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Full Length Research Paper

Organic and conventional vegetables: Comparison of the physical and chemical characteristics and antioxidant activity

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The objective of this research was to compare the physical and chemical characteristics and antioxidant activity of organic and conventional carrot (*Daucus carota*), green pepper (*Capsicum annuum*) and lettuce (*Lactuca sativa*). Five representative samples of conventional vegetables, certified organic and non-certified organic vegetables were gotten from farms and supermarkets in the city of Rio de Janeiro. The result shows that the organic carrot showed higher acidity (0.11 g % citric acid) and total sugar (5.68 g %) than those found in standard samples and certified organic ones ($p<0.05$). Regarding the density analysis and total soluble solids, there was no statistical difference between carrots, green peppers and lettuce from all types ($p>0.05$). It was observed that the vitamin C levels in carrot samples levels had no significant difference between the different forms of production ($p>0.05$). Conventional lettuce and certified organic pepper showed higher vitamin C than the other samples ($p<0.05$). The antioxidant activity of the samples was analysed by the capacity to reduce the DPPH (1,1-diphenyl-2-picryl- hydrazyl) radical, in which carrot and conventional pepper showed lower antioxidant activity ($p<0.05$) when compared to organic samples. There were no significant differences among the different forms of production in the lettuce samples ($p>0.05$). Carrot and green pepper, with seal certification or not, showed higher capacity to reduce DPPH than the conventional ones, this suggests that the form of cultivation has a direct relationship with the nutritional values of the vegetables.

Key words: Carrot, lettuce, green pepper; organic, conventional, antioxidant activity, physico-chemical analysis.

INTRODUCTION

Some diseases such as hypertension, cardiovascular disease and stroke could be prevented by a regular daily consumption of fruits and vegetables (WHO, 2004). Composite diets such as DASH diets and the Mediterranean diet, being rich in fruits, vegetables but with reduced fat content, have been associated with the

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reduced risk of developing these diseases (Reddy et al., 2004; Gulçin, 2012). The role of antioxidants in the prevention of various chronic diseases, delaying and inhibiting the oxidation of lipids and other molecules has been an established fact. They neutralize free radicals and thereby prevent many non-communicable diseases such as cancer, diabetes and cardiovascular diseases. Fruits and vegetables are good sources of hydroxylase aromatic compounds called polyphenols plants (Pandey and Rizvi, 2009; Cartea et al., 2011). Besides, there are many herbs and spices that are also a rich source of antioxidants (Waman and Karanjalker, 2010) and play pivotal role in the prevention of such diseases.

Production of vegetables may be performed by adopting different systems (Mattheis and Fellman, 1999). The conventional system is characterized by a high use of chemical pesticides, in which the use of these inputs is justified in order to increase productivity, quality and resistance to pests and diseases (Aktar et al., 2009; Oates and Cohen, 2009). Possible toxic effects in humans are related to the use of pesticides in conventional agriculture (Brouwer et al*.,* 1999). These products have in their composition hundreds of active ingredients that are linked to those effects (Kopke, 2000). In the conventional system, when the technical recomendations are not followed appropriately, it can endanger humans health and contaminate the environment.

Organic production entails growing of crops without synthetic pesticides and should be produced from organisms that have not been genetically modified or have undergone ionizing radiation (Ferreira et al., 2015). Moreover, it should use sustainable practices to enable the reduction of contamination and degradation of th soil, water and air (Dangour et al., 2009). Organic producers utilize a wide range of alternative inputs and cultural practices for managing the cultures in a manner believed to be safer for the environment and better for the consumer (Amodio et al., 2007; Knap et al., 2014).

The organic system is an alternative, but is considered to be more expensive than the conventional (Engindeniz and Tuzel, 2006). From the moment that producers begin to produce more organic foods and become more accustomed to this type of production the tendency is that these foods become cheaper. Then the cost can be reduced even more over time with increase in production scale and the organization of these producers. The certification of organic products can be made internationally or nationally and is recognized in the market with an organic certification seal. Organic food produced against the standards of the country, where the product is sold is identified as fraud (FAO, 2009).

Antioxidant activity has been reported to increase due to cultivation following organic methods, as such crops are known to produce higher amounts of protective substances, such as polyphenols, etc. A study by Castro et al. (2014) showed that plants grown on organic system were more exposed to the environment and had higher antioxidant capacity. Another study by Roghelia and Patel (2015) concluded that antioxidant potential, flavonoid and total phenol levels were higher in the system without fertilizers as against the conventional system. The connection between plant stress levels and the production of secondary metabolites, including many polyphenols and antioxidants, it is justified because relatively higher levels of antioxidant are produced by plants in response to stress and pest attack (Benbrook, 2005). A study by Dumas et al*.* (2003) still reports that inorganic fertilizer reduce the antioxidant levels, while organic fertilizer has been proven to enhance antioxidant content in plants. In addition, modifications caused by the use of highly toxic soluble chemical fertilizers and agricultural chemicals can cause imbalances in food and reduce protein production or increase the degradation of these substances.

Some agricultural practices increase the emissions of gases such as frequent interventions, mineral fertilization and intensive tillage. Müller-Lindenlauf (2009), in FAO report, analyzed the possibilities of organic farming be considered a significant activity for carbon sequestration. The carbon is one of those responsible for greenhouse gases and the high level of carbon capture in organic production systems contribute to the increase of carbon stock in soil and biomass United Nations, 2003). Pelletier et al. (2008) also showed that the production system without fertilizer generated lower emissions than the conventional production, mainly involving use of nitrogen fertilizers in conventional farming. Furthermore, the yield and power consumption of an organic production is nearly the same as that of conventional production.

Organic production has been discussed and evaluated in the modern world as an alternative to the conventional way, being characterized as adequate for health and capable of reducing environmental degradation. Due to increased consumer interest in organic food, it becomes necessary to know, based on scientific studies, the quality of the different organic systems. The present study concerned comparison of the physical and chemical characteristics and antioxidant capacity of organic and conventional carrot, green pepper and lettuce.

MATERIALS AND METHODS

Samples

Samples of carrot (*Daucus carota*), green pepper (*Capsicum annuum*) and smooth lettuce (*Lactuca sativa*) were purchased from the supermarkets and farmer's markets in Rio de Janeiro, Brazil. The carrot samples were analyzed between August and December 2012, the green pepper from January to April 2013 and the lettuce between April and July 2013. Initially, a survey was conducted to identify producers of the organic vegetables and the establishments that sell them. The vegetables were chosen based on a survey of commercial establishments in the city of Rio de Janeiro, which sell organic food. The carrot along with lettuce and green pepper were the most found organic vegetables on the areas surveyed. Therefore these foods were chosen for comparison of their physical

and chemical characteristics and antioxidant activity. All samples were obtained according to their similar sensorial characteristics (appearance, color and texture), including the conventional ones. The vegetables were purchased and immediately analyzed in the Laboratory of Nutritional Biochemistry at the Federal University of the State of Rio de Janeiro, in Brazil.

Sample preparation

The samples were washed in tap water to eliminate any surface contaminants. Analysis of vitamin C and antioxidant activity were made immediately after the acquisition of the products, to avoid any changes in these characteristics. Approximately 300 g of each products were put in a comercial juice extractor (Samson GB- 9001, Greenbison Inc., USA) to obtain a more liquid extract to be used subsequently in all analyses. Fluid extract was stored in the refrigerator (5°C) and other analyses were made five days after purchase. Five representative samples for each vegetables (certified organic, non-certificate organic and conventional) were obtained and analyzed *in triplicate*.

Quality parameters

The reducing sugars analysis was performed using 20 ml of the sample, which was first heated to 90°C followed by acid hydrolysis. For this, 1 ml of concentrated HCl was added and it was maintained in a water bath for 30 min. The mixture was removed, cooled at room temperature and neutralized using sodium hydroxide (40%). The mixture was transferred to a 100 ml volumetric flask and volume was made up to 100 ml using distilled water (Institute Adolfo Lutz, 2008).

The total soluble solids analysis was performed by reading the refractometer at 25°C, in which three drops of juice from each sample were placed and the results were expressed in °Brix. The density was analyzed with the use of pycnometer at 25°C. The titratable acidity was determined by a neutralization titration with 0.01 N NaOH using a phenolphthalein indicator (pH 8.1 to 10.0). The volume of the NaOH used in the analysis was measured after the color change. The total acidity was expressed as citric acid (Institute Adolfo Lutz, 2008).

N-Bromosuccamide method (NBS) was used to determine the amount of ascorbic acid (vitamin C) in the samples. The sample was weighed (10 g) and transferred to 100 ml volumetric flask. This mixture was transferred to an Erlenmeyer flask to which was added 10 ml oxalic acid, 4 ml KI (potassium iodide) and drops of starch. Then it was titrated with NBS solution to form a blue color, indicating signal the end point. The results were expressed in g/100 g of citric acid (Institute Adolfo Lutz, 2008).

Total antioxidant capacity of 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The DPPH (2,2-diphenyl-hydrazyl 1-picryl-) analysis was performed following the method described by Brand-Williams et al. (1995) and modified by Miliauskas et al. (2004). In test tubes, the extracts of the samples (5, 10, 15 and 25 µL) and 3 ml of methanol solution of DPPH were placed and left to stand for half an hour. Using a spectrophotometer (Sequoia - Turner^{TM} 340) absorbance was recorded at 515 nm. The antioxidant capacity was expressed as percentage of DPPH consumed, calculated according to the following expression:

% consumed = (Control absorbance – Sample absorbance) × 100 / Control absorbance

The results were expressed as IC_{50} which is the concentration of the sample required to reduce 50% of the radical DPPH.

Phenolics

The Folin-Ciocalteu assay (FCR) is one of the oldest methods of quantification of phenols in a sample therefore also known as total phenols assay. Through the spectrophotometric method it was possible to perform the analysis of phenolic compounds (Singleton et al., 1999). Three solutions were prepared: 10ml Folin-Ciocalteau (10%) and water; 0,1g gallic acid and water; 20 g sodium carbonate (4%) and water. In a thermostat the samples were placed and then the absorbance was read using a spectrophotometer at λ^{max} = 750 nm. For the analysis were used three extractors: methanol 70% (M), ethanol 70% (E) and water (W). For each extractor were dissolved five grams of sample (5 g of sample + 15 ml of extractor). This was then filtered through a filter paper and 25 ml of sample were transferred to a volumetric flask and it was completed with the respective extractor. Gallic acid solution was subjected to the same process and calibration curve was obtained. By the absorbance the concentration of phenolics was read (mg/ml) from this line and the phenolics content in extracts was expressed in gallic acid equivalent (mg GAE/100 g).

Statistical analysis

Results were expressed as means ± SD and all the analysis were perfomed *in triplicate*. The results were submitted to analysis of variance (ANOVA) and the means were seperated by Tukey's test at 5% probability, using the GraphPadPrism 4.0 and Statistical 6.0. Differences were considered significant when $p < 0.05$ and not significant when $p > 0.05$. In addition, a correlation was taken between the antioxidant capacity, phenolic compounds and vitamin C through the same statistic program.

RESULTS AND DISCUSSION

Physico-chemical analysis

Carrot (*Daucus carota*)

The results of total acidity were 0.08 ± 0.00 g% for conventional samples, 0.08 ± 0.00 for organic certified and 0.11 ± 0.34 g% for organic samples, so organic carrot presented values higher than organic certified and conventional ($p < 0.05$). The Nutrition and Public Health Intervention Research Unit London School of Hygiene and Tropical Medicine (July, 2009) conducted a review of studies in which those with classified as satisfactory analysis showed a higher titratable acidity in organic crops, that goes with this present study and confirm the results (Table 1).

Soluble solids (^{0}Brix) did not differ significantly in all samples. These results agree with Bender et al. (2009) in which the contents of soluble solids was the same among carrots cultivation systems. Total sugar content in carrot of organic non-certified and organic with seal showed significantly higher mean values, when compared with conventional ones. The same result was found in the study by Rembialkovska and Hallmann (2007).

The values of density and ascorbic acid were not

Table 1. Physico-chemical analysis of conventional and organically grown of carrot (*Daucus carota*).

Parameter	Sample	Acidity (g%)	Total sugar (g%)	Reducing sugar (g%)	Density (g/cm^3)	Brix(^{o})	Ascorbic acid (mg%)
Organic	O1	0.10±0.00	6.42±0.50	3.17±0.08	1.02	8.15	2.41±0.60
	O2	0.07±0.01	6.32±0.41	4.80±0.17	1.03	9.56	2.93±0.79
	O3	0.09±1.69	4.52±0.35	2.45±0.08	1.02	6.83	3.00±0.53
	O4	0.13±0.01	4.75±0.25	4.08±0.19	1.02	6.83	3.23±0.39
	O5	0.16±0.00	6.40±0.28	2.33±0.07	1.04	8.81	2.67±0.23
	Mean ± SD	0.11±0.34^a	5.68±0.35^a	3.36±0.11^b	1.02±0.01^a	8.03±1.20^a	2.84±0.50^a
Certified Organic	O1	0.06±0.00	4.71±0.31	2.26±0.13	1.04	7.49	3.49±0.21
	O2	0.09±0.00	5.10±0.62	3.17±0.11	1.03	8.15	3.60±0.54
	O3	0.07±0.00	5.32±0.21	3.07±0.04	1.03	7.49	3.05±0.65
	O4	0.09±0.00	4.56±0.15	3.07±0.13	1.03	7.49	2.44±0.00
	O5	0.11±0.01	5.53±0.54	3.33±0.33	1.03	8.15	3.46±0.25
	Mean ± SD	0.08±0.00^b	5.04±0.36^a	2.36±0.14^b	1.03±0.00^a	7.75±0.36^a	3.20±0.33^a
Conventional	O1	0.08±0.01	3.45±0.12	4.35±1.00	1.04	8.81	3.58±0.77
	O2	0.08±0.00	5.34±0.49	4.63±0.22	1.04	8.81	4.39±1.18
	O3	0.08±0.00	5.27±0.73	3.83±0.70	1.03	8.15	3.25±0.22
	O4	0.08±0.01	3.17±0.06	3.17±0.06	1.04	8.15	2.09±0.42
	O5	0.08±0.00	3.62±0.26	3.62±0.26	1.04	9.46	3.01±0.68
	Mean ± SD	0.08±0.00^b	4.17±0.33^b	3.92±0.44^a	1.03±0.00^a	8.67±0.57^a	3.26±0.65^a

Data represent mean ± SD values of triplicate experiments. Tukey–Kramer Multiple Comparison test; Different letters indicate statistically significant differences at the 0.05 level.

significantly different ($p > 0.05$) in carrot samples, unlike the earlier study by Bender et al. (2009) in which the contents of β-carotene and ascorbic acid were higher in conventionally-grown than in organically-grown. Another study by Sikora et al. (2009) also showed that organic carrots contained significantly more ascorbic acid, carotenoids and phenolic acids in comparison to the conventional ones, which contradicted the findings of the present study.

Changes in the management of chemicals and agricultural practices are likely to affect the content of agricultural nutrients. So, the nutritional requirements and final nutritional contents of a particular crop may also vary depending on the cultivation (Hornick and Parr, 1987). Moreover, the nutritional composition is often more dependent on different weather conditions, and this influence interferes in the possible effect of cultivation system (Bender et al., 2015).

Green Pepper (*Capsicum Annuum*)

For total titratable acidity, total sugar, density and soluble solutes the results obtained showed that there was no significant differences between conventional, organic and certified organic samples ($p > 0.05$) (Table 2).

In case of ascorbic acid, the certified organic samples presented higher average values (33.48 ± 2.99 mg%) than the conventional (19.25 ± 2.99 mg%) and organic samples (21.67 ± 2.14 mg%) at $p < 0.05$. Similar results have been reported by Hallmann and Rembialkowska (2012), which demonstrated that green peppers samples produced under an organic system presented higher average values of vitamin C.

Lettuce (*Lactuca sativa*)

The conventional lettuce samples had a higher tritratable acidity and ascorbic acid value than organic and certified organic samples ($p < 0.05$) (Table 3).

These results differ than those observed by Williams (2002) and Magkos et al. (2003), who reported that the vegetables produced under organic systems frequently had higher contents of vitamin C, when compared with those produced conventionally. Another study by Ismail and Fun (2003) also showed that the ascorbic acid content was found to be significantly lower in lettuce grown conventionally compared to the organically grown ones.

