination percentage of the sedes.

Seeds	Germination*	SE (±)
6 months		
L. leucocephala	75^{a}	
L. lanceolata	65^{b}	
L. diversifolia	75^{a}	0.01
L. macrophylla	71^{a}	
L. esculenta	63^{c}	
18 months		
L. leucocephala	27^{a}	
L. lanceolata	19^{b}	
L. diversifolia	26^{a}	0.01
L. macrophylla	24^{b}	
L. esculenta	16^{c}	

*Average values expressed in %. Equal letters do not differ at *P* ≤ 0.05.

longevity is direct. In this regard, Powell and Matthews (1981) express that seed ageing occurs much faster when they show high moisture content and are stored at high temperature, because the biochemical processes are affected (Amaral and Lemos, 2009).

While most of the cultivated and wild plants maintain their viability better when they are preserved with low moisture contents and at low temperatures, others, especially those from tropical regions, do not survive when that content is lower than a certain value (Gálvez (2012). For such reason, he distinguishes two seed categories, according to their response during storage: orthodox and recalcitrant. This aspect was also approached by Schmidt (2000), who places the species of the *Leucaena* genus in the first category. Its seeds can be satisfactorily kept exsitu, during long periods, under adequate conditions; they can be dried to low moisture levels, without suffering damage; and their longevity is increased with the decrease of humidity and temperature. According to Harrington (1972), in seeds stored under ambient conditions, the moisture content is considered optimum when it reaches between 10 and 12%; which coincides in a certain way with the results of this study. Likewise, Benkonva and Zakóva (2009) state that such values are appropriate for short- and medium-term conservation. On the other hand, Delouche et al. (1973) reports that with these values the physiological deterioration will depend directly on other processes inherent to the seeds (for example, the biochemical ones such as reactivation and synthesis of enzymes, respiration, absorption of O_2, consumption of carbohydrates, among others), and not on the moisture content itself. Similarly, Gálvez (2012) states that there should be a balance between the relative moisture of the seeds and that of the environment; however, it is valid to point out that the seeds with hard coats, such as those of *Leucaena,* are an exception.

The physiological quality of a seed lot implies that they fulfill the indispensable condition of viability; but, in addition to being alive, they must germinate and produce a seedling with its essential structures correctly developed. To start the germination process, they must be physiologically mature and, thus, the maturity status is considered an important characteristic of their quality. For such reason, the immature or not completely mature seeds, generally, show lower physiological quality with regards to the seeds that reached maturity (Del Valle, 2008). In this sense, significant differences (p ≤ 0.05) were found in germination (Table 3); there was a higher percentage in the seeds with lower storage time (6 months). A marked difference was also observed in the performance of *L. esculenta* (65 %) with regards to *L. leucocephala*, (between 80 and 85 %) which is perhaps conditioned by the more frequent use of the latter in breeding programs, or because it is more domesticated than the former. On the other hand, the 18-month seeds showed high percentages of hard, fresh and dead seeds (Figure 1); unlike the ones with lower physiological age (15 and 7 %, respectively). The germination test provides sufficient information on the performance of a seed lot. Although there are many factors that can affect germination and, thus, seedling emergence, temperature plays an important role (Nacimento, 2005; Aramendiz-Tatis et al., 2007). However, authors such as Lopes and Pereira (2005) agree that there is not a general optimum temperature, because each species has a particular optimum temperature range to germinate, and within that range marked differences can appear among cultivars and, seemingly, among species.

Physiological age is another intrinsic factor which can affect good germination; hence the 6-month seeds had a better performance in this indicator than the 18-month ones. According to reports by Courtis (2013), as they advance in age, seeds tend to absorb water faster. This

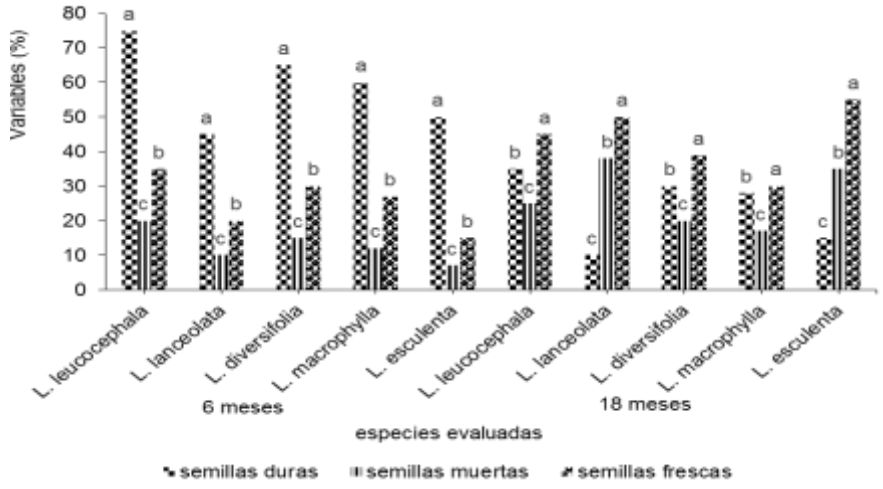

Figure 1. Performance of the variables during the germination test.

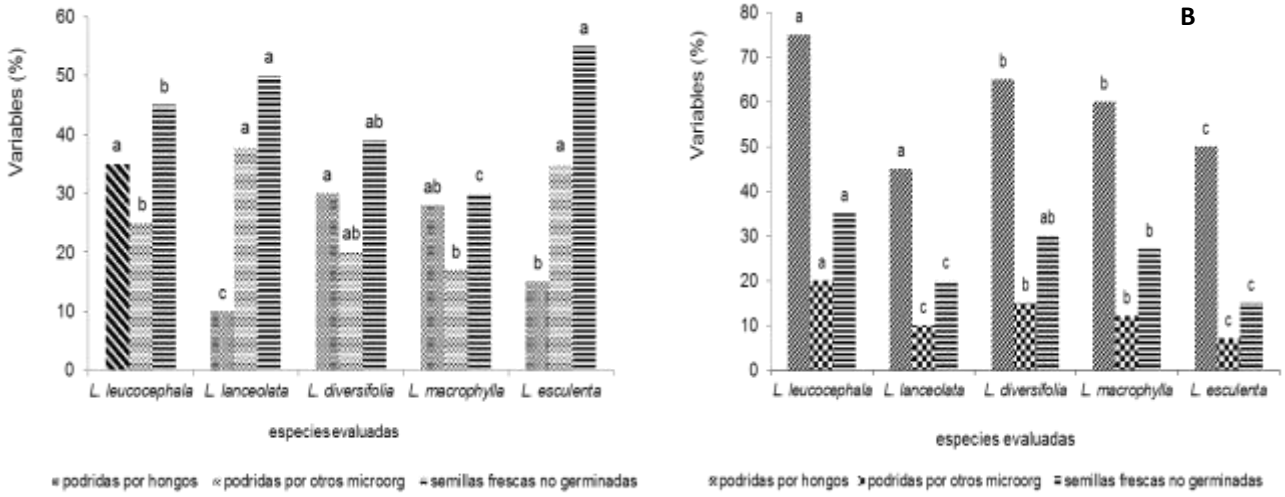

Figure 2. Performance of the species, according to the evaluated variables; a, 6 months; b, 18 months of storage.

phenomenon is considered associated to the loss of integrity of cell membranes. Another factor that could have influenced the decrease of germination is the maturity status of the seeds, which can be visually inferred through the observation of the absence or presence of chlorophyll pigments, which act as receptors of the light energy in the photosynthesis process. Nevertheless, in different situations they can be transformed into a negative factor, by reducing the germination capacity and the vigor of seeds (Scheeren and Tolentino, 2005). During the period of seed formation, and even after the plants reach the status of physiological maturity and of commercial maturity, the tissues of the pods and the coats of the seeds are contracted and elongated as a result of the humidity and temperature fluctuations that occur throughout the day (Arango et al., 2006). This hydration and dehydration process which occurs in the seed structures, during seed development, originates damages of different magnitude and affects, to a higher or lesser extent, the physiological quality. In this test the superiority of the *L. leucocephala* seeds with regards to those of *L. esculenta* was also observed, which could be conditioned by the higher use of the first species compared with the others; as well as by the presence of hard seed coats (Gálvez, 2012), which can cause some species to be harder than others; although, in general, the values were low. The *L. esculenta* seeds were the most affected ones in the higher storage time.