There were no significant differences ($p>0.05$) among the values of density, total sugar and soluble solids in lettuce samples. These results disagrees to those observed by Polat et al. (2008) who found that soluble

Table 2. Physico-chemical analysis of conventional and organically grown of green Pepper (*Capsicum Annuum*).

Parameter	Sample	Acidity (g%)	Total sugar (g%)	Reducing sugar (g%)	Density (g/cm^3)	Brix(^{o})	Ascorbic acid (mg%)
Organic	O1	0.15±0.00	2.43±0.09	2.14±0.10	1.02	4.14	28.55±1.90
	O2	0.13±0.00	1.96±0.02	2.27±0.05	1.01	4.82	14.63±2.57
	O3	0.09±0.00	2.80±0.08	2.61±0.08	1.01	5.49	17.02±1.70
	O4	0.15±0.00	2.59±0.07	2.74±0.16	1.01	5.49	19.16±1.91
	O5	0.13±0.00	2.45±0.06	2.31±0.06	1.01	5.49	29.02±2.63
	Mean ± SD	0.13±0.00^a	2.44±0.06^a	2.41±0.09^a	1.01±0.00^a	5.08±0.60^a	21.67±2.14^a
Certified Organic	O1	0.10±0.00	3.06±0.12	1.82±0.08	1.02	3.46	63.97±2.35
	O2	0.10±0.00	2.35±0.07	2.29±0.02	1.01	4.14	22.48±2.04
	O3	0.14±0.00	3.15±0.20	1.81±0.03	1.02	4.14	35.33±4.76
	O4	0.11±0.00	2.23±0.05	2.68±0.12	1.03	4.14	19.03±3.88
	O5	0.11±0.00	2.64±0.05	2.58±0.11	1.02	4.14	26.62±1.94
	Mean ± SD	0.11±0.00^a	2.69±0.10^a	2.23±0.07^a	1.02±0.00^a	4.14±0.48^a	33.48±2.99^b
Conventional	O1	0.17±0.00	2.62±0.07	2.45±0.13	1.01	4.14	19.75±2.66
	O2	0.13±0.00	2.94±0.11	2.17±0.13	1.02	4.82	20.27±2.26
	O3	0.12±0.01	2.720.08	2.29±0.04	1.01	4.82	22.81±2.98
	O4	0.12±0.00	2.25±0.05	2.13±0.05	1.02	4.14	20.29±0.0
	O5	0.1±0.00	2.12±0.04	2.34±0.14	1.02	4.82	13.63±1.07
	Mean ± SD	0.12±0.00^a	2.53±0.07^a	2.27±0.09^a	1.01±0.0^a	4.55±0.37^a	19.35±2.99^a

Data represent mean ± SD values of triplicate experiments. Tukey–Kramer Multiple Comparison test; Different letters indicate statistically significant differences at the 0.05 level

Table 3. Physico-chemical analysis of conventional and organically grown of lettuce (*Lactuca sativa*).

Parameter	Sample	Acidity (g%)	Total sugar (g%)	Reducing sugar (g%)	Density (g/cm^3)	Brix(^{o})	Ascorbic acid (mg%)
Organic	O1	0.03±0.00	0.79±0.01	1.46±0.02	1	2.09	0.29±0.03
	O2	0.04±0.00	0.75±0.00	1.39±0.02	1	0.7	0.27±0.04
	O3	0.04±0.00	0.78±0.01	1.42±0.01	1	2.09	0.44±0.02
	O4	0.03±0.00	0.8±0.01	1.44±0.01	1	1.4	0.39±0.00
	O5	0.04±0.00	0.79±0.01	1.49±0.01	1	1.4	0.29±0.06
	Mean ± SD	0.04±0.00^a	0.78±0.01^a	1.44±0.01^a	1±0.00^a	1.53±0.57^a	0.33±0.03^a
Certified Organic	O1	0.04±0.00	0.75±0.00	1.36±0.03	1.01	2.09	0.28±0.02
	O2	0.03±0.00	0.82±0.01	1.40±0.03	1.01	1.4	0.26±0.04
	O3	0.04±0.00	0.85±0.01	1.38±0.01	1	2.09	0.34±0.00
	O4	0.03±0.00	0.79±0.09	1.35±0.01	1.01	0.7	0.32±0.06
	O5	0.04±0.00	0.82±0.01	1.44±0.01	1.01	2.77	0.21±0.03
	Mean ± SD	0.04±0.00^a	0.81±0.03^a	1.39±0.02^a	1±0.00^a	1.81±0.78^a	0.28±0.01^a
Conventional	O1	0.04±0.00	0.75±0.01	1.45±0.01	1	1.4	0.24±0.06
	O2	0.04±0.00	0.79±0.01	1.29±0.01	1.15	0.7	0.34±0.02
	O3	0.06±0.00	0.66±0.00	1.49±0.01	1	1.4	0.71±0.14
	O4	0.03±0.00	0.80±0.01	1.36±0.06	1	2.77	0.86±0.11
	O5	0.73±0.00	0.70±0.11	1.51±0.01	1	1.4	0.76±0.05
	Mean ± SD	0.32±0.00^b	0.74±0.06^b	1.42±0.02^a	1.03±0.0^a	1.53±0.75^a	0.58±0.07^b

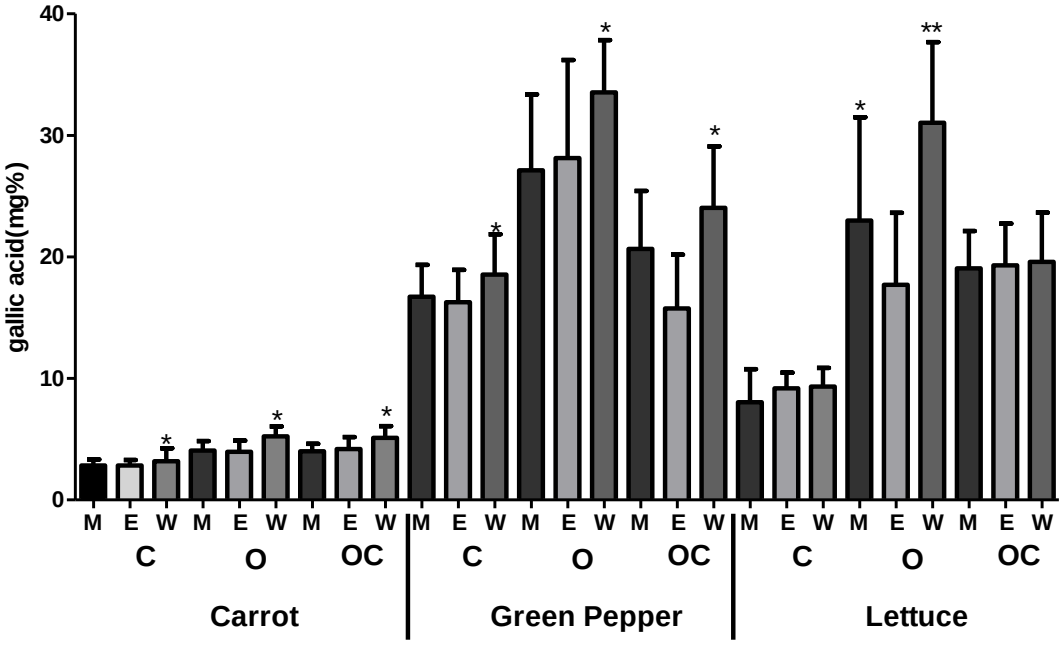

Figure 1. Total Phenolic Compounds in samples of carrots, peppers and lettuce organic (O) , conventional (C) and certified organic (OC) extracted by methanol, ethanol and water. *statistically different ($p < 0.05$).

sugar contents of lettuce growing on applicantions were significant.

Organic acid, soluble sugars and pigments are important components that may contribute to flavor and nutritional value of vegetables, indicating the quality of these products. However the production system, maturation stage, the environmental temperature (Guevara-Figueroa et al., 2015), incident radiation (Caldwell and Britz, 2006) and soil type (Pinto et al., 2014) may change their chemical characteristcs.

Antioxidant activity and phenolic compounds

Plants produce their own means of defense against external agents such as pathogens, predators and even ultraviolet radiation. Phenolic compounds are examples of these means of defense which provide protection against such stresses (Dai and Mumper, 2010; Mittler, 2002). Moreover they take part in processes responsible for color, aroma and astringency in various vegetables. Polyphenolics, thiols, carotenoids, tocopherols and glucosinolates are frequently found in fruits, vegetables and grains. The determination of total phenolic compounds revealed that the use of water as extractor was more efficient compared to others extractors used (Figure 1). Considering this extractor, certified organic and organic samples showed higher total phenolic compounds ($p<0.05$) than those conventional in all samples analyzed vegetables (Table 4). The production of secondary metabolites in plants are influenced by environmental factors because they have an important role in the adaptation of plants and studies have hypothesized that the organic system had higher levels of phenolics (Young et al., 2005).

Young et al. (2005) reported no significant differences in phenolic compounds in organic crops of lettuce and cabbage samples, however, the total phenolic content in conventional pak choi (*Brassica rapa subsp. Chinensis*) samples, measured by Folin- Ciocalteu assay, was significantly lower than the organic samples. This seems to be associated with an increased attack plants in organic plots by insects. On his study using samples of organic green peppers, Amor et al. (2008) observed higher values of phenolic compounds, when compared to the conventional samples. That goes with this present study and confirm the results.

Antioxidants are neutralizing free radical mechanism and act by preventing the damage that these free radicals can cause, for example, damage human cells and DPPH assay has been a proven method to determine the antioxidant capacity (Hossain et al., 2013). Conventional samples of carrot and green pepper showed less antioxidant activity ($p < 0.05$) when compared with the organic samples (Figure 2). A study by Kazimierczak et al. (2008) showed that the antioxidant capacity was lower in conventional currant when compared to organic. Leclerc et al. (1991) showed that organic carrots had more β –carotene and organic celery had more vitamin C than crops grown conventionally. Results of antioxidant

Table 4. Mean and standard deviation of total phenolic compounds (mg% GAE) in carrot samples, pepper and lettuce oganic (O), conventional (C) and certified organic (OC) extracted by water.

Samples	C	O	OC
Carrot	2.69±1.07[a]	5.40±0.81[b]	4.64±0.96[b]
Green Pepper	19.37±4.96[a]	32.79±9.60[b]	29.02±5.87[b]
Lettuce	10.77±7.15[a]	33.64±6.64[b]	19.31±3.19[b]

mg% GAE= gallic acid equivalent mg / 100 g.

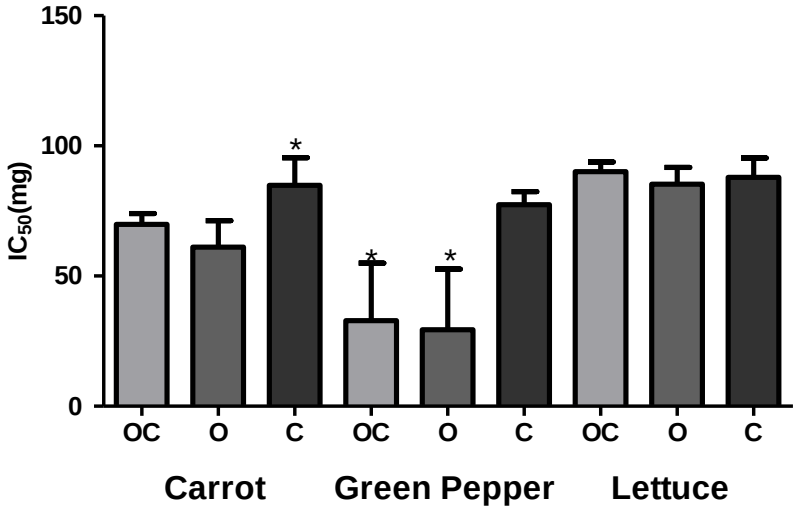

Figure 2. Antioxidant Activity by DPPH assay**.** IC_{50} value in samples of carrot , peppers and lettuce organic (O) Certified Organic (OC) and conventional (C) of different batches (*p <0.05 Tukey test).

activity in this study confirm earlier work (Hallmann and Rembialkowska, 2012; Worthington, 2004) that higher average values were obtained for samples of organic and certified organic green peppers as compared to the conventional ($p < 0.05$). There were no significant differences between the different forms of production in lettuce samples ($p>0.05$).

The effectiveness of the antioxidant capacity depends on the chemical structure and concentration of polyphenols in vegetables. This capacity in plant extracts can be determined by various analytical methods such as capture of the peroxyl radical (ORAC,TRAP), power metal reduction (FRAP,CUPRAC), the hydroxyl radical capture (deoxyribose method), capture of the organic radical (ABTS, DPPH) and the first thing to do is to isolate phenolic compounds from vegetables.

A study by Ribeiro et al. (2010) showed that the investigated extracts (methanol, methanol 50%, methanol acidified, methanol 50%: acetone 70% and acetone 70%) differed significantly ($p<0.05$) in their total phenolic content that is contributed to the different antioxidant activities (% DPPH reduction). Faller and Fialho (2010) analyzed the phenolic compounds and the antioxidant capacity of organically and conventionally grown vegetables, which used a extraction solution of 1.2 M HCl and 50% methanol. The conclusion was the ability of HCl to extract fraction of phenolic compounds bound and quantifies the free and bound phenolics, generating probably increased in phenolic content and antioxidant activity.

According to Wu and Prior (2005) there is not a most appropriate or better extractor for analysis of phenolic compounds but an issue that should be considered is that most phenolic compounds are water soluble. Due to the low viscosity of solvents, their density becomes smaller and therefore the ease of diffusion of the bioactive compounds becomes greater (Naczk and Shahidi, 2006).

Through the correlation between the total content of phenolic compounds and vitamin C with antioxidant activity in carrot samples, organic pepper and lettuce, certified organic and conventional, it was found that the only identified correlation was between phenolic compounds and antioxidant activity in green pepper samples ($p = 0.0433$) (Table 5).

Antioxidant activity depends mainly on phenolics, which are considered more potent antioxidants as compared to

Table 5. Correlation coefficient between the total content of phenolic compounds and vitamin C with antioxidant activity carrot , peppers and lettuce organic , certified organic and conventional.

Vegetable	Parameter	Correlation coefficient	p-value	N
Carrot	Phenolics x Antioxidant activity	0.7049	0.4356	5
	Vitamin C x Antioxidant activity	0.3371	0.7811	5
Green Pepper	Phenolics x Antioxidant activity	0.9977	0.0433*	5
	Vitamin C x Antioxidant activity	0.8289	0.378	5
Lettuce	Phenolics x Antioxidant activity	0.6465	0.5525	5
	Vitamin C x Antioxidant activity	0.2759	0.822	5

vitamins (Koleva et al., 2002; Usenik et al., 2008). The non-existence of correlation in carrots can be explained because the bioactive compounds with recognized antioxidant activity present in the carrot are mainly, according to Singh et al. (2012), β-carotene, α-carotene, lycopene and lutein. The peppers are rich in capsaicinoids which are responsible for the pungent taste and which have a direct relationship with the antioxidant activity of these foods (Materska and Perucka, 2005). From the analysis of the antioxidant activity was observed that the different types of lettuce cultivation was not significant difference so it influenced the results of this correlation.

Conclusion

Differences were observed in chemical characteristics between of organic and conventional cultivation of vegetables, mainly due to the great variety among the lots. On the other hand, the organic samples had higher antioxidant capacity and amounts of phenolic compounds superior when compared to conventional samples. Thus, the data reinforce the need for greater standardization in production of vegetables without loss of nutritional quality and maintenance of bioactive characteristics. Environmental factors such as weather and soil conditions of different regions can cause variations in the physical and chemical characteristics of the plants. So more studies are required at different locations to prove the superiority of the organic system over conventional one.

Conflict of Interests

The authors have not declared any conflict of interest.

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Oxidative enzymes in coconut cultivars in response to *Raoiella indica* feeding

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The increase in oxidative enzyme activities is related to diminished mite infestation. Some biological aspects of *Raoiella indica* Hirst reared on the coconut cultivars ('Jamaican Tall' (JT), 'Malayan Yellow Dwarf' (MYD), Niu Leka (NL) and a hybrid JT x MYD) were studied under laboratory conditions. Aditionally, changes in oxidative enzyme activities (peroxidase and polyphenol oxidase) as response to *R. indica* feeding were studied in the cultivars where red palm mites showed highest and lowest biological parameters values. Longer time spans and lower oviposition rates observed on the JT suggest this cultivar to be more resistant to *R. indica* feeding. Cultivar JT showed the highest value in PPO/POX ratio, being about twice the value shown by MYD in the infested plants. The observed enzyme activity ratios in both genotypes showed a slight increase 24 h after mite infestation, suggesting these enzymes could be related to plant resistance to *R. indica*. However, this relationship is still unclear. The biological parameters of *R. indica* together with higher enzyme activity, particularly on JT suggest this cultivar could be considered as a more resistant cultivar as compared to MYD. More detailed studies are required to determine the effect of these enzymes on coconut resistance to red palm mites.

Key words: Coconut, peroxidase, polyphenol oxidase, red palm mite.

INTRODUCTION

Plant defense mechanisms can be expressed permanently, without the presence of any stress factor (constitutive resistance), or can be induced in response to biotic or abiotic environmental stresses (induced

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resistance) (Agrawal and Karban, 2000; Kessler and Baldwin, 2002; Agrawal, 2005; Sunoj et al., 2014). Both permanent and induced responses are crucial for arthropod resistance management (Kant, 2006). Plant induced resistance involves defense mechanisms including structural barriers, increase of toxic substance level (Grubb and Abel, 2006), and protease inhibitors (Chen et al., 2005). However, some of these compounds obtained as a function of induced resistance can be auto-toxic (Gog et al., 2005) or activated relatively late in the interacting plant-herbivore (Morris et al., 2006), thus involving a high metabolic cost for the plant (Walters and Boyle, 2005). Oxidative stress is a complex chemical and physiological phenomenon that accompanies virtually all biotic and abiotic stresses in higher plants and develops as a result of overproduction and accumulation of reactive oxygen species (ROS) (Demidchik, 2015). Different ROS types are able to evoke oxidative damage to proteins, DNA and lipids (Apel and Hirt, 2004). The cellular damage by ROS appears to be due to their conversion into more reactive species such as the formation of •OH, which is dependent on both H_2O_2 and $O_2^{\bullet-}$ and, thus, its formation is subject to inhibition by both superoxide dismutase (SOD) and catalase (CAT) (Sharma et al., 2012). Besides SOD and CAT, there is a complex of enzymatic components of the antioxidative defense system that comprise several antioxidant enzymes such as guaiacol peroxidase (GPX), enzymes of ascorbateglutathione (AsA-GSH) cycle ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Noctor and Foyer, 1998). Peroxidases (POX) are involved in many physiological processes in plants, involving responses to biotic and abiotic stresses, the biosynthesis of lignin in the polymerization of the precursors of lignin, and in the scavenging of reactive oxygen species (ROS). The ROS are partially reduced forms of atmospheric oxygen, highly reactive and capable of causing oxidative damage to the cell, and can either scavenge or be a source of hydrogen peroxide (H_2O_2) (Vicuña, 2005). Also, peroxidases may be involved in defense against pathogens (López-Curto et al., 2006) or insects (Dowd and Lagrimini, 1998). The expression of different peroxidase isoenzymes depends upon the plant developmental stage and on environmental stimuli (Valério et al., 2004). The increase in POX activity during pathogen/herbivore attack has been associated with phenolic compounds binding to the cell wall in soybeans and beans (Lamb and Dixon, 1997). POX activity has been shown to increase in tomato or hop after *Tetranychus urticae* Koch or *Tetranychus cinnabarinus* Boisduval feeding (Stout et al., 1994; Kielkiewicz, 2002; Trevisan et al., 2003). Similarly, higher oxidative enzyme activity has been associated with lower *Steneotarsonemus spinki* Smiley density on tolerant rice varieties (Fernández et al., 2005). Polyphenol oxidase (PPO) is considered an important oxidative enzyme involved in several physiological functions; however, greater activity has been reported in damaged tissue, therefore PPO's are also considered plant defense proteins (Pinto et al., 2008). Sunoj et al. (2014) demonstrated that reduced activity of PPO and an increased membrane stability index in some coconut seedlings indicate that even under abiotic stress, oxidative stress was reduced by the enzymatic protection mechanisms in operation, suggesting that coconut seedlings were able to maintain membrane stability. Previous studies showed a negative relationship between PPO activity and developmental rate of *Heliothis zea* (Boddie) in tomato leaves, probably due to chelating of amino acids and leaf proteins, with subsequent nutritional quality reduced in the infested foliage (Felton et al., 1989). More recently, caterpillars of several noctuid species (*Spodoptera exigua* (Hübner), *Spodoptera litura* (F.) and *Helicoverpa armigera* Hübner) showed decreased weight gains and consumption rates when feeding on transgenic tomato lines showing PPO expression (Mahanil et al., 2008; Bhonwong et al., 2009).

The red palm mite, *Raoiella indica* Hirst, has been considered a serious pest for coconut (*Cocos nucifera* L.) and Areca palms (*Areca catechu* L.) in India (Daniel, 1981; NageshaChandra and Channabasavanna, 1984), and date palms (*Phoenix dactylifera* L.) in Egypt (Zaher et al., 1969). After being reported in the Caribbean in 2004, *R. indica* quickly spread through that region, reaching Florida (USA) and the northern area of South America (Gondim Jr. et al., 2012; Vásquez and Moraes, 2013). *R. indica* inflicted serious damage to Arecaceae, primarily to the coconut trees, but also to Musaceae and other botanical families (Carrillo et al., 2012; Rodrigues and Irish, 2012). It has been observed that coconut seedlings may die from pest attack, while older plants show discoloration and consequent yield reduction. This information has not been systematically quantified (Welbourn, 2005; Peña et al., 2006). Considering the economic impact of *R. indica* in the Caribbean area, resistance in coconut cultivars should be addressed in order to improve knowledge on how sustainable strategies could contribute in red palm mite population management. For this reason, peroxidase and polyphenol oxidase activities in response to *R. indica* feeding were evaluated in different coconut palm cultivars used commercially in Venezuela.

MATERIALS AND METHODS

Plant material

A study was conducted at the Universidad Centroccidental Lisandro Alvarado, in the state of Lara, Venezuela (10°01'04" N; 69°17'03"W) during 2012. Forty 1-2 years-old plants from each of the coconut cultivars were planted in plastic containers (60×40 cm) containing a substrate of ground soil + rice hulls + sand (1:1:1). One month before the test was initiated, the plants were fertilized with NPK (15-20-20) and treated with Mancozeb (3 g) in 300 ml

Table 1. Mean (±SD) developmental time (days) and oviposition rate of *Raoiella indica* on different coconut cultivars (29 ± 1.0°C, 60±10% RH and 12 h light photoperiod).

	Developmental time					Oviposition
	Egg	Larva	Protonymph	Deutonymph	Egg-adult	Mean egg number/female/day
Jamaican Tall	6.7±1.8^{b}	5.8±0.7^{a}	4.7±0.5^{a}	4.8±0.7^{a}	21.9±2.7^{a}	1.7 ± 1.3^{b}
MYD x JT	7.6±0.5^{a}	4.4±0.5^{b}	3.8±0.8^{b}	4.0±0.8^{b}	19.8±1.0^{b}	2.4 ± 1.5^{a}
Niu Leka	7.7±0.5^{a}	3.7±0.7^{c}	3.8±0.9^{b}	3.9±0.9^{b}	19.0±1.3^{b}	2.2 ± 2.1^{ab}
Malayan Yellow Dwarf	6.0±0.5^{b}	4.3±0.5^{b}	2.6±0.8^{c}	4.0±0.6^{b}	16.9±0.9^{c}	2.4 ± 1.9^{a}

Means followed by different letters within each column are significantly different according to the Tukey test (P< 0.05).

water per palm. Then, plants of each cultivar were divided into two groups; the first group being females infested with 25 *R. indica* on each one of the three leaflets of well-developed middle leaves, while the second group was kept mite free and used as a control. A leaf section (about 4 cm^2) was taken from each of five infested plants per cultivar, and from the control group at 0, 24, 72, 120 and 264 h after mite infestation. Samples were wrapped in a piece of foil and brought in an icebox to the laboratory. Leaf samples were weighed and stored at -20°C until being processed.

Biological aspects of *R. indica* on several coconut genotypes

The biological cycle of *R. indica* was studied using rearing units on four distinct coconut cultivars. The cultivars studied were: the Jamaican Tall (JT), Malayan Yellow Dwarf (MYD), the Niu Leka (NL), and a hybrid cultivar (JT x MYD) provided by the Instituto Nacional de Investigaciones Agrícolas (INIA), Irapa, Sucre state, Venezuela. Each rearing unit consisted of a coconut leaf disc (3 cm diameter) placed with the lower surface on a polyurethane layer, continuously maintained wet by the daily addition of distilled water (Vásquez et al., 2015). One 3-5 day old female was put on each of the thirty rearing units of each cultivar to obtain one egg per rearing unit. After 24 h, the females were removed and just eggs were kept in rearing units. The units continued to be examined in 12-h intervals to determine the duration of each developmental stage. Leaf disks were replaced by new disks every 3-4 days to ensure a physiologically adequate rearing substrate throughout the work. Oviposition was studied in 30 mated-females for each cultivar. The study was carried out under room conditions (29 ± 1.0°C, 60 ± 10 % RH and 12 h photoperiod).

Biochemical changes in coconut cultivars induced by *R. indica* feeding

Total proteins (TP)

Content of total protein was determined using the Bradford (1976) method. Absorbance of each dilution was measured using a spectrophotometer (GENESYS 10S UV-Vis) at 595 nm. Induced biochemical response to *R. indica* feeding were determined on coconut cultivars in which *R. indica* showed the lowest and highest biotic potential in the experiment above, those being JT and MYD. Biochemical responses in leaf tissue included total protein (TP) content, polyphenol oxidase (PPO), peroxidase (POX) and lipid peroxidation.

Enzyme extract

Enzyme extract was done following Martínez et al. (2013), with some modifications. 300 mg of leaf sample from each cultivar (infested and uninfested plants) was ground in liquid nitrogen and homogenized with 50 mM Tris-HCl buffer (pH 5.7), containing 1% polyvinylpyrrolidone (PVP) and 1 mM EDTA. Plant extract was centrifuged at 12,000 rpm, 4°C for 20 min, and the supernatant was used to determine total proteins and enzyme activity.

Quantification of peroxidase (POX) activity

POX activity was measured by spectrophotometry. The guaiacol oxidation rate by POX mediated by H_2O_2 was measured on 470 nm absorbance of the light spectrum absorbed by oxidized guaiacol. Changes in optical density were determined every 15 s for 1 min. Enzymatic activity was expressed in mM of tetraguaiacol min^{-1} μg^{-1} of protein.

Quantification of polyphenol oxidase activity (PPO)

PPO activity was quantified by the oxidation rate of pyrogallol (Alexander et al., 1964). Pyrogallol was prepared in a buffer solution of sodium acetate (50 mM, pH 5.5). Enzyme activity was measured at 15 s intervals for 3 min in a spectrophotometer at 420 nm and expressed in mM of quinone min^{-1} μg^{-1} protein.

The PPO/POX ratio was used as an indirect measurement to visualize the behavior of the genotypes after herbivore feeding. This decision was made due to the lack of information on which specific PPOs or POXs are induced.

Statistical analysis

The results were subjected to variance analysis and mean values were compared by Tukey test at p< 0.05. POX and PPO activities were correlated with the number of mites per leaflet, using Statistix software version 8.0.

RESULTS AND DISCUSSION

Biological aspects of *R. indica* reared on different coconut cultivars

Life cycle of *R. indica* was influenced by the coconut cultivar tested (Table 1). Higher developmental time was observed on JT cultivar (21.9 days), while it was about 23% lower on MYD cultivar. Developmental time was intermediate on JT×MYD hybrid and NL, being reduced by 9.6 and 13.4%, respectively in relation to JT. Furthermore, effect of coconut cultivar on *R. indica*

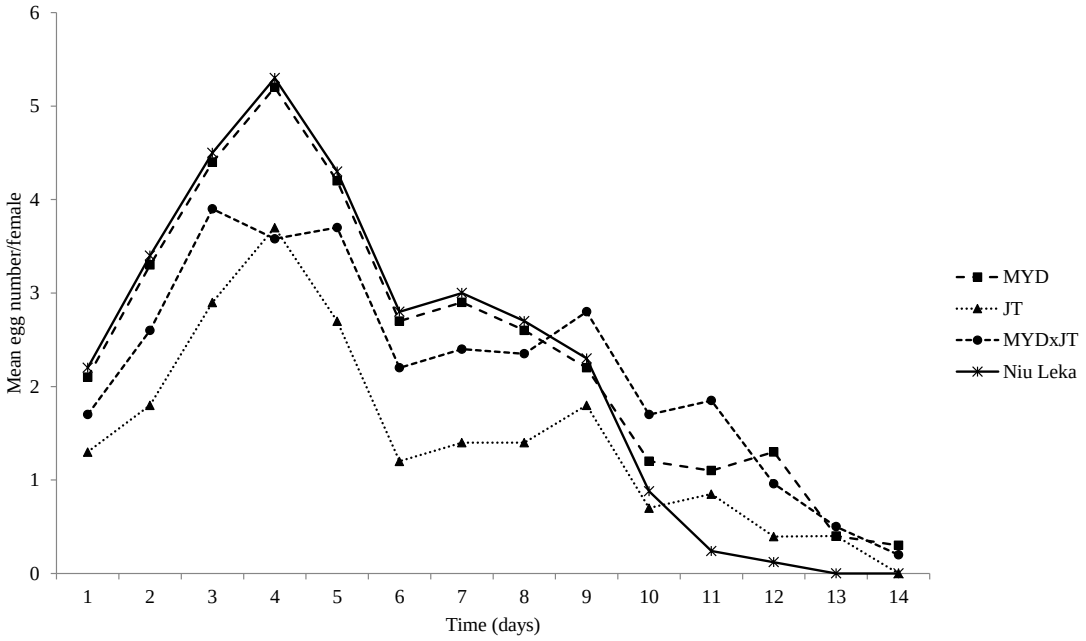

Figure 1. Daily oviposition of *R. indica* reared on different coconut genotypes leaf disk (29 ± 1.0°C, 60 ± 10% RH and 12 h light photoperiod).

oviposition was also observed (Table 1 and Figure 1). The highest oviposition rate was observed in *R. indica* females reared on MYD and on hybrid MYD×JT leaf disks (2.4 eggs female^{-1}day^{-1}), while the oviposition rate on the JT cultivar was about 30% lower than on MYD. Daily oviposition was intermediate on NL.

Host plant effect on phytophagous mite species reproduction was previously shown as growing, without effect, or decreasing (Ribeiro et al., 1988; Hilker and Meiners, 2002; Praslička and Huszár, 2004). Differences in *R. indica* developmental time was observed when reared on coconut cultivars, being 21.5 and 19.8 d on JT (in Trinidad) and on a hybrid MYDxJT (in Venezuela), respectively (Vásquez et al., 2015). Vásquez et al. (2008) hypothesized that reproductive parameters of *Oligonychus punicae* appeared to be negatively associated with flavonoid content in grape cultivars. These phenolic compounds can be synthesized in grapevine leaves and fruits in response to biotic or abiotic stress (Morrissey and Osbourn, 1999) and these compounds may act synergistically with tannins to provide plant resistance (Harborne, 1994; Bernards and Båstrup-Spohr, 2008).

Biochemical changes in coconut cultivars induced by *R. indica* feeding

Total proteins

TP content was significantly higher in JT cultivar as compared to MYD during the evaluation period (Figure 2). Although no significant variations were observed in TP content in JT after *R. indica* feeding, higher TP content was observed 72 h after infestation. In MYD, total protein content varied significantly ($p<0.01$, $F= 0.000$; $df=14$), being greater 120 h after infestation. Likewise, previous studies have shown TP increasing in response to different types of abiotic (García et al., 2003) or biotic stress (Kamal et al., 2010; Wang et al., 2011). This response has been considered as a protective strategy against stress factors, which may be associated with specific gene expression favoring induction of proteins only synthesized under non-optimal conditions (Pérez et al., 1997). Polyphenol oxidase (PPO) and peroxidase (POX) catalyze oxidation of phenols and consequently, quinones formed by oxidation of phenols, bind covalently to leaf proteins, and inhibit the protein digestion in herbivores (War et al., 2012). Consenquently, the conserved TP content in JT suggests this cultivar might be considered tolerant to the stress caused by mite feeding.