In this sense, another factor that perhaps influenced the decrease of germination (Figure 2) is seed rot, which reached the highest values in the seeds with more time of storage and physiological age (75% of affectation). Román-Miranda et al. (2013) obtained similar results in

Table 4. Viability percentage in the analyzed samples.

Seeds	Viability*	SE (±)
6 months		
L. leucocephala	95[a]	
L. lanceolata	65[b]	
L. diversifolia	85[a]	0.01
L. macrophylla	71[a]	
L. esculenta	63[c]	
18 months		
L. leucocephala	65[a]	
L. lanceolata	19[b]	
L. diversifolia	56[a]	0.01
L. macrophylla	34[b]	
L. esculenta	16[c]	

*Average values expressed in percentage. Equal letters do not differ at $p \leq 0.05$.

seeds of *L. lanceolata*. In tropical countries, where temperature and RH always reach high and continuous values, the presence of potentially pest insects and microorganisms is favored. Thus, according to studies conducted by Cerovich and Miranda (2004) for good storage under ambient conditions, it is essential to maintain the moisture content of grains and seeds low. According to reports by the mentioned researchers, this high affectation may depend more on the relative humidity and temperature of the environment, than on the moisture content of the seeds. In addition, the moisture content the seeds of other species of the genus should have in order to be stored is not accurately known. The highest affectations occurred in the *L. esculenta* seeds. This superiority could have been influenced by the storage period, which was evidently higher at 18 months, and also by the environmental conditions; and to a lesser extent, the affectations provoked by other causes appeared; in them it was observed that although there were no significant differences, there was an increase in the seeds with higher physiological age.

Along with the above-mentioned factors, others should also be considered that somehow have incidence on seed storage, such as: genetic characteristics of the species to be stored, pre-harvest history of the crop, structure and chemical composition of the seed, degree of maturity, presence of dormancy, vigor and mechanical damage (Cerovich and Miranda, 2004). Similarly, the performance of the hard, fresh and dead seeds may not be due only to the depletion of nutritional reserves –which they preserve mostly, even after the loss of their germination capacity–; but also to the damage suffered by the cell membranes as well as the membranes of intracellular organelles (mitochondria, plastids, among others), which is known as natural aging during storage under ambient conditions, among other causes (Jaramillo et al., 2012). In this sense, Bustamante (2010) states that seed deterioration is a complex mechanism of which all the physiological and biochemical processes that participate in it are not accurately known; while the compensation of the damage undergone in storage occurs during imbibition, which causes the delay of germination and vigor loss or, even, if the damage is very important, the seeds are incapable of germinating or the seedling dies after germination. The viability percentage (Table 4) decreased as the storage time increased (95 vs. 65,7 %, for the seeds with 6 and 18 months, respectively), which coincides with the report by Benkonva and Zakóva (2009) about the trend of this quality indicator during the storage. In this case, the seeds of *L. esculenta* were also the most affected.

The viability loss that occurred in the 18-month seeds could have been due to the action of fungi or other microorganisms. According to Gálvez (2012), when this occurs other processes are affected, such as: enzymatic activity; synthesis or metabolism of proteins, carbohydrates or lipids; cell respiration; and increase of the volatile components which are toxic for the seeds, the chromosomal material and the DNA synthesis. This deterioration does not occur in a uniform way, but, in general, starts in meristematic areas, especially in the root meristem. Likewise, it depends on each species and the group to which it belongs (recalcitrant or orthodox seed). Equally, although it was not considered in the research, the inadequate collection processing and transportation (to the storehouse, until the moment of seeding) could have influenced the decrease or loss of viability. In general, the seeds showed an acceptable performance during the study, if it is considered that the increase of relative humidity and temperature, associated to the storage time, lead to a progressive decrease of vigor, due to the deterioration caused by the loss of membrane integrity (Delouche, 2002). It is important to mention that in the consulted literature there are very few studies about the seeds of species of the *Leucaena* genus: only some accessions of *L. leucocephala* are mentioned, while regarding the others their use as parents in breeding programs is reported (*L. diversifolia* and *L. macrophylla*).

Conclusions

(1) There was variation in terms of the physical, physiological and sanitary quality in both lots; although it was more marked in the one with 18 months of storage. The most affected were the seeds of the species *L. esculenta*.
(2) There was an increase in seed rot as the storage period under ambient conditions increased; the most affected were the ones of the species *L. esculenta*.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Abundance and distribution of Ixodid tick species infesting cattle reared under traditional farming systems in Tanzania

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Ticks and tick-borne diseases are serious constraints to livestock production in Tanzania and other sub-Saharan African countries. Despite this, knowledge on the abundance of tick species infesting cattle in most parts of Tanzania is insufficient or lacking. This study was conducted to identify species and establish the abundance of ticks infesting cattle in Mara, Singida and Mbeya regions of Tanzania. The ticks were collected from one side of the body, counted and identified, based on morphological characteristics; to species level. The mean tick count per animal was significantly higher in Mara (35.8±4.3, p=0.0001) as compared to Singida (12.9±2.1) and Mbeya (7.0±0.4) regions. Young animals in Mara (24.7±6.0, p=0.0395) and Mbeya (5.4±0.3, p=0.0252) exhibited relatively lower mean tick counts compared to the weaners (Mara = 33.8±6.5, Mbeya = 7.2±0.7) and adult animals (Mara = 46.3±8.4, Mbeya = 7.8±0.7). Seven tick species from three different genera, namely *Ambylomma*, *Hyalomma*, *Rhipicephalus* (including the subgenus *Boophilus*), were identified. However, only five species (*A. lepidum*, *A. variegatum, R. decoloratus, R. microplus* and *H. rufipes*) were observed in all the three regions. *R. appendiculatus* and *R. evertsi* were not found in Mbeya and Mara respectively. The most prevalent species in Mara, Singida and Mbeya were *R. appendiculatus* (50.5%), *A. lepidum* (31.2%) and *R. evertsi* (35.6%), respectively. This study showed the existence of a variety of tick species, most of them being of veterinary importance. Therefore, strategic planning and cost-effective tick control measures should be implemented in order to reduce losses caused by ticks and tick borne diseases in the study area.

Key words: Ixodid ticks, abundance, distribution, cattle, Tanzania

INTRODUCTION

Livestock keeping is one of the primary economic activities in Tanzania and contributes greatly to food security and income of small holder farmers. The livestock sector in 2014 contributed 4.4% to the National GDP of Tanzania (MLFD, 2014). Tanzania has 22.8 million cattle, 15.6 million goats, 35.5 million indigenous chickens, 24.5 million commercial chickens and 2.01 million pigs (MLFD, 2014) and 90% of agricultural households keep livestock of some kind. In importance, cattle come top followed by goats. In Tanzania 95% of the cattle population is reared under traditional agro-pastoral and pastoral husbandry systems (Msechu, 2001). Under such traditional rearing systems cattle are extensively grazed in pastures and forests and, hence, exposed to a high risk of tick infestation (Swai et al., 2005; Kwak et al., 2014; Laisser et al., 2014).