Enzyme activity

The PPO/POX ratio was similar in both non-infested genotypes in JT and MYD at 0 h. This ratio tended to decrease both in infested or non-infested JT plants, showing an increase after 72 h on infested plants. Ratio values were relatively similar along evaluation periods in MYD infested plants, ranging from 2.1 to 2.94 after 24 and 72 h, respectively (Figure 3). Mayer (2006) stated

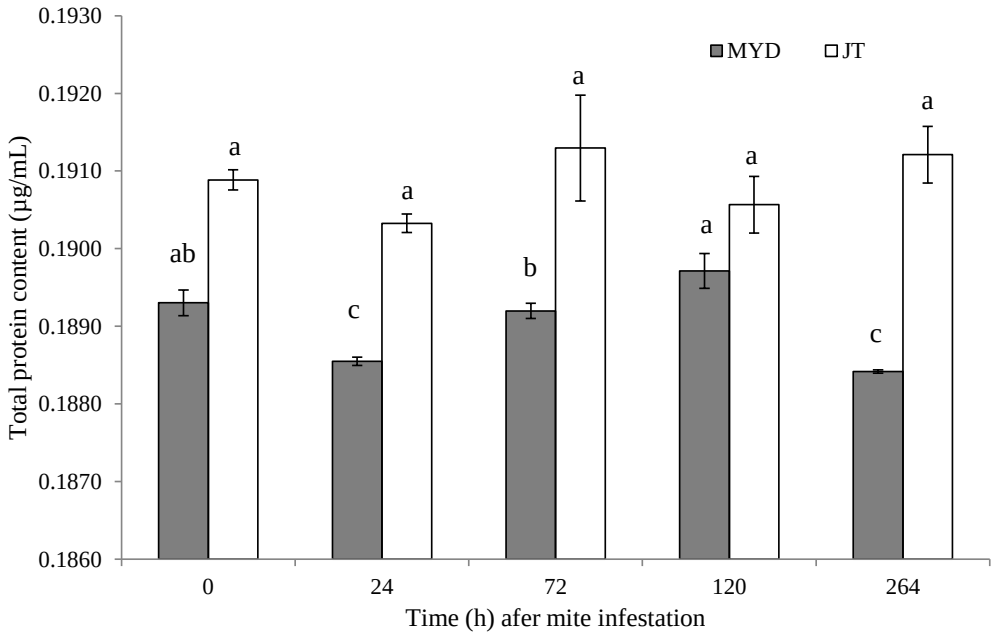

Figure 2. Variation in total protein content in MYD and JT coconut cultivars in response to *R. indica* feeding.

that resistant genotypes had localized elevated levels of PPO formation which was rapidly induced following infection. Susceptible cultivars failed to accumulate PPO even after considerable time. These results suggest that increases in PPO/POX ratio in response to *R. indica* feeding could be considered as the first evidence of resistance expressed by coconut cultivars to mite feeding. It is still unclear whether PPO may be involved in resistance to the red palm mite in coconut palms; however, the observed enzyme activity ratios in both genotypes show a slight increase 24 h after mite infestation. Simultaneously, the biological parameters of *R. indica* together with the above, particularly on JT (Table 1 and Figure 1), suggest this cultivar could be considered as a more resistant cultivar as compared to MYD.

Changes in total protein content and levels of oxidative enzymes are considered the first plant response to feeding herbivores (Felton et al., 1994; Ni et al., 2001). These biochemical responses are in function to plant growth stages and stress intensity (Constabel and Barbehenn, 2008). Furthermore, POX activity is influenced by plant species and sampling time and it reaches higher levels during the first 3 days and tends to diminish as stress decreases (Ni et al., 2001). Peroxidases and polyphenol oxidases are involved in plant defense against phytophagous mites and insects, by the production and polymerization of phenolics and lignification and hypersensitive responses in injured tissues (Kielkiewicz, 2002).

The production of PPO as a defense response to herbivores involves a complex sequence of reactions starting with gene expression and then leading to the formation and activation of enzymes for substrate production (Mayer, 2006). However, the lower mRNA levels associated with this enzyme in some species suggests that its role in defense has evolved only in a few species (Constabel et al., 2000). Results associated with PPO activity and arthropod herbivore performance, using plant genotypes that vary in resistance to herbivory and ontogenetic variation in PPO activity within the plant and leaves treated with PPO, have been contradictory (Constabel and Barbehenn, 2008).

Previous studies dealing with the relationship between the plant resistance and the activity of POD and PPO are intriguing. Most of the results have shown that higher POX or PPO levels are associated with plant resistance to *Steneotarsonemus spinki* Smiley in rice cultivars (Fernández et al., 2005), *T. urticae* in strawberry (Steinite and Ievinsh, 2002) and hop (Trevisan et al. 2003), *T. cinnabarinus* (Kielkiewicz, 2002), common cutworm (*Spodoptera litura*) and the cotton bollworm (*Heliothis armigera*) in tomato (Thipyapong et al., 2006). More recently, Samsone et al. (2012) observed that high *Vasates quadripes* Shrimer infestation levels could evoke increases in POX activity in *Acer saccharinum* leaves. Conversely, higher levels of PPO in coffee leaves apparently was not associated with resistance to the coffee leaf miner (*Leucoptera coffeella*) (Melo et al., 2006; Ramiro et al., 2006). The induction of phenolic activity, and the enzymes peroxidase and polyphenol oxidase in response to insect attack might not be

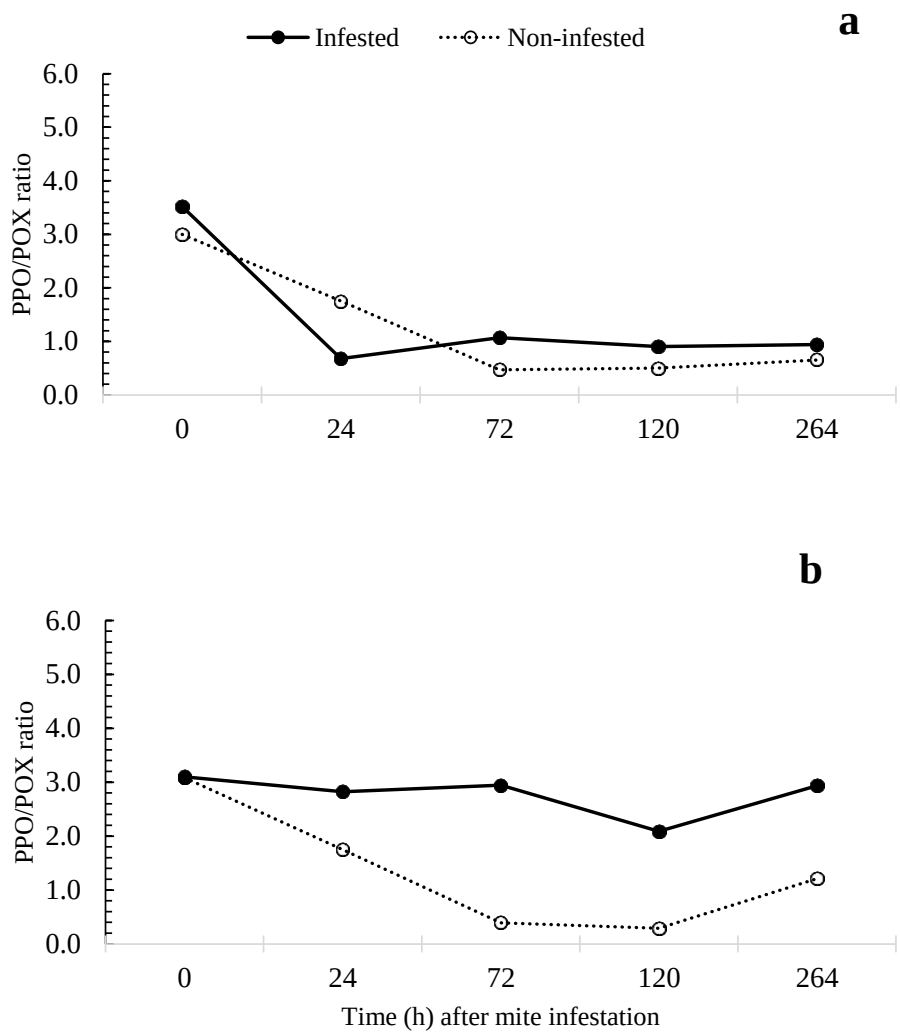

Figure 3. PPO/POX ratio in coconut cultivars Jamaican tall (a) and Malayan Yellow Dwarf (b) after *Raoiella indica* feeding.

concrete evidence that these substances participate directly in plant defense mechanisms (Ramiro et al., 2006). In addition, *Manduca quinquemaculata* caterpillars surprisingly showed greater performance on younger tobacco leaves, which contain higher PPO levels (Kessler and Baldwin, 2002). Given the tremendous variation in PPO expression patterns, activity levels, and potential substrates in different species, similar variation in the adaptive roles played by PPO in defense and other processes may be anticipated. Thus, correlations of PPO activity with defense may be confounded by the complexity of PPO gene families (Constabel and Barbehenn, 2008).

Similar to PPO production in response to plant-arthropod interaction, peroxidases catalyze synthesis of products with antimicrobial activity in plants, suggesting a role in plant defense by participating in phytoalexin synthesis (Almagro et al., 2009). Peroxidases are also involved in the binding of cell wall components. Extensin, phenolic compounds and polysaccharides act as a mechanical barrier for pathogen penetration (Brisson et al., 1994). In addition, these mechanical barriers, formed as result of strengthening cell walls, have been reported as a resistance mechanism in pericarp (García-Lara et al., 2004) and embryo in maize grain (García-Lara et al., 2007) to *Sitophilus zeamais*. Moreover, quinone oxidation in the developing grain pericarp regulated by peroxidases may contribute to plant resistance reducing digestibility for insect pests (García-Lara et al., 2007).

The observed enzyme activity increase soon after mite infestation, suggesting that the role of this enzyme should be further investigated. In this regard, more detailed studies are required to better understand mechanisms of plant response to arthropod herbivores and thus use this information for crop protection and sustainable crop production.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Ovarian follicular dynamics in purebred and crossbred Boran cows in Ethiopia

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Boran is an endangered breed of cattle indigenous to Ethiopia and the relatively poor understanding of its reproductive physiology has impeded efforts to maximize reproductive performance of the breed. This study characterized ovarian follicular dynamics in 9 purebred Boran and 8 Boran×Holstein (B×H) crossbred cows. Ovaries of all 17 cows were examined once per day for 61 consecutive days (encompassing three periods of estrus) using transrectal ultrasonography. The mean (±standard error of mean) inter-ovulatory interval (IOI) was similar (P>0.05) in Boran (19.4 ± 0.2 days) and B×H cows (20.1 ± 0.4 days). Two (in 79% of estrous cycles) or three (in 21% of cycles) follicular waves per IOI were observed and IOI was shorter (P<0.001) for cows with two follicular waves per estrous cycle (n=27; 19.5 ± 0.9 days) than for cows with three (n=7; 20.9 ± 2.1 days). All Boran cows (100%) exhibited two waves of follicular growth per estrous cycle and this was higher (P<0.001) than the proportion observed in B×H cows (56%). Mean diameter of the dominant follicle was similar (P>0.10) in both genotypes (15.8 ± 1.5 mm in Boran and 19.4 ± 2.9 mm in B×H). Boran cows possessed a greater (P<0.001) total number of ovarian follicles than B×H cows and both genotypes displayed more (P<0.05) activity on their right than left ovary. Results of our study have provided novel insights into the normal reproductive physiology of the Boran breed.

Key words: Boran, estrous cycle length, follicular dynamics, follicular waves, dominant follicle.

INTRODUCTION

Various researchers have documented that zebu cattle (*Bos indicus*) show several morphological and physiological differences from European taurine cattle. Follicular dynamics, one of the most important subjects in ovarian physiology, has been studied mostly in European breeds (Savio et al., 1988; Sirois and Fortune, 1988;

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Ginther et al., 1989; Roche and Boland, 1991; Badinga et al., 1994). The appropriate manifestation of estrus resulting from normal ovarian follicular dynamics is crucial to successful reproductive performance in cattle. A strong understanding of basic reproductive physiology enables appropriate application of controlled reproduction techniques (such as synchronization of estrus, synchronization of ovulation, and embryo transfer). Use of these reproductive biotechnologies can bring about sizeable improvements in the biological and economic efficiency of cattle breeding. Ultrasonography has enhanced the scientific understanding of the basic physiological processes in the estrous cycle including growth and regression of ovarian follicles and ovulation (Zacarias et al., 2015).

Studies in *Bos taurus* cattle have indicated the occurrence of two to four ovarian follicular waves during the estrous cycle; two waves are predominant, but four waves are rare (Sirois and Fortune, 1988; Rhodes et al., 1995; Bo et al., 1995; Adams, 1999; Townson et al., 2002; Evans, 2003; Sartori et al., 2004). Information available for zebu cattle showed a predominance of three ovarian follicular waves per estrous cycle with some observations of two, four, and even five (Zeitoun et al., 1996; Figeuiredo et al., 1997; Gambini et al., 1998; Viana et al., 2000; Mollo et al., 2007). Ethiopian zebu cattle exhibit a short duration/low intensity period of estrus, have a complete absence of behavioral estrus/receptivity to a bull in some cases, may refrain from repeated breeding attempts, and often ovulate without displaying overt signs of estrus (Tegegne et al., 1989; Tegegne et al., 1991; Bekele et al., 1991). Specific information on ovarian follicular dynamics in the Boran and other indigenous cattle breeds in Ethiopia, however, is absent. The objective of this study was to characterize ovarian follicular dynamics in purebred Boran and F_1 crossbred Boran cattle (created previously by insemination of Boran cows with Holstein semen in a national program aimed to increase milk production). It was hypothesized that purebred Boran cattle, adapted to Ethiopian conditions for centuries and reported by farmers as having greater fertility than crossbreds, would exhibit a different pattern of ovarian follicular waves than Boran×Holstein crossbred cows.

MATERIALS AND METHODS

This study was carried out for three consecutive cycles at the Debre Zeit Agricultural Research Center (DZARC) of the Ethiopian Institute of Agricultural Research, located about 45 km east of Addis Ababa (8°46′13.57"N, 38°59′50.45"E; 1900 m above sea level). The average annual temperature for the last five years was 18.5°C and average annual rainfall was 757 mm (DZARC Agro-meteorology, 2015). Seventeen cows (9 purebred Boran and 8 Boran×Holstein [B×H] crossbred) were used for this study after screening for normal reproductive anatomy through clinical and gynecological examinations. To monitor ovarian follicular dynamics, all cows underwent transrectal real-time B-mode ultrasonographic examinations using a 5.0 to 7.5 MHz linear array rectal transducer (Mindray, Hong Kong). The diameters of the three largest follicles were measured using the internal electronic calipers. Follicle sizes were categorized as small (1 to 3 mm), medium (4 to 6 mm), or large (≥7 mm) as previously described (Muasa, 2010). All follicles ≥4 mm diameter were counted on each ovary to determine follicular populations. The inter-ovulatory interval (IOI) was defined as the number of days between two consecutive ovulations in the same female. Ovulation was confirmed based on the sudden disappearance of the largest diameter follicle and subsequent appearance of a corpus luteum (CL) whose diameter was measured. The growth rate of the dominant follicle was computed from the date of divergence until ovulation.

Data were summarized using descriptive statistics and analysis of variance (PROC MIXED; SAS, 2004) was used to determine genotype effects. Mean comparisons were made using the least significant difference (LSD) procedure. Kendall's tau was computed to show rank correlations using the PROC CORR procedure of SAS. Differences were considered statistically significant at a P-value of $P<0.05$.

RESULTS

The mean (±standard error of mean [SEM]) IOI was 19.8 ± 0.2 days (n=34) and was not affected by genotype ($P>0.10$). Table 1 depicts results (segregated by genotype) of IOI and other parameters associated with ovarian follicular dynamics. Forty-five percent Boran cows had an IOI of 20 days (22% had 18 days, 22% had 19 days, and 11% had 21 days), whereas 38% of B×H crossbred cows had a 21-day IOI (range of 18 to 22 days).

The cows in this study exhibited two or three follicular waves during the IOI (Figures 1 and 2). A higher ($P=0.002$) proportion of Boran cows (100%) exhibited two follicular waves per estrous cycle than B×H crossbreds (56%). The remaining 44% of B×H crosses exhibited three follicular waves per estrous cycle; there were none with four or five. The mean (±SE) IOI for cows with two waves per cycle was 19.5 ± 0.9 days (Figure 3) and was shorter ($P=0.001$) than the 20.9 ± 2.1 days exhibited by cows with three waves.

The mean (± SEM) maximum diameter of the largest ovarian follicle was 17.0 ± 2.9 mm and this diameter was not affected ($P>0.10$) by genotype (16.9 ± 1.5 mm for Boran and 17.1 ± 2.9 for B×H crosses) (Table 2). However, purebred Boran cows had more ($P<0.001$) ovarian follicles than B×H crossbred (Table 2 and Figure 3), despite having a similar range in total number of follicles per cow (11 to 28 for Boran and 10 to 28 for B×H crosses). More ($P<0.001$) follicles were found on the right than the left ovaries in both breeds (Table 3). Representative ultrasonographic images of the ovaries of purebred Boran cows are shown in Plate 1.

DISCUSSION

Ultrasonography has been a highly useful tool to study reproductive events such as follicular wave emergence, dominant follicle selection, and ovulation (Gimenes et al.,

Table 1. Inter-ovulatory interval, number of follicular waves, and diameter of the dominant and subordinate follicles in purebred Boran and Boran×Holstein crossbred cows.

Parameter evaluated	Breed	N	Mean (±SEM)	Range
Inter-ovulatory Interval (IOI) [days]	Boran	18	19.44±0.23	18-21
	Boran×Holstein cross	16	20.13±0.35	18-21
Diameter of preovulatory dominant follicle (mm)	Boran	18	16.28±0.71	14-27
	Boran×Holstein cross	16	17.75±0.29	15-20
	Overall	34	16.97±0.415	14-27
Diameter of dominant follicle at day of divergence	Boran	18	9.28±0.311	8-13
	Boran×Holstein cross	16	9.75±0.348	8-12
	Total	34	9.50±0.232	8-13
Growth rate (mm/day)	Boran	18	1.14±0.15	0.50-3.2
	Boran×Holstein cross	16	1.46±0.12	0.71-2.3
	Overall	34	1.29±0.10	0.50-3.2
Diameter of largest subordinate follicle at ovulation (mm)	Boran	18	9.17±0.493	7-16
	Boran×Holstein cross	16	8.88±0.315	6-11
	Total	34	9.03±0.297	6-16
Diameter of second largest subordinate follicle at divergence (mm)	Boran	18	7.39±0.293	6-10
	Boran×Holstein cross	16	7.44±0.223	5-8
	Total	34	7.41±0.185	5-8

SEM, Standard error of mean.

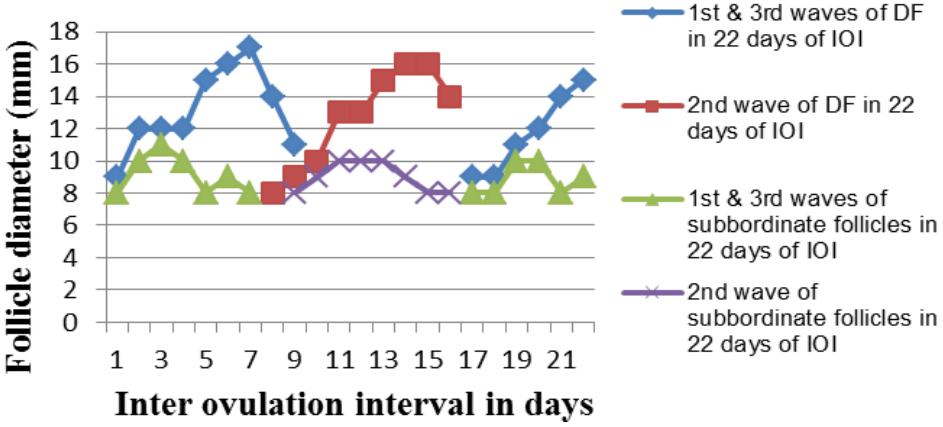

Figure 1. Ovarian follicular growth patterns of Boran×Holstein crossbred cows exhibiting three follicular waves per estrous cycle.

2008). A major benefit of ultrasound is that it facilitates the study of reproductive function without interrupting or distorting that function (as can occur when studying the same events via laparotomy or laparoscopy). The use of ultrasonography was reported herein to obtain the most detailed data recorded at present on ovarian follicular dynamics of the indigenous Ethiopian Boran breed of cattle.

The IOI for purebred Boran cows in the current study was shorter than the IOI reported by Bo et al. (2003) for in a review of *B. indicus* reproductive performance and by Sartori et al. (2004) for Nelore cows in Brazil. The IOI of

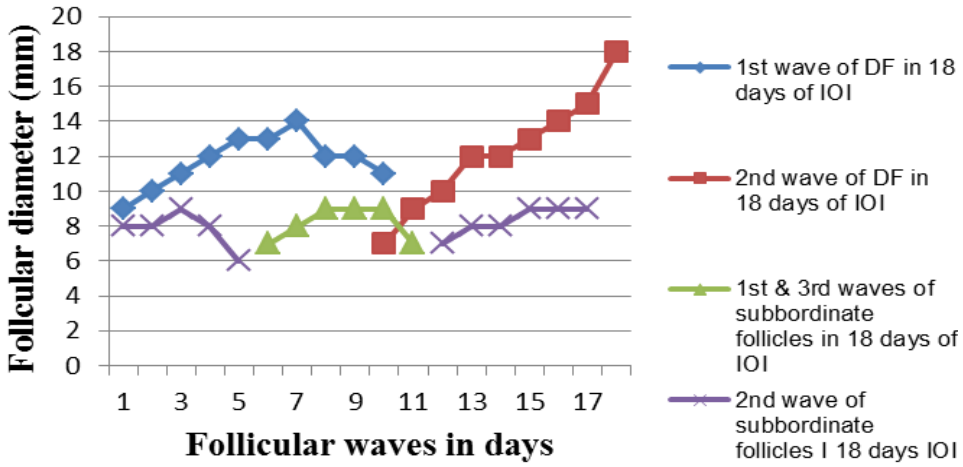

Figure 2. Ovarian follicular dynamics in purebred Boran & Boran*Holstein crossbred cows exhibiting two follicular waves per 18-day inter-ovulatory interval (IOI).

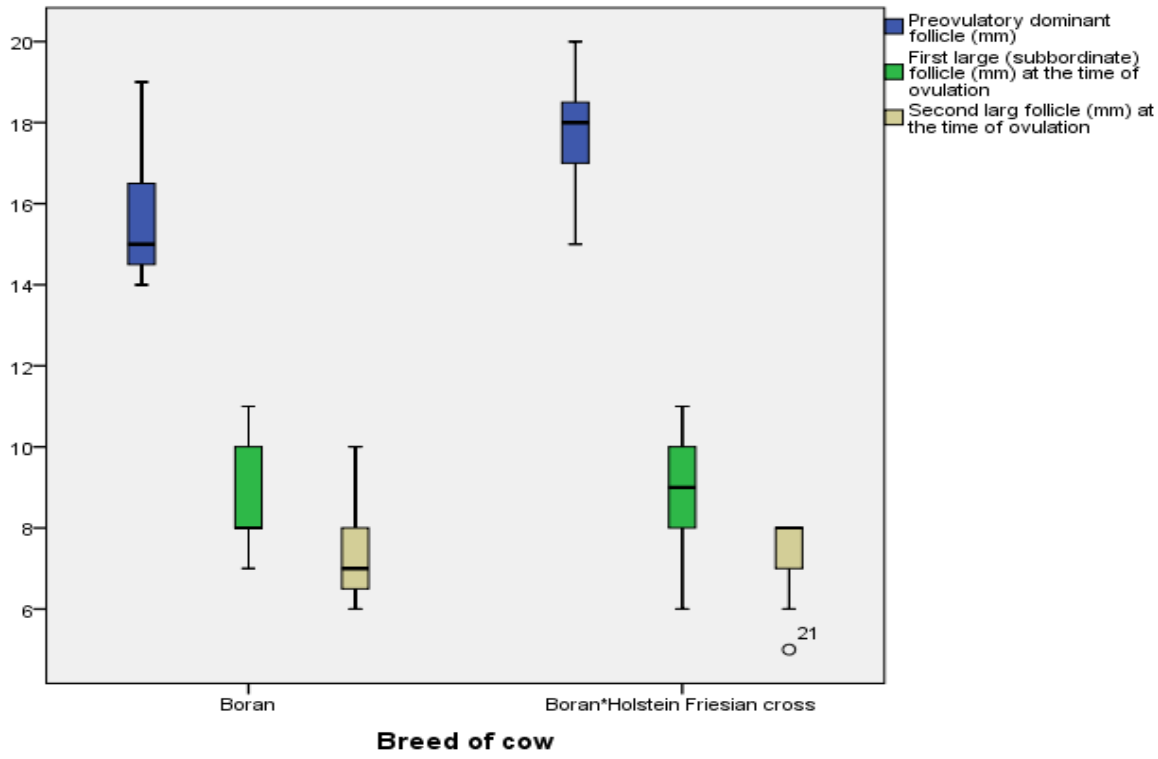

Figure 3. The size of the preovulatory dominant follicle (DF), largest subordinate follicle and second largest subordinate follicle in purebred Boran and Boran×Holstein crossbred cows.

the Ethiopian Boran in our study was also shorter than that reported for Kenyan Boran (Muraya, 2013), suggesting adaptation of the Boran to differing climates in these neighboring east African countries and/or a greater incidence of Kenyan Boran cows with three follicular waves per estrous cycle. The IOI of Nelore heifers in Brazil (Corte et al., 2012), however, was quite similar to our findings.

Early studies on ovarian follicular dynamics in *B. taurus* cattle (Savio et al., 1988; Savio et al., 1990; Sirois and Fortune, 1988), as well as later studies (Alvarez et al., 2000; Townson et al., 2002; Sartori et al., 2004), reported

Table 2. Total number of follicles ≥ 4 mm diameter on the right, left or both ovaries in purebred Boran and Boran×Holstein crossbred cows.

Breed	N	Total follicles ≥ 4 mm on right ovary [Mean (±SEM)]	Total follicles ≥ 4 mm on left ovary [Mean (±SEM)]	Total follicles ≥ 4 mm on both ovaries [Mean (±SEM)]
Boran	549	9.14±0.091 (Range=4-17)	9.20±0.083 (Range=5-14)	18.34±0.145[a] (Range=11-28)
Boran×Holstein Friesian cross	488	8.69±0.089 (Range=5-17)	8.87±0.084 (Range=4-14)	17.56±0.141[b] (Range=10-28)
Total	1037	8.93±0.064 (Range=4-17)	9.05±0.059 (Range=4-14)	17.98±0.102 (Range=10-28)

[a,b]Means within a column with unlike superscripts are different (P<0.001).

Table 3. Diameter of the dominant, largest subordinate and second largest subordinate ovarian follicle and total number of ovarian follicles in the right and left ovaries in purebred Boran and Boran×Holstein crossbred cows.

Breed	Ovary	N	Mean	SD
		Dominant follicle		
Boran	Right	16	16.19	3.103
	Left	2	17.00	2.828
	Total	18	16.28	3.006
Boran×Holstein cross	Right	10	17.50	1.354
	Left	6	18.17	0.753
	Total	16	17.75	1.183
Total	Right	26	16.69	2.619
	Left	8	17.88	1.356
	Total	34	16.97	2.418
		Largest subordinate follicle		
Boran	Right	16	9.19	2.198
	Left	2	9.00	1.414
	Total	18	9.17	2.093
Boran×Holstein cross	Right	10	8.90	1.287
	Left	6	8.83	1.329
	Total	16	8.88	1.258
Total	Right	26	9.08	1.875
	Left	8	8.88	1.246
	Total	34	9.03	1.732
		Second largest subordinate follicle		
Boran	Right	16	7.38	1.310
	Left	2	7.50	0.707
	Total	18	7.39	1.243
Boran×Holstein cross	Right	10	7.50	0.972
	Left	6	7.33	0.816
	Total	16	7.44	0.892
Total	Right	26	7.42	1.172
	Left	8	7.38	0.744
	Total	34	7.41	1.076

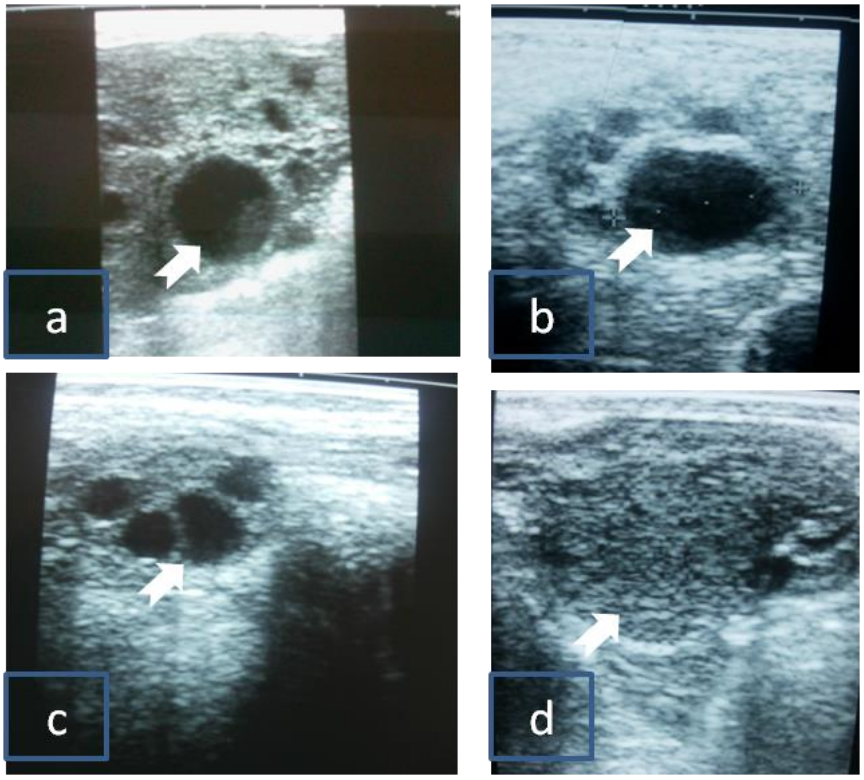

Plate 1. Ultrasonographic images of ovarian structures in purebred Boran cows: Panel a illustrates a dominant follicle; panel b shows several small ovarian follicles plus a large ovarian follicle; panel c depicts medium and large ovarian follicles; panel d illustrates a mature corpus luteum.

that cows with two follicular waves per estrous cycle had a shorter IOI than cows with three follicular waves. Our study revealed the same is true with purebred Boran and crossbred B×H cows raised in Ethiopia, even though cows in our study had a longer IOI than Boran cows in Kenya (Muraya, 2013). Reasons for the difference between Ethiopian and Kenyan Borans are likely due to genetic drift in the different populations (animals selected to live in different climates) and/or difference in feed resources (which under some circumstances could reduce reproductive performance). Our results are in agreement with those reported for the Gir breed in Brazil (Viana et al., 2000).

The results of our study showed fewer waves of follicular growth in Boran than B×H crossbred cows, and these results are in complete agreement with other research groups that investigated *B. indicus* breeds such as the Nelore (Figueiredo et al., 1997; Mollo et al., 2007), the Gir (Gambini et al., 1998; Viana et al., 2000), or Brahman (Zeitoun et al., 1996; Alvarez et al., 2000). Although the wave patterns in Zebu cows are shown to be influenced by parity (Figueiredo et al., 1997), our study was not sufficiently large to directly assess this effect.