Ticks are the main vectors for disease causing agents (protozoa, bacteria, fungi and viruses) to humans, livestock and wild animals all over the world (Aydin et al., 2015). Ixodid ticks are harmful blood-sucking ectoparasites of cattle (Tsegaye et al., 2013) and are important vectors for tick-borne diseases (TBDs) such as East Coast fever, babesiosis, anaplasmosis and heartwater which affect cattle in Tanzania and other sub-Saharan African countries (Makala et al., 2003; Kivaria, 2006; Swai et al., 2007). The economic losses caused by the direct effects of these ticks include: Reduced cattle productivity, that is, milk yield, low quality of hides and skin and increased susceptibility to other diseases due to secondary infections (Tsegaye et al., 2013). About 80% of cattle populations in the world are at risk of tick infestation and TBDs (De Castro, 1997). It was already estimated at the end of the 20th century that the annual global cost associated with tick and TBDs in cattle ranges between 13.9 and 18.7 billion USD (De Castro, 1997). Ticks of the genus *Rhipicephalus* have been documented in Australia to cause reductions in cattle live-weight of between 600 and 900 g per animal during the entire period (three weeks) of feeding under conditions of low infestation and more than 2 kg during the same period under conditions of medium to high infestation (Sutherst et al., 1983; Johnson, 2006). In Africa, *R. appendiculatus* and *A. variegatum* have been reported to cause reductions of up to 4 and 46-61 g of live-weight gain per tick, respectively, during the entire period of feeding (Pegram et al., 2000).

A number of tick species are widely distributed all over the world, predominantly in tropical and subtropical countries (FAO, 1984). There are 840 well established species of ticks found worldwide parasitizing livestock, wild animals and human (Walker et al., 2003). In Turkey for example, more than 30 tick species have been identified (Dumanli et al., 2012). Of the 30 tick species identified, 15 of them namely *Rhipicephalus (Boophilus) annulatus, R. bursa, R. sanguineus, R. turanicus, Dermacentor marginatus, Hyalomma aegytium, H. anatolicum, H. detritum, H. Excavatum, H. marginatum, Haemaphysalis parva, Hae Puncatata, Hae Sulcata, Argas percicus* and *Ornithodorus lahorensis* have been observed in all parts of Turkey.

In East Africa more than 79 different tick species have been identified and documented, though most of these appear to be of little or no economic importance (Cumming, 1999). In Tanzania, Ixodid ticks of the genera *Rhipicephalus* and *Amblyomma* are the most important and widely distributed species found in many parts of the country where cattle are raised (Yeoman and Walker, 1967; Lynen et al., 2007; Kwak et al., 2014; Laisser et al., 2014).

Tick abundance varies with time, habitat and agro-ecological zones due to interaction of diverse factors such as host diversity and resistance, climate, absence of control measures and managerial activities that may affect the host behavior (Lightfoot and Norval, 1982; Punya and Hassan, 1992). Knowledge of tick abundance and species composition gives helpful information on tick population dynamics, disease transmission dynamics and estimates of resistance of different hosts (Norval et al., 1992). Thus, in order to establish effective control measures of tick borne diseases (TBDs), knowledge of tick abundance and species present in a particular area is very important as it helps in predicting the occurrence of definite TBDs in such an area. Studies aiming at quantifying tick species, tick control strategies and distribution among agro-pastoral and pastoral cattle populations in Mara, Singida and Mbeya regions are still limited. Existing information on ticks infesting cattle in Tanzania is somewhat obsolete (Yeoman and Walker, 1967; Kagaruki, 1991, 1996) and some is derived from studies based on knowledge and perception of livestock keepers on ticks available in their localities (Chenyambuga et al., 2010; Laisser et al., 2015). The published studies related to tick abundance and species composition was conducted in Ngorongoro (Swai et al., 2005), Iringa and Maswa (Kwak et al., 2014) districts of Tanzania. The most recent published information on tick abundance in Mara region was conducted by Laisser et al. (2014). However, their information is restricted to

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genus level.

The current study provides broad information on tickabundance and species infecting cattle in three regions of Tanzania (that is, Mara, Singida and Mbeya). Mara is situated in the lake zone between latitudes 1° 0' and 2° 31' south of the equator and longitudes 33° 10' and 35° 15' east of Greenwich and it is humid with annual mean rainfall of 1200 mm and altitude ranging from 1120 to 1300 m above sea level. Mbeya is located in the southern highlands between latitudes 7° and 9° south of the equator and between longitudes 32° and 35° East of Greenwich and has an annual mean rainfall of 1650 mm and an altitude of up to 2600 m above sea level. Singida is found in the semi-arid zone between longitudes 33° 27' 5" and 35° 20' east of Greenwich and latitudes 3° 52' and 7°34' south of the equator and is characterized by annual mean rainfall of 650 mm and altitude ranging from 1000 to 1500 m above sea level (MRSP, 1997; SRSP, 1997; MRUF, 2013). Furthermore, the results of the present study will highlight the various tick species present in the study areas. The presence of these ticks will also help to inform management decisions as to what are the probable tick-borne diseases that can be present in the study areas and many other areas that utilize pastoral and agro-pastoral cattle production systems and as such influence the design of effective control measures which can result into the improvement of the physical, economic and social wellbeing of livestock and livestock keepers in rural areas.

MATERIALS AND METHODS

Study area

This study was conducted in three regions of Tanzania, namely Mbeya, Mara and Singida. Mbeya is located in the western corner of the southern highlands of Tanzania and lies between latitudes 7° and 9° south of the equator and between longitudes 32° and 35° East of Greenwich (MRSP, 1997) (Figure 1). The region covers an area of 63,420 km^2 and is divided into eight administrative districts namely; Chunya, Ileje, Kyela, Mbarali, Mbeya, Mbozi, Momba and Rungwe. In this study, sampling was performed in Mbarali and Momba districts. Mean temperatures range between 16°C in the highlands and 25°C in the lowlands. Annual rainfall varies from 650 to 2600 mm and usually starts in October and goes through to May yearly (MRSP, 1997). The vegetation comprises of Miombo woodland dominated by *Broschystegion* and *Julbernardia* species, wooded grassland and bush lands of dense thickets of acacia and thorny trees. The region also supports evergreen forests and bamboo thickets. The region has 870,218 cattle, 544,473 goats, 98,222 sheep and 346,466 pigs and out of 870,218 cattle, 0.08 and 0.01% are improved dairy cattle and beef, respectively (NBS, 2012).

Mara region has a total surface area of 30,150 km^2 and lies between latitudes 1° 0' and 2° 31' south of the equator and longitudes 33° 10' and 35° 15' east of Greenwich (MRUF, 2013). Administratively the region is divided into six) districts, namely: Bunda, Butiama, Musoma, Rorya, Serengeti and Tarime. In this study sampling was undertaken in Tarime and Serengeti districts. The area is scaled with wide valleys, savannah vegetation and receives rainfall between 900 and1500 mm (MRUF, 2013) with mean temperatures ranging from 18 to 35°C. About 90% of residents in the region depend on agriculture as a main source oftheir livelihood. Livestock in Mara comprises 1,691,118 cattle, 913,524 goats, 418,077 sheep and 1741 pigs (NBS, 2012).

Singida region covers an area of 49,341 km^2 and is located in the central zone of Tanzania between longitudes 33° 27' 5" and 35° 20' east of Greenwich and latitudes 3° 52' and 7°34' south of the equator (SRSP, 1997). The region is divided into six (6) districts (Iramba, Ikungi, Manyoni, Mkalama, Singida district and Singida municipality). Sampling was carried out in Iramba and Mkalama districts. Most parts of the region are arid with annual rainfall ranging between 500 and 800 mm. The region has mean annual temperatures ranging between 15 and 30°C depending on season and altitude (SRSP, 1997). The vegetation is mainly dominated by *Acacia commiphora, Hyparrhenia* spp. grassland, *Brachystegia julbernadia* woodland and *Pseudoprosopis fischeri* bush thickets (Itigi thickets) (SRSP, 1997). The livestock population comprises 1,588,837 cattle, 839,169 goats, 477,772 sheep and 48935 pigs (NBS, 2012).

Research design

Sampling was carried out during the dry season, 2015 in the agro-pastoral and pastoral communities with the purpose of determining tick burden, species composition and their associated risk factors in cattle.