The diameter of the dominant follicle (DF) in Boran cows in our study was equivalent to that reported in *B. taurus* cows by Fortune et al. (1988), Savio et al. (1988), and Ginther et al. (1989). However, the diameter of the DF of Boran cows in our study was greater than that reported in other *B. indicus* breeds by Figueiredo et al. (1997) and Sartorelli et al. (2005). The follicular size at the point of deviation in the current study for the Boran is much higher than that reported for *B. taurus* breeds (Ginther et al., 1996; Sartori et al., 2001) and for Zebu cattle (Figueiredo et al., 1997; Sartorelli et al., 2005; Castilho et al., 2007; Gimenes et al., 2008). There is no definitive explanation for this difference. Follicular deviation is characterized by a decrease in the growth rate of the largest subordinate follicle and an increase in the growth rate of the DF (Ginther et al., 2001), and this phenomenon was observed quite clear in purebred Boran as well as B×H crossbred cows.

The total number of follicles greater than 4 mm in diameter in Boran cows was comparable to the number of follicles reported in *B. taurus* breeds (Ginther et al., 1996; Alvarez et al., 2000; Carvalho et al., 2008; Bastos et al., 2010) and lower than the number of follicles reported for Nelore (Buratini Jr et al., 2000; Carvalho et al., 2008; Gimenes et al., 2009; Bastos et al., 2010), as well as Brahman and Senepol (Alvarez et al., 2000). Differences between our results and those of other studies are likely due to differences in climate and

available feed resources. Because Boran originated from an area where feed resources typically are not abundant on a year-round basis, perhaps, it developed the ability to grow large numbers of ovarian follicles on a restricted plane of nutrition.

Theoretically, one would expect the function of the left and right ovaries to be comparable. However, in a number of species, including the bovine, the number of ovarian follicles present on (and/or the number of ovulations from) the right ovary typically exceed that of the left ovary by a few percent (Giraldo et al., 2010). Our observation of the right ovaries being more active than the left ovaries in Boran and B×H cows confirms reports of others. It is also in complete agreement with Muraya (2013) who stated that "higher incidence of ovulatory DFs in the right ovary is attributed to the fact that the right ovary receives more blood supply compared to the left one and it is clinically observed to be more active than the right ovary".

Conclusion

This was the first detailed study of ovarian follicular dynamics of the Boran breed of cattle in Ethiopia. The inter-ovulatory interval of the Boran lies in the range reported for other zebu breeds. However, unlike other zebu breeds where the maximum size of the dominant follicle is typically smaller than that of *B. indicus* cows (Sartori and Barros, 2011), the maximum diameter of the preovulatory dominant follicles were larger and similar to that seen in F_1 crossbred B×H cows. The uniformity of two waves of follicular growth per estrous cycle in the Boran would likely permit more predictable and efficacious application of reproductive biotechnologies such as synchronization of estrus or embryo transfer (Degefa et al., 2016). In addition, the greater number of growing ovarian follicles in the Boran would be ideal for ovum pick-up and subsequent *in vitro* embryo production. Great opportunity exists to capitalize on the reproductive potential of the Boran, and further investigations on advanced reproductive biotechnologies (especially embryo transfer and *in vitro* embryo production) are warranted. It is important to develop effective and sustainable genetic improvement schemes for this indigenous cattle breed of Ethiopia and to ensure the availability of this breed for future use through creation of an embryo cryobank.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Micropropagation of caçari under different nutritive culture media, antioxidants, and levels of agar and pH

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The caçari (*Myrciaria dubia*) is a native fruit tree from Amazon with high concentrations of vitamin C. This study aimed to adjust a culture medium that meets the nutritional needs for the *in vitro* development of caçari, evaluating the effect of different concentrations and nutritive culture media, antioxidant, and levels of agar and pH. Three experiments were carried out in a completely randomized design: 1 - woody plant medium (WPM), Murashige and Skoog (MS) and Juan, Antonio, Diva and Silvia medium (JADS) nutritive media with 25, 50, 75 and 100% concentrations; 2 - pH (3.7, 4.7, 5.7 and 6.7) and agar concentrations (0, 3.5, 7.0 and 10.5 g.L^{-1}); 3 - Antioxidants [(ascorbic acid (AA), citric acid (CA), polyvinylpyrrolidone (PVP)] and concentrations (0, 100, 200, 300 mg.L^{-1}) on the control of phenolic oxidation of stem segments. After collection, the explants were disinfested in a laminar flow chamber, dipping in 70% ethanol for 3 min and 1.5% sodium hypochlorite for 12 min, followed by three washes in distilled and autoclaved water. After disinfestation, the explants were inoculated in 15 × 125 mm test tubes containing 30 mL culture medium, according to each experiment and their respective treatments. After 90 days, the number and length of sprouts (cm) and the oxidation were evaluated. The best results were obtained using the WPM medium at a concentration of 100% with 7 g.L^{-1} agar, and pH adjusted to 5.7. The use of antioxidants in the tested conditions did not contribute to decrease in oxidation in explants, indicating that there is no need of adding them into the culture medium.

Key words: Camu-camu, *in vitro* culture, *Myrciaria dubia,* organogenesis.

INTRODUCTION

The caçari (*Myrciaria dubia* (Kunth.) McVaugh) is a native fruit tree from Amazon, widely distributed in the conditions of the Northern Amazon. This species has aroused a great interest of the national and international

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market due to its high content of vitamin C, which may reach 7,355.20 mg per 100^{-1} g of ascorbic acid pulp (Chagas et al., 2015), and nutraceutical compounds important for our health (Neves et al., 2015).

Because the species is still in domestication process (Chagas et al., 2012), there is little information about its micropropagation. This technique is important for use in breeding programs of the species, multiplication of high clones with difficult rooting and obtaining plants free from pests and diseases and with high quality. Thus, one of the most important factors to be studied, that most influences and determines the success of the *in vitro* culture, is the nutritional balance of the culture medium and its interaction with the genetic material or explant. In most cases the culture medium is empirically defined, using as a basis the protocoled culture media, showing no specificity to the species nutritional requirements, the limiting factor of the morphogenetic processes of a given species (Correia, 2006).

Many culture media formulations have been made in the last 100 years, such as the Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), to optimize the culture of roots, cells, calli, among others, representing one of the most widespread and used media for herbaceous species. This medium has a high total ionic concentration, with a high concentration of nitrogen, potassium, zinc and chlorine; in comparison to other media (Leifert et al., 1995). For woody species, media with more diluted mineral balance have been more used, such as the WPM culture medium (Lloyd and Mccown, 1981) which has lower salt concentrations, in particular, the nitrogen and potassium (Rocha, 2005). The Juan, Antonio, Diva and Silvia (JADS) medium (Correia et al., 1995), similar to the Woody plant medium (WPM), also has low nitrogen, potassium, and molybdenum content.

The culture medium consistency is another important factor for the *in vitro* culture and plays a fundamental effect on the morphogenesis and growth of sprouts, and may cause serious trouble to the expected development of the explant if its basic requirements are not met (Karasawa et al., 2002). The agar is recognized for its gelling action; therefore, it is used to give support to the plant when it is placed in the culture medium. The agar may also be a controller of vitrification and hyper-hydration phenomena (Williams and Leopold, 1989).

The pH is another critical and very important factor for the culture medium as it influences both the availability of nutrients and phytoregulators and the degree of agar solidification. If the pH is well adjusted, it may promote a higher and better use of the nutrients by the explant. Therefore, the cells and tissues of plants require an appropriate pH range for the growth and development *in vitro*. Thus, the pH of the culture media are generally adjusted to 5.6 to 5.8 because in these conditions all ions are in solution and readily available for the cells.

Furthermore, it is a pH value close to those, which under natural conditions involve plant cells (Bhatia and Ashwath, 2005; Canhoto, 2010).

The oxidation is another important factor for the *in vitro* culture, this process is caused by the reaction of polyphenoloxidases on phenolic compounds, and may cause the death of the stem apexes in the early stages of development, or it may affect the performance of the multiplication phase (Souza et al., 2000). To reduce the phenolic oxidation, some procedures may be adopted, such as the use of antioxidant substances, reduce the mechanical and chemical damages, wash the vegetative propagules under running water, use more diluted basic media, remove the phenolic substances, among others (Xavier et al., 2009).

With regards to the antioxidant effect, it consists of the inactivation of free radicals, complexation of metabolic ions, or the reduction of peroxides for products unable to form free radicals with oxidative potential (Araújo, 1985). Among the substances with antioxidant effects, we may mention the ascorbic acid, citric acid, polyvinylpyrrolidone (PVP), activated carbon, L-cysteine, dithiothreitol, thiourea, coconut water, and bovine serum albumin. These substances may act by inhibiting the synthesis or action of enzymes related to the oxidation of polyphenols, or act as adsorbents of these substances (Goulart et al., 2010).

In this context, this study aimed to adjust a culture medium that meets the nutritional needs for the *in vitro* development of caçari, evaluating the effect of different concentrations, culture media, antioxidants, and agar and pH levels.

MATERIALS AND METHODS

The experiment was performed at the Tissue Culture Laboratory of Embrapa Roraima, in Boa Vista (Roraima State, Brazil). Stem segments with 4 pairs of axillary buds (± 6 cm length) from matrix plants of caçari with good phytosanitary health were used; these plants were kept in a greenhouse under daily irrigation, nutrition, and health care. Before obtaining the explants, the plants were pruned to induce new sprouting; then, they were pulverized with 2 $ml.L^{-1}$ fungicide (Nativo®) for 30 days, with an interval of seven days between one application and another.

After collection, the segments were taken to the laboratory and were submitted to a pre-cleaning process, excising the leaves and stem excess, in order to obtain a stem with ±1 to 1.5 cm with a pair of buds and ready to be inoculated, after the disinfestation process. Then, they were washed in running water for partial leaching of phenolic compounds released as a result of the tissue cutting. Afterward, they stayed for 24 h immersed in a solution composed of a mixture of $ml.L^{-1}$ fungicidal (Nativo®) and $2ml.L^{-1}$ bactericide (Kasumin®). Subsequently, the explants were taken to an inoculation room and disinfested in a laminar flow hood, immersed in a solution of alcohol (70%) for 1 min and, after that, they were immersed in sodium hypochlorite (1.5%) for 12 min, followed by three washes in distilled, deionized and autoclaved water (DDA water) for a complete removal of the products from the surface of the tissues.

After disinfestation, the explants were inoculated in 15 × 125 mm test tubes containing 30 mL culture medium, according to each experiment and treatments, described as follows.

Determination of the culture medium and concentration for the *in vitro* regeneration of caçari

After disinfestation, the explants were cultured in different media (WPM, MS and JADS), combined with four concentrations (25, 50, 75 and 100%) of the composition of mineral salts present in the culture media; 3 $g.L^{-1}$ activated carbon were added to all treatments, solidified with 7 $g.L^{-1}$ agar, and the pH was adjusted to 5.8, before autoclaving at 121°C and 1 atm for 20 min.

The experimental design was completely randomized in a 3×4 factorial scheme with five replications and five explants per replication were used for each treatment.

Influence of pH and agar concentration in the *in vitro* regeneration of caçari

The explants were cultured in WPM medium with different agar concentrations (0, 3.5, 7.0 and 10.5 $g.L^{-1}$), combined with different pH values (3.7, 4.7, 5.7 and 6.7), added with 3 $g.L^{-1}$ activated carbon, and autoclaved at 121°C and 1 atm for 20 min, before the pH adjustment.

The experimental design was completely randomized in a 4×4 factorial scheme with five replications and five explants per replication were used for each treatment.

Effect of different antioxidants and concentrations on the control of phenolic oxidation in stem segments of caçari

The explants were cultured in WPM medium containing different antioxidants (ascorbic acid (AA), citric acid (CA), polyvinylpyrrolidone (PVP)), combined with their concentrations (0, 100, 200, 300 $mg.L^{-1}$). It was used the WPM as a standard medium, with 4 $g.L^{-1}$ activated carbon, solidified with 7 $g.L^{-1}$ agar and pH adjusted to 5.8, before autoclaving at 121°C and 1 atm for 20 min.

The experimental design was completely randomized in a 3×4 factorial scheme with five replications and five explants per replication were used for each treatment.

For all experiments, after inoculation, the explants stayed for 15 days in darkness; then, they were transferred to a growth chamber and submitted to 16 h of photoperiod, at a temperature of 25 ± 2°C and luminosity of 32 $\mu mol.m^{-2}.s^{-1}$. After 90 days, the number and length of sprouts, and the oxidation were evaluated.

The results of the variables evaluated were submitted to the variance analysis by the statistic program Sisvar (Ferreira, 2011), performing the regression analysis for the quantitative factor and Tukey's test for the qualitative factor, at 5% of probability.

RESULTS AND DISCUSSION

Optimum culture medium and concentration for the *in vitro* regeneration of caçari

The analysis of variance showed an interaction between the culture media and concentrations established in this study for the sprout length and oxidation value. For the number of sprouts, there was a significant difference just for the culture media.

A greater number of sprouts were obtained in the JADS medium with a mean of 2.70 sprouts, followed by the MS medium (2.15) which did not significantly differ from JADS medium. The WPM medium provided a lower number of sprouts, but it did not differ from MS medium (Table 1). Similar results were found by other authors, who found that the JADS and MS media provided a sprout rate of 5.3 and 4.9, respectively, for the cultivation of *Eucalyptus* clones (Borges et al., 2011; Andrade et al., 2006). These two media provided similar increments in the number of sprouts in *Eucalyptus* clones. A similar behavior was observed for the number of sprouts obtained in our study.

Table 1. Number of sprouts from stem segments of caçari cultured *in vitro* using different culture media.

Culture media	Number of sprouts
WPM	1.68^{b}
JADS	2.70^{a}
MS	2.15^{ab}
CV (%)	45.5

Means followed by the same letter in the column do not differ from another by the Tukey test level of 5% probability.

The MS is a medium with a high content of nutrients (Leifert et al., 1995) and the JADS medium has a more diluted nutrient content in comparison to the MS medium. Therefore, by comparing the MS, JADS, and WPM media, it was observed that the MS and JADS have higher concentrations of macro and micronutrients than the WPM medium (Borges et al., 2011). Thus, there is an intimate correlation between the MS and JADS media; this fact may explain the best results regarding the number of sprouts by using these two media. Therefore, the nutritional similarity may have influenced the results, which showed no significant difference between the MS and JADS media.

Leitzke et al. (2010), studying the mulberry Xavante (*Rubus* sp.), also observed a higher number of sprouts using the MS medium (2.5 sprouts). A similar result was also obtained by Villa et al. (2005), who found a higher number of sprouts for the MS medium.

The vegetative growth of the sprouts and oxidation of the explants, submitted to different culture media and concentrations, are shown in Figure 1A and B, respectively.