Study animals (local East African zebu) were divided into the following age groups, young (≤ 12 months), weaners (12 - 36 months) and adults (> 36 months). A total of 648 cattle (that is, 216 animals from each of the three regions), were investigated in this study. In each region two districts were purposively selected and four villages from each district were randomly selected, making a total of 24 villages from all the three regions. In each village, 9 households, 3 cattle from each household were randomly selected making a total of 27 animals per village. The required sample size (622) in all the three regions was determined as described by Chulaluk (2009) with an expected prevalence of 30, 96.4 confidence interval and an absolute error of 3.6%. However, in order to increase precision a total of 648 cattle were sampled.

Sampling procedure

Purposive sampling procedure was employed to select regions and districts. Selection of herds and villages was performed using a simple random technique after developing a list of all villages rearing indigenous cattle. Only households having at least ten cattle, including all age groups were included in this study.

Physical examination

The cattle were manually restrained by herdsmen to allow physical examination and sample collection. Each animal was subjected to physical examination such as evaluation of body condition score (BCS) and determination of animal age. Evaluation of BCS was based on 1 to 5 point scale as previously documented by Nicholson and Butterworth. (1986). We classified body condition scores into three groups as follows, BCS 1 and 2, BCS 3, BCS 4 and 5 for poor, average and good, respectively. Age of each animal was determined according to De-Lahunta and Habel. (1986). In addition, information on sex, frequency of tick control and tick control method used in each household was recorded.

Tick counting and collection

Tick burden on each animal was assessed by counting the number

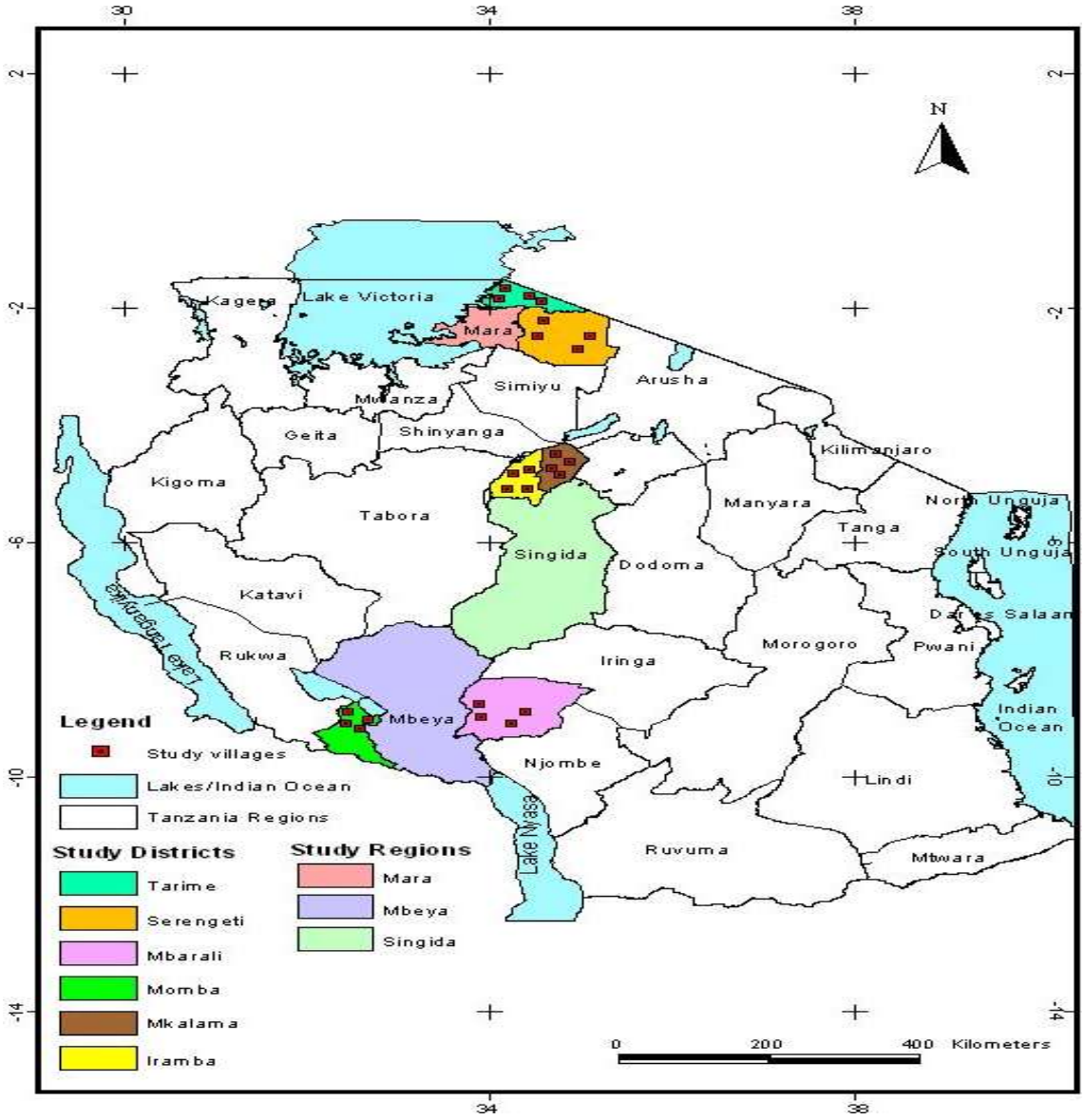

Figure 1. Map of the study area. Sampling was conducted in three regions (Mara, Singida and Mbeya). In each region two districts were purposively selected and four villages from each district were randomly selected, making a total of 24 villages from all the three regions.

of ticks (adult and nymph) from one side of the body and the result multiplied by two to represent the whole body of the animal. After counting, adult ticks were collected from each animal and stored in separate pre-labeled bottles containing 70% of ethanol. The ticks were identified based on morphological characteristics as described by Walker et al. (2003).

Data analysis

Data were initially entered into a Microsoft excel spread sheet before analysis. Graphical representation was also performed in Microsoft excel for windows (version 4.0). Mean tick counts and mean tick load for each species and their standard error (± SE) with 95% confidence interval (CI) were assed using General Linear Model (GLM) procedure of Statistical Analysis System (SAS) proprietary Software, Release 9.1 (SAS Institute Inc).

To determine the difference of means among categorical variables in each region, one way ANOVA was employed. Cumulative tick counts were statistically compared according to associated risk factors (age group, body condition score, sex, tick control method and frequency of tick control) in each region using Fisher's least significant different test. Values of $p \leq 0.05$ were considered significant.

RESULTS

Out of the 648 cattle examined, 392 (149, 114 and 129

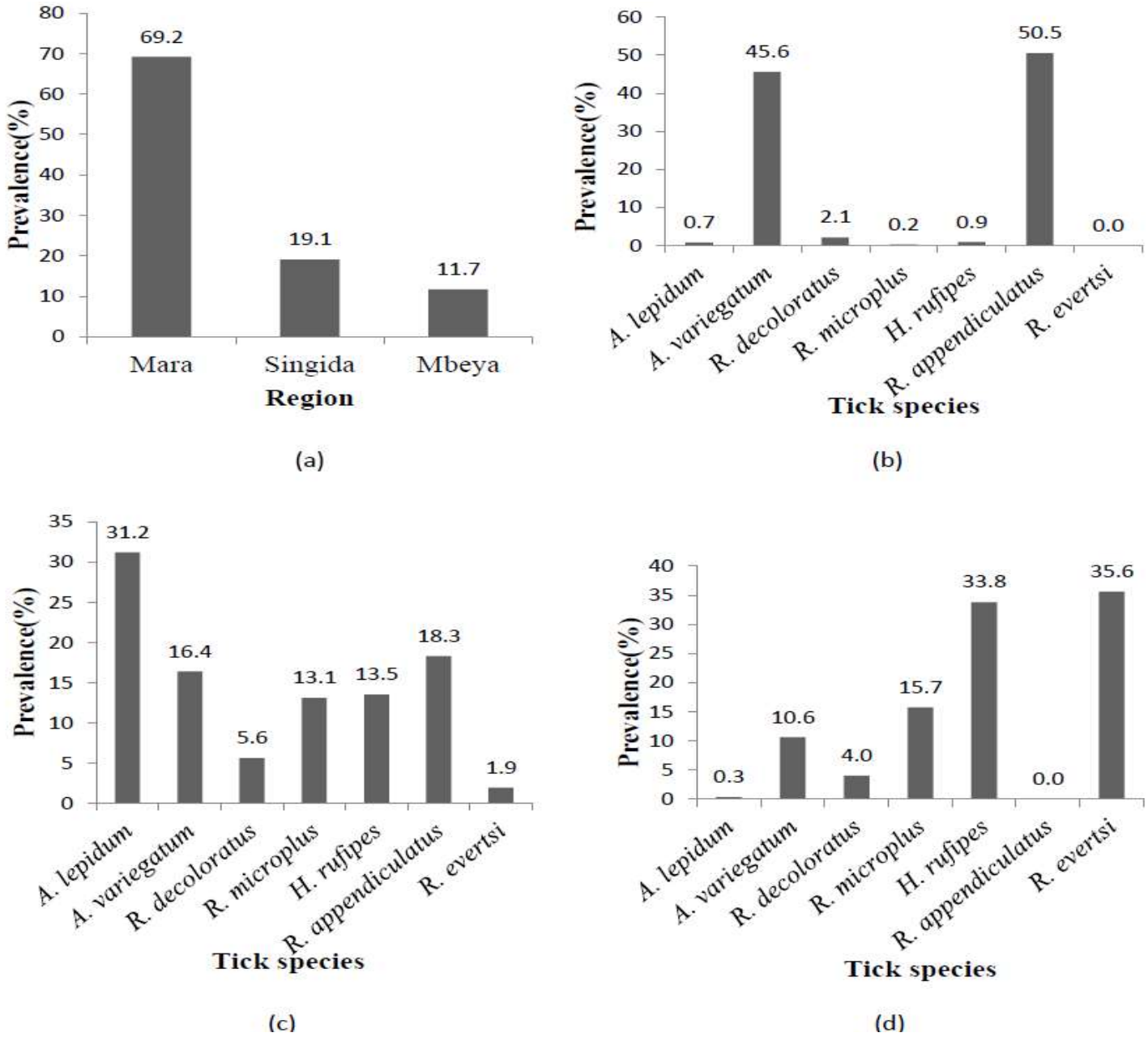

Figure 2. (a) Prevalence of total tick count by region (Mara, Singida and Mbeya), (b) prevalence of different tick species in Mara, (c) prevalence of different tick species in Singida, (d) prevalence of different tick species in Mbeya. The prevalence of ticks in Mara region (p=0.0001) was significantly higher than in Singaida and Mbeya regions. In Mara, *R. appendiculatus* (50.5%) was found to be the most abundant species followed by *A. variegatum* (45.6%). *Rhipicephalus microplus* (0.2%) was the least abundant species in this region. *Amblyomma lepidum* (31.2%) was the most prevalent tick species in Singida followed by *R. appendiculatus* (18.3%). *Rhipicephalus evertsi* (1.9%) was observed to be the least abundant species in the region. In Mbeya *R. evertsi* (35.6%) was the most abundant species followed by *H. rufipes* (33.8%). *Amblyomma lepidum* (0.3%) was the least abundant tick species observed in this region.

from Mara, Singida and Mbeya) were infested with ticks. A total of 7,705 ticks were observed in cattle from all the three regions. Of the 7,705 ticks, 69.2, 19.1 and 11.7%were observed in Mara, Singida and Mbeya regions respectively (Figure 2a). Mean tick count per animal was significantly higher in Mara region (35.3±4.3) and lower in Mbeya region (7.0±0.4) (Table 1). Three methods (dipping, hand spray and hand picking) of tick control were reported in the study area as depicted in Table 2. In Mara region all the three methods were practiced with dipping (54.2%) being the most common tick control method used in the region. Hand spraying

Table1. Number of infested cattle, Total number of ticks, and minimum, maximum and mean number of ticks per animal ± standard error (SE) across the regions based on General linear model

Risk Factor	Cattle	Tick count	Mean tick count/animal ± SE	Minimum	Maximum	p Value
Region						<0.0001
Mara	149	5331	35.8±4.3[a]	4	342	
Singida	114	1470	12.9±2.1[b]	4	216	
Mbeya	129	904	7.0±0.4[b]	4	24	

[a, b] values in the same column with the same superscript do not differ significantly ($p \leq 0.05$) N= Number of cattle.

Table 2. Frequency and tick control methods practiced by households in each region based on descriptive statistics generated from frequency procedure of Statistical Analysis System (SAS).

	.....Mara.....			.....Singida.....			.....Mbeya.....			.....Overall.....		
Risk factor	**Household**	**%**	**P value**	**Household**	**%**	**P value**	**Household**	**%**	**P value**	**Household**	**%**	**P value**
Tick control method			< 0.0001			< 0.0001			< 0.0001			< 0.0001
Dipping	39	54.2		0	0		14	19.4		53	24.5	
Hand spray	20	27.8		62	86.1		22	30.6		104	48.1	
Hand picking	3	4.2		0	0		0	0		3	1.4	
No tick control	10	13.9		10	13.9		36	50		56	25.9	
Total	**72**	**100.0**		**72**	**100**		**72**	**100**		**216**	**100.0**	
Frequency of Tick control			< 0.0001			< 0.0001			< 0.0001			< 0.0001
Weekly	5	8.1		0	0		6	16.7		11	6.8	
Bi-Weekly	5	8.1		10	16.1		30	83.3		45	28.0	
Monthly	10	15.6		17	27.4		0	0		27	16.8	
Occasionally	42	68.2		36	56.5		0	0		78	48.4	
Total	**62**	**100**		**63**	**100**		**36**	**100**		161	**100**	

was the only method used to control ticks in Singida region and was practiced by 86.1% of the farmers whereas the remaining 13.9% did not use any tick control method. In Mbeya region hand spraying (30.6%) and dipping (19.4%) were the methods practiced by most farmers in controlling ticks, however 50% of farmers did not employ any tick control methods.

For tick control frequency, four practices were observed in the study area, namely weekly, bi-weekly, monthly and occasionally. In Mara region, the majority (68.2%) of the farmers controlled ticks occasionally, and these were followed by those (15.6%) who controlled ticks on a monthly basis. The remaining 16.2% either controlled ticks on a weekly basis or after every two weeks. In Singida, 56.6% of farmers controlled ticks occasionally whereas the remaining 16.1 and 27.4% controlled ticks bi-weekly and once per month, respectively. In Mbeya farmers were either controlling ticks on a weekly basis (16.7%) or after every two weeks (83.7%) (Table 2).

Mean tick counts per animal in relation to associated risk factors (age category, body condition score, sex, tick control method and frequency of tick control) for each region are presented in Table 3.

Significantly lower mean tick counts were observed in Mara and Mbeya region on younger animals than on other age groups. However, in Singida region no such statistical variation in mean tick count was observed among animals of different age groups. Cattle with poor body conditions exhibited higher mean tick counts than those with better body conditions in Mara and Singida regions. No significant difference was noted in mean tick numbers between animals with different body condition scores in Mbeya region. On the other hand, no significant difference was observed in mean tick counts between males and female cattle across all the three regions. Higher mean tick counts were observed in Mara (46.5±5.8) and Mbeya (9.7±1.4) in cattle managed under dipping system while hand sprayed animals had a low tick count. In Mara a lower mean tick count was observed on cattle which were under tick control practice done on a weekly and bi-weekly basis. In Mbeya where tick control was practiced on a weekly basis, animals exhibited a lower mean tick count than in those which were exposed to tick control every two weeks. However, no significant difference was observed on the mean tick count among cattle on different tick control frequency in Singida region.