With regards to the length of sprouts (Figure 1A), there was an interaction between the culture media and salt concentrations. The WPM medium provided a linear growth of sprouts with the increase of salt concentrations in the medium, promoting a higher average growth of sprouts (2.15 cm). Regarding the MS medium, there was a reduction in the sprout length, up to the concentration of 57% salts, obtaining sprouts with a mean of 0.99 cm; above this concentration, there was an increase in the sprout length with the increase of salt concentration. However, for the JADS medium, an opposite behavior was observed. There was an increase in the sprout length with the increase of the concentration of salts up to 50%, obtaining sprouts with a mean of 0.92 cm, but above this concentration, there was a reduction in the

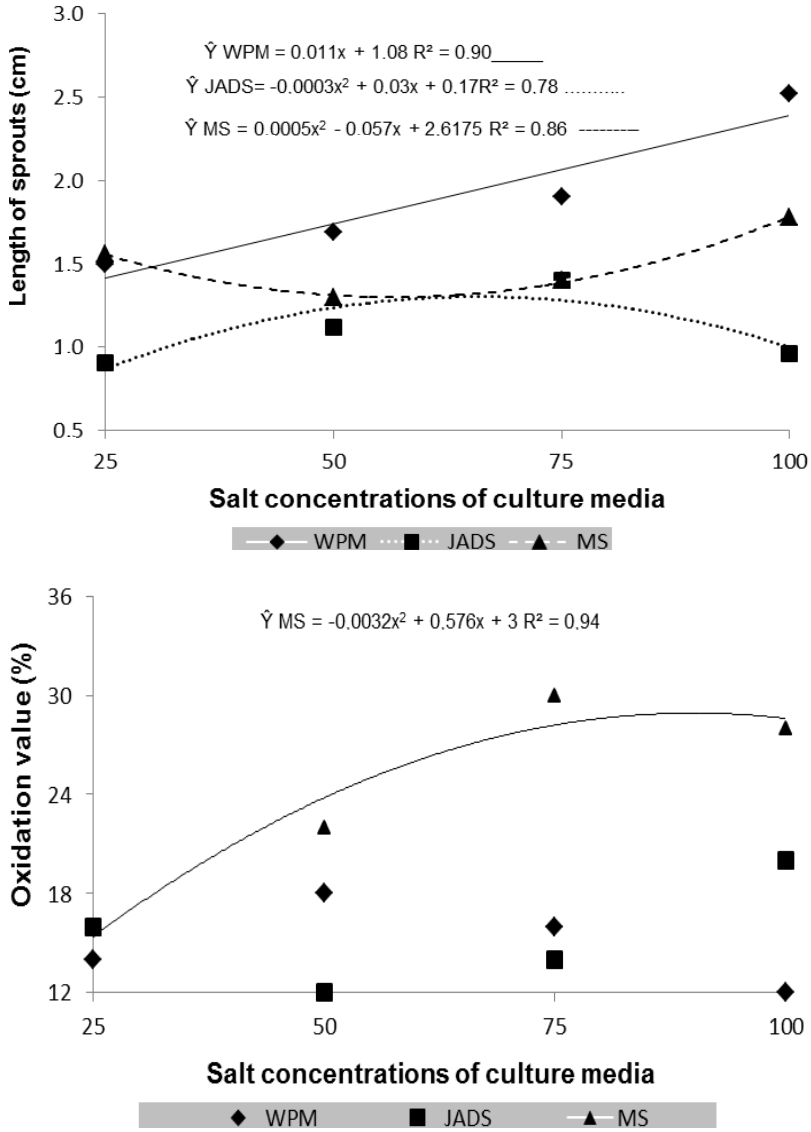

Figure 1. Length of sprouts (cm) (1A) and oxidation value (1B) of stem segments of caçari cultured in vitro in different culture media and salt concentrations.

sprout growth cultivated *in vitro*.

The WPM medium, in comparison to the MS and JADS media, has more diluted concentrations of macro and micronutrients. This medium has also been the most widely used in studies involving woody species, such as the caçari. Thus, probably, despite having provided a lower number of sprouts in comparison to the MS and JADS media (Table 1), the WPM provided a higher growth rate (Figure 1A). Therefore, if the aim is the *in vitro* propagation of a species, the obtainment of longer and more vigorous sprouts is better than a high number of small sprouts with lower capacity to regenerate or retard the multiplication process. It is noteworthy that for the production of conventional seedlings, the caçari is a rustic species and grows best in nutritionally poor substrates (Chagas et al., 2013). It is also evidenced that under the *in vitro* conditions, the best result for sprout length was obtained by using the culture medium with lower concentrations of nutrients, particularly the nitrogen and potassium.

Other authors also reported satisfactory results studying the *in vitro* culture with WPM for *Ficus carica* (fig tree), a woody plant. These authors found that the WPM medium promoted an optimal growth, reaching 6 cm length (Brum et al., 2002; Palú et al., 2014). Several studies have confirmed the efficiency of WPM in the *in vitro* culture of woody species using nodal segments and apical buds, such as *Eugenia involucrata* (Golle et al., 2012), *Tectona grandis* L. (Fermino Júnior et al., 2014), and coffee tree (*Coffea arabica*) (Rezende et al., 2008;

Jesus et al., 2010).

With regards to the oxidation (Figure 1B), after the interaction unfolding, we observed that only the MS medium and its salt concentrations showed a significant difference. Regarding the WPM and JADS media, there was no significant difference, so in Figure 1B, we did not insert the trend line at the curve points of these media.

For the MS medium, the lowest oxidation value was observed at the concentration of 25%, however, as the increase of the salt concentrations up to 90%, there was a higher oxidation (29%). For the WPM and JADS, a total mean of 12 and 15.5% of oxidized explants, respectively was verified, with no influence of the salt concentrations since there was no significant difference between the media and concentrations (Figure 1B). Therefore, it is noteworthy that the three media showed low oxidation.

Different results were found by Fagundes et al. (2012) studying stem explants of *Campomanesia guazumifolia* (Myrtaceae). These authors found that the WPM medium led to a higher oxidation rate in comparison to the MS medium (98.33 and 92.50%, respectively). According to Teixeira (2001), some species of plants are more susceptible to oxidation than other species. Golle et al. (2012), studying the *Eugenia involucrata,* also observed an oxidation process, however, these processes did not affect the establishment and development of the cultures.

Similar results to those presented in this study were found in other studies. Pelegrini et al. (2013), studying zygotic embryonic axis of a forest species (*Ocotea porosa*), found that the MS medium promoted a higher oxidation (80%), in comparison to the WPM (30%). For other forest species, these authors, studying the axillary buds of "jacaranda da Bahia", cultured in MS and WPM, found no significant differences between these two media, finding a mean of 70% of oxidation, for both MS and WPM (Sartor et al., 2013). Thus, the oxidation values found in this study is lower than those found by other authors. Therefore, it was observed that the lowest oxidation values were obtained for the WPM and JADS media, with a mean of 12 and 15% of oxidation, respectively, even at a concentration of 100% salts, evidencing that the WPM favored the *in vitro* regeneration process of caçari, probably due to provide the best nutritional balance for this species.

Influence of pH and agar concentration in the *in vitro* regeneration of caçari

There was a significant interaction between the factors tested for the number and length of sprouts. With regards to the oxidation, there was a significant difference only for the agar factor. Figure 2 show the number (Figure 2A) and length (Figure 2B) of sprouts. Best results were observed for the concentration of 7 $g.L^{-1}$ of agar. At this concentration, there was higher number of sprouts with the increase of the pH of the culture medium, up to the concentration of 6.04, obtaining 7.6 sprouts. This probably occurred because the concentration of 7 $g.L^{-1}$ promotes a support and availability of suitable nutrients for the growth of the plant since it is the agar concentration most commonly used for most species cultured *in vitro*. At the concentration of 3.5 $g.L^{-1}$, there was a linear increase in the number of sprouts as the increase of the pH of the culture medium, resulting in better means when combined with a pH of 6.7. However, for the concentration equal to zero, there was a different behavior in comparison to the previous concentrations since there was a decrease in the number of sprouts as the increase of the medium pH. Regarding the concentration of 10.5 $g.L^{-1}$ agar, there was no significant difference. This occurred because a higher concentration of agar (10.5 $g.L^{-1}$) provides a stiffer medium and hinders the absorption of nutrients and development of the sprouts.

Similar results to those obtained in this experiment were observed by Pereira (2014), who evaluated the pH and nitrogen source effects in the *in vitro* propagation of "medonheiro" (*Arbutus unedo* L.), finding a preference for pH close to neutrality (5.7 to 6.5) for the proliferation of sprouts. Karim et al. (2007), evaluating the effect of sucrose concentration and pH levels in the *in vitro* regeneration of *Araria elata*, observed a higher number of sprouts in pH ranging from 4.5 to 5.8, thus corroborating the results obtained in the present study. Nair and Seeni (2003) also observed a best multiplication rate of *Calophyllum apetalum* with the medium pH adjusted to 5.8.

However, different results were found by Naik et al. (2010), studying the *Bacopa monnieri*. These authors obtained a higher number of sprouts in more acidified culture media, and the best results were observed at pH of 4.5, obtaining 151 sprouts. Similar results were reported by Bhatia and Ashwath (2005), who found the best sprout *in vitro* regeneration, for tomato (*Solanum lycopersicum* L.), using media with more acidified pH, in comparison to more alkaline pH media.

With regards to the length of sprouts (Figure 2B), there was a significant interaction only for the concentrations of 3.5 and 7 $g.L^{-1}$ agar and pH levels. For the concentration of 3.5 $g.L^{-1}$ agar, there was a linear increase in the sprout length as the increase of the pH levels. For the concentration of 7 $g.L^{-1}$ agar, there was an increase in the growth of sprouts as the increase of the medium pH up to 4.95, resulting in a sprout growth of 4.43 cm. Regarding the concentrations of 3.5 and 7.0 $g.L^{-1}$ agar, the higher the pH the higher the average growth of sprouts. For the concentrations of 0 and 10.5 $g.L^{-1}$ agar, the data were not significant, indicating that the caçari has a better development in culture media closer to neutrality than when it is cultured in an acidic medium with a pH below 4.5 since more acidic culture media hamper the availability of nutrients for the explant.

Karim et al. (2007), evaluating the sucrose

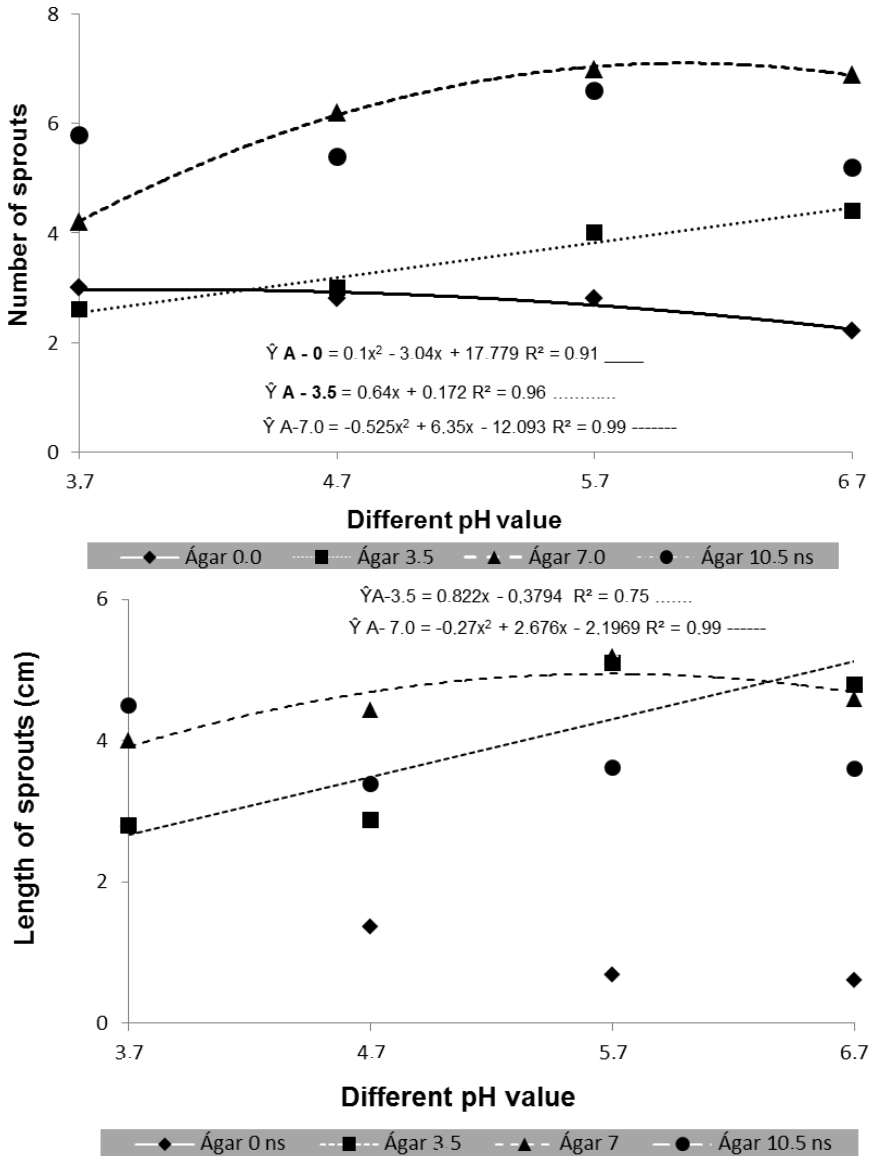

Figure 2. pH and agar effect on the number (A) and length (B) of caçari sprouts cultured *in vitro*.

concentration effect and pH levels on the *in vitro* regeneration of *Araria elata*, also observed a higher sprout growth at pH of 5.8, obtaining sprouts of 5.9 cm. However, pH above 5.8 significantly reduced the development of the sprouts *in vitro*. On the other hand, Pasqual et al. (2002) observed a greater height of the aerial part (2.3 cm) in tangelo tangerine (*Citrus reticulata.*), with pH adjusted to 4.7 and 9.3 $g.L^{-1}$ agar. Subsequently, there was a decrease in this variable as the increase of agar concentration.

Suthar et al. (2011), evaluating the influence of agar on the sprouting and sprout length of *Boswellia serrata*, observed better results in liquid culture medium and low agar concentrations; these results differ from those observed in the present study, wherein the best results were obtained in the concentration of 7 $g.L^{-1}$ agar.

Effect of different antioxidants and concentrations in the control of phenolic oxidation in stem segments of caçari

There was a significant difference in the interaction between the antioxidants and concentrations used for all analyzed variables.

Figure 3 shows the vegetative growth through the variables number (Figure 3A) and length (Figure 3B) of sprouts.

Regarding the number of sprouts, there was no significant effect of the citric acid (antioxidant), therefore,

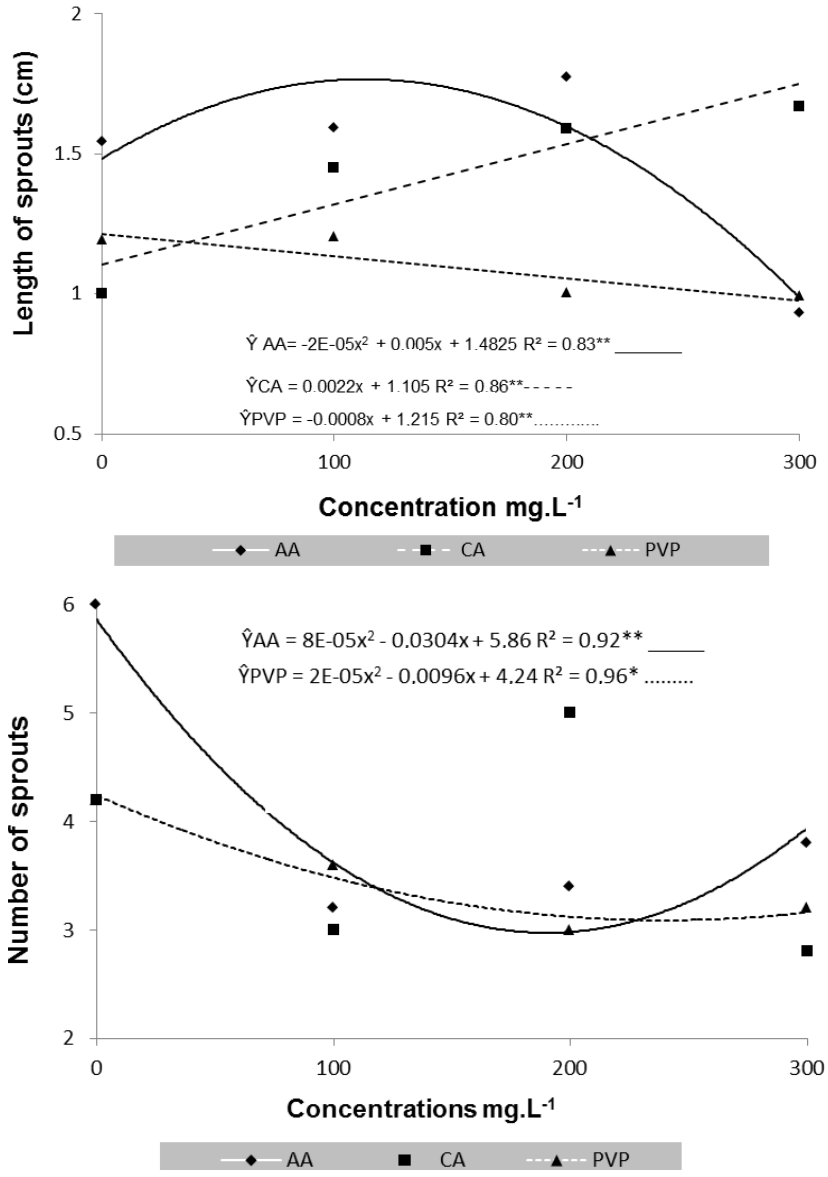

Figure 3. Effect of ascorbic acid, citric acid, and polyvinylpyrrolidone (PVP), combined with different concentrations, on the number (A) and growth of sprouts (B) of stem segments of caçari cultured *in vitro*.

we did not insert a trend line for it, exposing only the sprouting number mean. However, for the ascorbic acid and PVP, a similar behavior was found; there was a decrease in the number of sprouts as the increased of these antioxidant concentrations, in the culture medium, up to the concentration of 190 and 240 $mg.L^{-1}$, obtaining means of 2.97 and 3.08 sprouts, respectively. From these concentrations, there was a small increase in the number of new sprouts. However, the highest values (6 and 4.2 sprouts) for this variable were observed in the absence of the tested antioxidants (Figure 3A). Probably, the high antioxidant concentrations were toxic to the explants, so the number of explant sprouts was affected.