When data from all the three regions were combined; young stock appeared to be associated with a lower mean tick count compared to other age groups, though statistically the differences were not significant (Table 3). Cattle with poor body condition score demonstrated higher mean tick count ($p = 0.0021$) than those with average and good body condition scores. Moreover, cattle exposed to frequent tick control measures (weekly and bi-weekly), were associated with lower mean tick counts ($p < 0.0001$) than those exposed to infrequent tick control practices (monthly and occasionally).

Furthermore, when all data were analyzed as a single population, dipping was associated with larger mean tick count per animal (41.6±5.1) than the other methods of tick control. On the other hand, hand spraying exhibited lower mean tick counts (10.4±1.5) compared to other tick control measures across the regions. Sex did not show any significant effect on the mean tick count per animal across the regions (Table 3).

A total of 3,784 adult ixodid ticks from 392 cattle were collected from three regions for identification purposes. Out of 3784 ticks, 2742 were males and 1042 were females with a Male: Female ratio of 2.6: 1. In Mara, Singida and Mbeya regions the total number of ticks collected were 1922, 1070 and 792, respectively. Three different genera (that is, *Ambylomma*, *Hyalomma* and *Rhipicephalus* (including the *Boophilus* subgenus) and seven tick species (*Amblyomma lepidum* (Dönitz, 1909), *Amblyomma variegatum* (Fabricius, 1794), *Rhipicephalus decoloratus* (Koch, 1844), *Rhipicephalus microplus* (Canestrini, 1888), *Hyalomma rufipes* Koch, 1844, *Rhipicephalus appendiculatus* (Neumann, 1901) and *Rhipicephalus evertsi* (Neumann, 1897) were identified. However, out of the seven tick species observed in the study area only five species (*A. lepidum*, *A. variegatum, R. decoloralus, R. microplus* and *H. rufipes*) were observed in all the three regions. Adults of the remaining two species, namely *R. appendiculatus* and *R. evertsi*, were restricted to Mara and Singida, and Mbeya and Singida regions, respectively (Table 4).

In Mara, *R. appendiculatus* (50.5%) was found to be the most abundant species (Figure 2b) with 12.1 ± 0.8 mean number of ticks per animal, followed by *A. variegatum* (45.6%) with a mean number of ticks per animal of 7.2 ± 0.4. *R. microplus* (0.2%) was the least abundant species in this region with the mean of 2.6 ± 1.1 ticks per animal. *A. lepidum* (31.2%) was the most prevalent tick species in Singida region (Figure 2c) with the mean of 6.0 ± 0.4 ticks per animal, followed by *R. appendiculatus* (18.3%) with 5.1 ± 1.2 ticks per animal. *R. evertsi* (1.9%) was observed to be the least abundant species in the region with the mean of 4.0 ± 0.8 ticks per animal. In Mbeya region *R. evertsi* (35.6%) was the most abundant species (Fig 2-d) with the mean tick number of 4.2 ± 0.2 per animal, followed by *H. rufipes* (33.8%) with the mean of 3.6 ± 0.3 ticks per animal. *A. lepidum* (0.3%) was the least abundant tick species observed in this region with the mean tick load of 2.0 ± 3.3 per cattle (Table 4).

When comparison was made across the regions, *A. lepidum* exhibited significantly higher mean tick count per animal in Singida region compared to the other regions. Mean tick numbers per animal for *A. variegatum* and *R. appendiculatus* were higher in Mara region than in the other regions. *H. rufipes* showed significantly higher number of ticks per animal in Singida region compared to other regions. However, the mean tick counts per animal for *R. decoloralus, R. microplus* and *R. evertsi* did not differ significantly across the three regions (Table 4). Sex ratio (Male: Female) was also recorded as shown in Table 4. *A. lepidum* exhibited the highest number of males compared to females in Mara (14: 0) and Singida (17.6: 1) regions. In Mbeya region only two females of A. lepidum were recorded. For *A. variegatum*, more males than females (3.3:1, 11.6:1 and 4.3:1 male: female ratio in Mara, Singida and Mbeya regions, respectively) were observed. With regard to *R. decoloratus* and *R. microplus*, larger numbers of females than males were observed in all the three regions. It was also observed that the number of males of *H. rufipes* was larger in comparison to that of females in all the three regions. For *R. appendiculatus* more males than females were observed in Mara and Singida regions. Only *R. evertsi* males were found in Singida and Mbeya regions.

DISCUSSION

In order to establish effective control measures of ticks and the diseases they transmit, knowledge of tick abundance and species present in a particular

Table 3. Amount of encountered ticks, Mean tick count/animal ± SE as influenced by associated risk factors in each region and across the regions.

Risk factor	….....….Mara region……			……....Singida region……..…			……..…..Mbeya region……..			…Across the regions (Overall)…		
	N	Tick count	Mean tick count/animal ± SE	N	Tick count	Mean tick count/animal ± SE	N	Tick count	Mean tick count/animal ± SE	N	Tick count	Mean tick count/animal ± SE
Age category												
Young	44	1086	24.7±6.0^{a}	21	172	8.2±1.7	31	168	5.4±0.3^{a}	96	1426	14.9±2.9
Weaner	49	1654	33.8±6.5^{ab}	38	628	16.6±6.0	46	330	7.2±0.7^{b}	133	2612	19.6±3.1
Adult	56	2591	46.3±8.4^{b}	55	670	12.2±1.2	52	406	7.8±0.7^{b}	163	3667	22.5±3.2
p value			0.0395			0.3822			0.0252			0.2696
Body condition score												
Poor	112	4559	40.7±5.5^{a}	56	908	16.2±4.2^{a}	71	458	6.5±0.5	239	5925	24.8±2.9^{a}
Average	37	772	20.9±3.7 ^{b}	55	538	9.8±1.0^{b}	54	428	7.9±0.7	146	1738	11.9±1.1^{b}
Good	0	0	0	3	24	8.0±2.3^{b}	4	14	4.7±0.7	7	42	6.0±1.2^{b}
p value			0.0438			0.0407			0.1517			0.0021
Sex												
Male	55	1690	30.7±7.8	50	700	14.0±4.3	55	348	6.3±0.4	160	2738	17.1±3.1
Female	94	3641	38.7±5.0	64	770	12.0±1.7	74	556	7.5±0.6	232	4967	21.4±2.3
p value			0.3662			0.6468			0.1575			0.2545
Tick control method												
Dipping	104	4835	46.5±5.8^{a}	0	0	0	16	156	9.7±1.4^{a}	120	4991	41.6±5.1^{a}
Hand spray	24	152	6.3±1.0^{b}	99	1280	12.9±2.4^{a}	38	238	6.3±0.6^{b}	161	1670	10.4±1.5^{c}
Hand picking	4	62	15.5±10.8^{c}	0	0	0	0	0	0	4	62	15.5±10.8^{bc}
No tick control	17	282	16.6±2.9 ^{c}	15	192	12.7±2.8^{a}	75	510	6.8±0.6^{b}	107	982	19.2±0.8^{b}
p value			0.0014			0.9668			0.0368			<0.0001
Tick control frequency												
Weekly	15	48	6.0±1.3^{b}	0	0	0	18	32	4.0±0.0^{b}	16	80	5±0.7^{a}
Bi-Weekly	15	34	4.9±0.9^{b}	30	92	7.7±1.1	90	362	7.9±0.7^{a}	65	488	7.5±0.5^{a}
Monthly	29	718	34.2±7.1^{a}	51	332	11.4±1.5	0	0	0	50	1050	21.0±3.5^{bc}
Occasionally	127	4249	44.3±6.2^{a}	105	856	14.8±4.0	0	0	0	154	5105	33.2±4.3^{b}
			0.0043			0.6024			0.0255			<0.0001

$^{a, b,c}$ values in the same column with the same superscript in each region do not differ significantly ($p<0.05$); N= Number of observed animals.

Table 4. Tick species count, mean tick load ±Standard error (SE) and Male (M) to Female (F) ratio by region based on General linear model

Tick species	.....Mara region.....					.....Singida region.....					.....Mbeya region.....					
	Total ticks	Mean tick loads ±SE	M	F	M:F	Total ticks	Mean tick loads ±SE	M	F	M:F	Total ticks	Mean tick loads ±SE	M	F	M:F	P value
Amblyomma lepidum	14	2.8±0.5[a]	14	0	14:00	334	6.0±0.5[b]	316	18	17.6:1	2	2.0±0.0[ab]	0	2	00:02	0.0428
Amblyomma variegatum	876	7.0±0.5[a]	674	202	3.3:1	176	4.5±0.4[b]	162	14	11.6:1	84	3.1±0.3[b]	68	16	4.3:1	<0.0001
Rhipicephalus decoloratus	40	2.9±0.3	0	40	00:40	60	4.6±0.9	0	60	0.6	32	3.6±0.6	10	22	0.6:1	0.1668
Rhipicephalus microplus	4	4.0±0.0	0	4	00:04	140	4.8±0.7	2	138	0.01:1	124	3.5±0.4	44	80	0.6:1	0.2501
Hyalomma rufipes	18	2.6±0.4[a]	14	4	3.5:1	144	5.5±1.0[b]	130	14	9.3:1	268	3.4±0.2[ac]	258	10	25.8:1	0.0038
Rhipicephalus appendiculatus	970	12.1±1.0[a]	576	394	1.5:1	196	5.0±0.8[b]	172	24	7.2:1	0	0	0	0	0	<0.0001
Rhipicephalus evertsi	0	0	0	0	0	20	4.0±1.3	20	0	20:00	282	4.2±0.2	282	0	282:00:00	0.8088

[a, b,c] values in the same column with the same superscript in each region do not differ significantly ($p<0.05$)

area is very important as it helps in predicting the occurrence of definite TBDs in such an area. The aim of this study was to assess the burden and species of ticks parasitizing cattle in three regions of Tanzania, namely Mara, Singida and Mbeya. Mara region is located in the north-west part of Tanzania while Singida and Mbeya are found in central and southern parts of the country, respectively.

In Mara and Mbeya regions, young cattle (≤ 12 months of age) exhibited a lower number of ticks than weaners and adults. The observed lower mean tick counts in young cattle compared to that of other age groups agree with the findings reported by Swai et al. (2005) in Ngorongoro, Tanzania. Similar findings have also been reported in Central Nigeria (Lorusso et al., 2013). Lower number of ticks on young animals compared to other age groups may signify that young animals are protected by innate age-related resistance which makes them less attractive to ticks than adult animals (Wickel and Bergman, 1997; Sutherst and Schnitzerling, 1982).

Furthermore, the possibility for ticks to attach on adult cattle than young stock when they are looking for a host is greater due to a larger surface area of the adult animals (Fivaz and Waal, 1993). Another reason can be attributed to progressive selective grooming of the calves' head, ears and neck from their respective dams (Fivaz and Waal, 1993). Additionally, in all the three regions investigated, young animals were grazed in areas close to the farmhouse while adult animals were grazed in grasslands and bushy areas located far away from the homestead as was the case in South Sudan (Kivaria et al., 2012), therefore reducing the risk of exposure of young stock to tick infestation. Adult animals continue to be at high risk of infestation with ticks due to prolonged mingling with other animals during grazing and watering since communal grazing lands are used. The observation in this study differs from the findings of Mwambene et al. (2012) who reported a different scenario in pastoral communities of southern part of Tanzania where all age groups grazed together and as a result all animals were subjected to an equal tick challenge.

From the three regions investigated, higher tick burdens on cattle were observed in Mara region compared to the others. This variability in tick burdens could be associated with geographical location (climate) and diverse tick control practices. Mara region ecologically favours growth and multiplication of ticks as it is more humid and receives more rainfall (MRUF, 2013). Despite the fact that more than 50% of livestock farmers reported that they dip their cattle for tick control, the prevalence of tick infestation was high because most of them dip their animals occassionally or when they observe severe tick infestation and this has accerelated the level of tick infestation on cattle in this region. On the other hand, Mbeya has an authoritarian community-managed dipping practice where it is mandatory for every famer to dip his/her animals at a designated frequency and this has significanly diminished the level of tick infestation in this region compared to Mara region where dipping is not compulsory. Hand spraying of cattle to control ticks was also practiced in all the three regions. In Mara and Mbeya, hand spraying significantly reduced the level of tick infestation in these two regions. A similar observation was reported by Simuunza et al. (2011) in Central, Eastern and Lusaka Provinces of Zambia whereby spraying was observed to be effective in the dry season.

In the current study, higher tick infestation was observed in animals with poor body condition scores in Mara and Singida and across the regions implying that cattle with medium and good body condition are less infested with ticks than those with poor body condition. Poor body condition in animals can be associated with poor management and nutritional status (Radostits, 2001). In particular, malnutrition can result into the lowering and deppression of the immune system and this, in turn, increases suceptibility of the animals to diseases and tick infestation, and failure to respond to vaccines and drugs (Radostits, 2001; Anderson et al., 2013). Our findings concur with other studies reported in Ethiopia (Tadesse et al., 2012; Onu and Sheferaw, 2013; Wogayehu et al., 2016). Futhermore, in the present study, sex had no significant effect on tick infestation among cattle studied, suggesting equal susceptibility of male and female animals to tick infestation. Similar findings have been reported in West Ethiopia (Amante et al., 2014) and South Western Ethiopia (Tadesse et al., 2012).

In the present study, only adult ticks were identified because immature ticks (larva and nymph) lack important morphological features required for identification to species level (Kaiser et al., 1982). The study observed a number of different tick species infesting cattle in the three regions. The three genera of ixodid cattle tick (that is, Ambylomma, Hyalomma and Rhipicephalus) identified in this study have also been reported by other researchers in some parts of Tanzania (Swai et al., 2005; Kwak et al., 2014; Laisser et al., 2014).

Similar findings have also been reported in Northwest Ethiopia (Moges et al., 2012), Adamawa and Northwest regions of Cameroon (Awaa et al., 2015) and central Nigeria (Lorusso et al., 2013). The presence of similar tick species in all the three regions in this study may be associated with unrestricted cattle movement from one area to another which is a common phenomenon in Tanzania. Of the tick species that were identified in this study, *R. appendiculatus* was the most abundant in Mara region, followed by *A. variegatum*. *R. appendiculatus* is a vector of *Theileria parva* which causes a fatal disease known as East Coast fever (ECF) in cattle (Mulumba et al., 2001; Konnai et al., 2006). East Coast fever has a considerable epidemiological and economic impact in the affected areas. In Tanzania the disease accounts for 68% of annual total losses due to TBDs in cattle (Kivaria, 2006). High abundance of *R. appendiculatus* in Mara region suggests the existence of ECF as previously reported by Laisser et al. (2014). Absence of *R appendiculatus* in Mbeya region has also been reported by Kwak et al. (2014) in Iringa region of Tanzania suggesting low risk of *T. parva* infection in the area.

Iringa and Mbeya regions are situated in the same agro ecological zone which is in the wet highland area with an annual mean rainfall of 1650 mm and an altitude of up to 2600 m above sea level (MRSP, 1997).

A. variegatum was the second abundant species in Mara region comprising 45.6% of the ticks observed. It was also observed across all the three regions. This species is the most common and widely distributed in Tanzania, covering sub-humid and low-to-high altitudes of the country (Lynen et al., 2007). The observations in our study are similar with the observations from a study by Swai et al. (2005) on tick management systems in Ngorongoro district of Tanzania. This species is of great veterinary importance because it is an efficient vector of *Ehrlichia ruminantium,* a causative agent of heartwater and stimulates the development of severe dermatophilosis, caused by *Dermatophilus congolensis* (Deem et al., 1996; Koney et al., 1996). Also *A. variegatum* has economic importance as it cause great damage to skin and hide due to its long mouth parts, lowering the value of the commodity on world market, especially when the number of ticks is large (Solomon et al., 2001). In addition, ulcers caused by this vector become complimentary location for secondary infection. *A. lepidum* was more abundant in Singida than Mara and Mbeya regions. Since *A. lepidum* needs more specialized environmental conditions, high abundance in this region could be attributed to its climatic condition which is semi-arid in nature with annual mean rainfall of 650 mm and an altitude ranging from 1000 to1500 m above sea level (SRSP, 1997). This species is an important vector of *Mycobacterium farcinogenes*, the causative agent of *Bovine farcy* (Hasabelrasoul et al., 2015), and it also transmits heart water.

Another important species found in the study area was *R. decoloratus* which was slightly more abundant in Singida region (5.6%) and Mbeya region (4.0%) and less abundant in Mara region (2.1%). Its relatively high abundance in these two regions compared to Mara region can be explained by its preference for highlands and sub-highlands receiving rainfall >800 mm annually (Pegram et al., 1981). This is in contrast to a study by Kwak et al. (2014) where *R. decoloratus* was not reported in Iringa region found in the southern highlands of Tanzania which is located in a similar agro-ecological zone as the Mbeya region.

According to Bekele (2002), relative abundance of *R. decoloratus* increases from lowland towards highland. Similar findings have been reported in Metekel Ranch of Ethiopia by Alekaw (1998).

R. microplus was also observed in all the three regions but the highest prevalence was noted in Mbeya region (15.7%). The higher prevalence of this species in Mbeya is of great interest because it is known to be a good vector of highly pathogenic *Babesia bovis* (Bock et al., 2004). In addition, this species in terms of control management is well-known to be resistant to numerous pyrethroid and organophosphate compounds (Baffi et al., 2008). Furthermore, *R. decoloratus* and *R. microplus* have great veterinary significance as they are important vectors of *Babesia bigemina* and *Babesia bovis,* which

cause a disease called *bovine babesiosis* (De Waal, 2000; Jongejan and Uilenberg, 2004; Kocan et al., 2004). They are also vectors of *Anaplasma marginale* which causes anaplasmosis in cattle. These two tick species do not occur together due to interspecies competition despite their similar requirements for temperature and rainfall (Estrada-Peña et al., 2006).

H. rufipes is another tick species that was recorded in all the three regions, but with the highest prevalence in Mbeya (33.8%). This observation does not concur with Kwak et al. (2014) who assessed ixodid tick infestation in Iringa and Maswa districts of Tanzania. In their study this species was not observed.

According to Hoogstraal (1956) *H. rufipes* is largely distributed in most of the arid parts of tropical Africa which receives 250 to 650 mm annual rainfall. The findings of Hoogstraal (1956) are not in agreement with our observation in Mbeya region which has a wet highland climate, but exhibited a high prevalence of *H. rufipes*. It was expected that higher prevalence of this species would be observed in Singida region which is semi-arid in nature. The presence of this species in the three regions of Tanzania, regardless of differences in prevalence, is of great veterinary importance as it is known to transmit *A. marginale,* a causative agent of anaplasmosis in cattle (Potgieter, 1979). This species is also a good vector of *Theileria annulata* and *Babesia occultans* (Jongejan et al., 1983; Blouin and Van Rensburg, 1988).

Another species belonging to the *Rhipicephalus* genus observed in this study was *R. evertsi* which showed the highest prevalence in Mbeya region. This can be explained by its preference for wet highlands (Singh et al., 2000). This species has also been reported by Kwak et al. (2014) to be least prevalent in wild animals. Abebe et al. (2010) reported a 10.9% of *R. evertsi* in the Somali Regional State, Ethiopia. This species is also of veterinary importance since it transmits *B. bigemina* in cattle (Bock et al., 2004).

The present study did not find any *R. appendiculatus* in Mbeya region, and no *R. evertsi* in Mara region. According to Yeoman and Walker (1967), both *R. appendiculatus* and *R. evertsi* were present in both of the two regions. The results of the current study could probably be due to a real change in the tick populations since that time. In addition, there have been enormous changes since 1967 in the conditions required by the various tick species: Decrease of wild hosts, increase in the human population and domestic hosts, habitat changes in vegetation and climate, changes in agricultural activities including possibly an increase in tick control regimes and its effectiveness, which together may well have had a great influence on the tick populations.

For all the species that were collected for identification, males were found, as usually, to be predominant compared to females, with the exception of *R. decoloratus* and *R. microplus*. The male: female ratios observed in the present study correspond well with those reported by other studies (Tatchell and Easton, 1986; Lorusso et al., 2013; Bedasso et al., 2014).

The high proportion of males compared to females observed for *A. variegatum* is moreover associated with its biology. This species has a tendency to localize in preferential body areas such as udder, groin and scrotum and as a result forms emblematic clusters with few females attached with many males (Macleod, 1975; Ndhlovu et al., 2009). This is due to the aggregation-attachment pheromones (AAP) discharged by *A. variegatum* males and as such attracts unfed males, resulting into higher concentration of males than females on the feeding sites (Norval and Rechav, 1979).

Large number of males in relation to females for *H. rufipes* and *Rhipicephalus* spp. is most likely due to the fact that males have a tendency to remain on the host for a long time, continue feeding and mating with other females before dropping off (Solomon et al., 2001). The females of *Boophilus* ticks outnumbered males. The greater number of females noted for *Boophilus* ticks in this study agrees with the findings of other studies (Lorusso et al., 2013; Amante et al., 2014). This could probably be due to small size of males which may be overlooked during collection.

Conclusion

Ticks cause severe economic losses either by transmitting numerous diseases or by significant damage of hides and skin to the animals. The present study has shown that the prevalence of tick infestation was lower in Mbeya region where routine dipping on weekly basis has been adopted but higher in Mara region where dipping is occasionally practiced. The results show that young cattle which are grazed in the areas around homesteads have lower level of tick infestation compared to adult animals which are grazed in communal areas far away from the homesteads. The results further show that animals with poor body condition score exhibit higher level of tick infestation than those with good body condition score. Results from this study show that ticks of economic importance exist throughout the study areas, but the prevalence of each species differs from region to region. The predominant species during the dry season were *R. appendiculatus* and *A. variegatum* in Mara region, *A. lepidum* and *R. appendiculatus* in Singida region and *R. evertsi* and *H. rufipes* in Mbeya region. In order to reduce losses caused by ticks and tick borne diseases, appropriate and cost effective control strategies should be implemented. This should be done hand in hand with the Government to subsidize acaricides to farmers making them affordable. Since the present cross sectional survey was carried out only during the dry season, the authors recommend further study to be conducted in the study area during the rainy season.

ABBREVIATIONS

AAP, Aggregation-attachment pheromones; **ACP**, Africa Caribbean and Pacific; **ANOVA**, analysis of variance; **BCS**, Body condition score; **CI**, Confidence interval; **ECF**, East Coast fever; **EPINAV**, Enhancing Pro-poor Innovation in Natural Resources and Agricultural Value Chains; **FAO**, Food and Agriculture Organization; **GDP**, gross domestic product; **GLM**, General linear model; **MLFD**, Ministry of Livestock and Fisheries Development; **MRSP**, Mbeya Region Socio-Economic Profile; **NORAD**, Norwegian Agency for International Development; **SAS**, statistical analysis system; **SE**, standard error; **SUA**, Sokoine University of Agriculture; **TBDs**, tick borne diseases; **UNZA**, University of Zambia; **USD**, United States dollar; **MRUF**, Mara Region-The Unique Features; **SRSP**, Singida Region Socio-Economic Profile; **NBS**, National Bureau of Statistics.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Ethics approval and consent to participate

The study was carried out with the full approval of households keeping cattle, district councils of the study areas, Sokoine University of Agriculture (SUA) and the University of Zambia (UNZA), School of Veterinary Medicine.

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