With regards to the sprout number, different results from those presented herein were found in a study on explants of paricá (*Schizolobium amazonicum*), in which the use of PVP (concentration of 0.2%) promoted a higher mean of sprouts (Cordeiro et al., 2014). On the other hand, Souza et al. (2014), testing different citric acid and ascorbic acid concentrations for the *in vitro* culture of *Schomburgkia crispa*, observed that a higher number of sprouts were obtained in the absence of antioxidants. Camolesi et al. (2007) also observed that the absence of antioxidant provided a higher number of sprouts in banana apexes (*Musa* spp.). Thus, it was found that the antioxidant effect, observed for the number

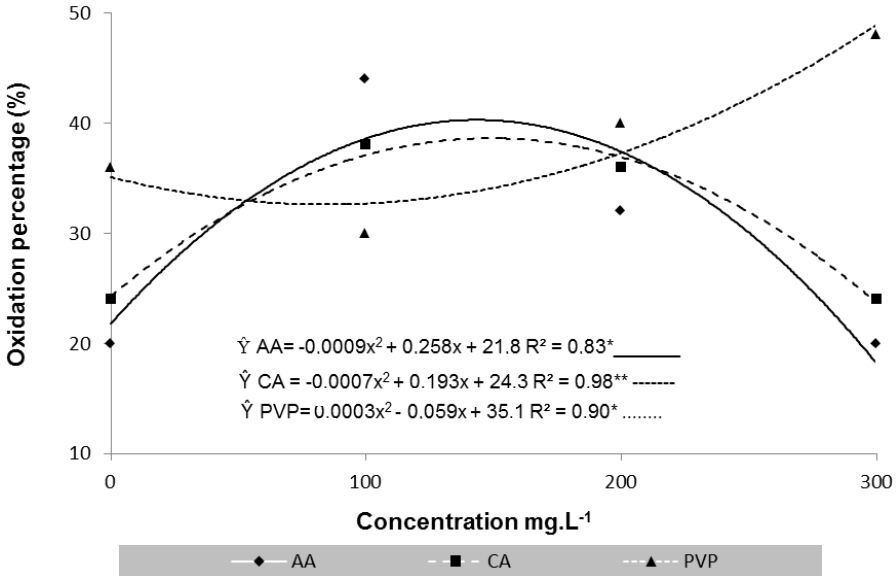

Figure 4. Effect of ascorbic acid, citric acid, polyvinylpyrrolidone, and different concentration, on the oxidation control of caçari stem segments cultured *in vitro*.

of explant sprouts of caçari cultured *in vitro* with different antioxidants and concentrations, is similar to those observed in explants of banana and *S. crispa*.

Concerning the sprout length, there were different behaviors for each antioxidant and concentrations (Figure 3B). For the ascorbic acid (AA), there was a great sprout growth (1.8 cm) up to the concentration of 125 $mg.L^{-1}$; above this concentration, there was a decrease in the sprout length. In the citric acid (CA), there was a linear sprout growth with the increase of this antioxidant concentrations, evidencing that, for this antioxidant, adjustments are needed to find an optimal concentration. For the PVP, there was an opposite behavior in comparison to the CA, with a decrease in the sprout length with the increase of the concentrations in the culture medium, and the higher sprout length was observed in the absence of an antioxidant. Probably it occurred because of the used material, given that the explants used *in vitro* are extremely small structures, which are very susceptible to dehydration and rapid oxidation since they belong to a woody and recalcitrant species. They may also exhibit different behaviors when submitted to treatment with high concentrations of antioxidants.

Camolesi et al. (2007) observed that the absence of antioxidant provided a greater growth of sprouts, in banana apexes. Similar results were reported by Souza et al. (2014), who tested different concentrations of citric acid and ascorbic acid in *S. crispa* Lindl, and found that a greater sprout length was observed in the absence of antioxidants.

For the oxidation, the use of AA antioxidant resulted in the oxidation increase, up to the concentration of 143.33 $mg.L^{-1}$, obtaining 40.2% of oxidation. For the CA antioxidant, there was a similar behavior, with an increase of the oxidation, up to the concentration of 138.1 $mg.L^{-1}$; resulting in 37.6% of oxidized explants. Whereas in the absence of these two types of antioxidants, there was a lower oxidation: 20% (AA) and 24% (CA). For the PVP, there was a decrease in the oxidation (38%), up to the concentration of 98 $mg.L^{-1}$; from this concentration, the oxidation rate tended to increase (Figure 4). The PVP was less effective to control the oxidation of caçari explants in comparison to the citric acid and ascorbic acid. This probably occurred by the fact the PVP may adsorb the phenolic compounds produced by the plant; whereas the CA and AA are reducing agents of oxidative enzymes, reducing the production of toxic substances.

Different from the results obtained in the present experiment, other studies were performed aimed to evaluate the PVP effect on the oxidation control of paricá (*Schizolobium amazonicum*) explants, in which the addition of 0.2 and 0.3% PVP, in the culture medium, controlled 100% of the oxidation, and the lowest value of PVP (0.1%) controlled only 80% of the oxidation. This effect shows the importance of antioxidants in the culture medium to control this limiting factor in micropropagation (Oliveira et al., 2011; Cordeiro et al., 2014).

In banana explants, it was observed that the pretreatment only immersing the explants in solution with antioxidant was enough to control the oxidation. However, by adding this antioxidant in the culture medium, the oxidation increased (Camolesi et al., 2007). This procedure is recommended by Anthony et al. (2004) and Grattapaglia and Machado (1998), who confirmed the results obtained for the banana. For the

Symonanthus bancroftii (Panaia et al., 2000) and *Conostephium pendulum* (Anthony et al., 2004), these authors described that the combination of 0.25 g L^{-1} citric acid with 0.75 g.L^{-1} potassium citrate, used in pretreatment and added to the culture medium, reduced the necrosis of the excised tissue, in addition to preventing the oxidation process.

Werner et al. (2009) tested some antioxidants such as ascorbic acid, citric acid, and activated carbon, in the calogenesis of *Caesalpinia echinata*. The activated carbon showed a better oxidation control, wherein 40% of the explants showed an oxidation ranging from low to moderate, but without callus formation, demonstrating that still there are factors that have to be studied, such as the addition of activated carbon to the culture medium and pretreatment of the tissues using antioxidant agents, instead of adding to the culture medium.

Conclusions

For the *in vitro* regeneration of caçari, the best results are obtained using the WPM medium at a concentration of 100% with 7 g.L^{-1} agar, and pH adjusted to 5.7. The use of antioxidants in the tested conditions did not contribute to decrease in oxidation in explants, indicating that there is no need of adding them into the culture medium.

Conflict of interests

The authors have not declared any conflict of interests.

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Abbreviations

WPM, Woody plant medium; **MS,** Murashige and Skoog medium; **AA,** ascorbic acid; **CA,** citric acid; **PVP,** polyvinylpyrrolidone, **JADS,** Juan, Antonio, Diva and Silvia medium.

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Plant growth-promotion by *Streptomyces* spp. in sorghum (*Sorghum bicolor* L.)

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Seven strains of *Streptomyces* spp.: BCA-546 (KF770898), BCA-659 (KF770889), BCA-667 (KF770888), BCA-689 (KF770899), BCA-698 (KF770900), CAI-133 (KF770895) and CAI-8 (KF770890), reported earlier to produce biocontrol and plant growth-promoting (PGP) substances were further evaluated for PGP traits in sorghum under greenhouse and field conditions. Under greenhouse conditions, plant height, leaf area and weight, root length and weight, shoot weight, panicle weight and seed weight were enhanced in plots inoculated with *Streptomyces* spp. than the un-inoculated control at 30, 60 days after sowing (DAS) and at final harvest. Similarly, treatment with *Streptomyces* spp. led to growth and yield enhancements under field conditions at 60 DAS and final harvest. Among the seven strains, BCA-698, BCA-689, BCA-546 and BCA-659 were found to be superior for PGP. Under field conditions, at both flowering and harvest stages, the soil organic C, available P and total N were also found to improve with *Streptomyces* spp. treatments. A scanning electron microscopic study showed extensive root colonization of sorghum. The gene expression profiles revealed up-regulation of β-1,3-glucanase, indole acetic acid (IAA) and siderophore genes. Based on the present findings, the seven selected *Streptomyces* strains could be employed to enhance plant growth and yield in sorghum.

Key words: Gene expression, plant growth-promotion, scanning electron microscopy, sorghum, *Streptomyces* spp.

INTRODUCTION

Sorghum (*Sorghum bicolor* L.) has been an important staple food in semi-arid tropics of Asia and Africa for centuries. It is the fifth most important cereal crop in the world. Sorghum is widely used as food, for production of alcoholic beverages, bio-fuel, starch, adhesives and paper. Lower yield in sorghum may be attributed to biotic and abiotic stresses in addition to lower yield potential of local landraces, poor agronomic practices, and low nutrient uptake and soil fertility.

Microorganisms can be beneficial to plant either by increasing the availability of both macro- and micro-elements such as nitrogen, phosphorus, iron and zinc in the rhizosphere (Cakmakci et al., 2006) or by producing plant growth-promoting (PGP) substances such as indole

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acetic acid (IAA) and siderophore (Vivas et al., 2006; Hanane et al., 2008). Soil microorganisms not only have the capability to produce compounds that are potentially promoting plant growth and yield but also inhibit phytopathogens by producing phthoxazolins, phosphinothricin and gougerotin (Murao and Hideo, 1983; Shiomi et al., 1995). Among the soil microorganisms, bacteria and fungi have received considerable attention as plant growth-promoters and biocontrol agents. For instance, plant growth-promoting *Pseudomonas chlororaphis* SRB 127, *Penicillium citrinum* VFI-51 and *Bacillus* spp. (Das et al., 2008; Haiyambo et al., 2015; Sreevidya and Gopalakrishnan, 2016) were shown to have antagonistic potential against *Macrophomina phaseolina*, a charcoal rot pathogen and other pathogens of sorghum.

Actinomycetes are important producers of bioactive compounds such as chitinase, β-1,3-glucanase and various antifungal substances (Rothrock and Gottlieb, 1984; Xiao et al., 2002; El-Tarabily and Sivasithamparam, 2006). Actinomycetes also produce extracellular active compounds such as IAA, phosphate solubilizing substances and intracellular siderophores, which induce germination of seeds and their growth (Hong et al., 2000; Zhang et al., 2000; Venkatachalam et al., 2010). Within actinomycetes, *Streptomyces* spp. have been investigated predominantly, mainly because of their dominance and the ease of isolation and their ample capacity for production of secondary metabolites, such as antibiotics and extracellular enzymes (El-Tarabily et al., 2000; Inbar et al., 2005; Carla et al., 2008; Sreevidya et al., 2015). Some of the *Streptomyces* sp. were also reported to have both PGP and antagonistic potentials against charcoal rot disease in sorghum (Ding et al., 2004; Gopalakrishnan et al., 2013a). Seven *Streptomyces* spp. (BCA-546, BCA-659, BCA-667, BCA-689, BCA-698, CAI-8 and CAI-133) were earlier reported to have PGP and biocontrol traits in chickpea (Alekhya and Gopalakrishnan, 2016). In the present investigation, the seven *Streptomyces* spp. were evaluated further for their PGP and yield enhancement potentialities in sorghum.

MATERIALS AND METHODS

PGP microbes

Seven strains of *Streptomyces* spp.: BCA-546 (KF770898), BCA-659 (KF770889), BCA-667 (KF770888), BCA-689 (KF770899), BCA-698 (KF770900), CAI-133 (KF770895) and CAI-8 (KF770890), reported earlier to have biocontrol and PGP properties in chickpea (Alekhya and Gopalakrishnan, 2016) were further studied in this investigation.

Greenhouse studies

All the seven *Streptomyces* spp. were evaluated for their PGP traits under greenhouse conditions. Soil mixture containing black soil, sand and farm yard manure (3:2:1) was prepared and filled in plastic pots (8"). A total of eight treatments (seven *Streptomyces* spp. and a control; without any inoculum) each with three replications were maintained. Sorghum seeds (SPV1411; maturing in 125 to 128 days) were surface-sterilized with 2.5% chlorox for 5 min, rinsed 8-10 times with sterilized water and incubated with *Streptomyces* treatment (10^7 cfu ml^{-1}; grown in starch casein broth-SCB) for 1 h before sowing. In each pot, three seeds were sown and thinned to one after germination. At 15, 30 and 45 days after germination (DAS), a booster dose of *Streptomyces* spp. (5 ml per pot, 10^7 cfu ml^{-1}) was applied on the soil together with watering. At 30 DAS, PGP parameters including the plant height, leaf area, leaf weight, shoot weight and root weight and length; and at 60 DAS, the plant height, leaf area, leaf weight, shoot weight and root weight were recorded. At final harvest, the panicle weight, seed weight, shoot weight and root weight were recorded.

Field studies

Field trials were performed in 2012 Rabi (post-rainy) season at ICRISAT, Patancheru (17°30.861'N; 78°16.080'E; altitude = 540 m) in the Telangana State of India. The experimental field soil is characterised as 51% clay, 22% silt and 26% sand with an organic carbon content of 0.4−0.5% and an alkaline pH of 7.5−8.1. Plots were composed of 4 × 3 m ridges arranged in a randomized complete block design (RCBD) with three replications. The seven selected strains of *Streptomyces* (BCA-546, BCA-659, BCA-667, BCA-689, BCA-698, CAI-133 and CAI-8) were grown in SCB for five days, soaked with sorghum seeds (SPV1411) just before sowing for 1 h and sown by hand at 5 cm depth. A booster dose of *Streptomyces* spp. (10^8 cfu ml^{-1}) was applied to soil at an interval of 15 DAS until flowering stage. The control plots contained no *Streptomyces* spp. Weeding was performed as and when required. No incidence of insect-pest or phytopathogens attack was observed during the cropping period. At 60 DAS, plant growth-parameters including the plant height, leaf area, root weight, shoot weight and leaf weight were recorded. During the final harvest, the growth and yield parameters including the plant height, panicle length, 1000 seed weight, grain yield and stover yield were recorded. Soil samples (from the 0 to 15 cm soil profile) were collected at flowering (60 DAS) and harvesting stages and analysed for organic carbon %, available P and total N using the standardized protocols described by Nelson and Sommers (1982), Olsen and Sommers (1982) and Novozamsky et al. (1983), respectively.

Colonization studies

Sorghum root colonization by *Streptomyces* spp. was studied by scanning electron microscopy (SEM) as per the protocols of Gopalakrishnan et al. (2015a). In brief, the seeds of sorghum (SPV1411) were surface-sterilized with 2.5% chlorox for 5 min followed by 70% ethanol in water for 5 min and rinsed with sterilized water (several times). The sterilized seeds were allowed to germinate on a Petri dish containing blotter paper for two days under dark conditions. The germinated seeds were treated with *Streptomyces* spp. (BCA-546, BCA-659, BCA-667, BCA-689, BCA-698, CAI-8 and CAI-133; 10^7 cfu ml^{-1}) for 1 h and sown in the pots containing sterilized coarse sand and incubated in a greenhouse for 15 days. At the end of the incubation, the root tips of the plants were fixed in 2.5% glutaraldehyde, 0.1 M phosphate buffer (pH 7.2) for 24 h at 4°C and post fixed in 2% aqueous osmium tetroxide for 4 h. The processed samples were mounted and coated with a thin layer of gold using an automated sputter coater (Model - JEOL JFC-1600) for 3 min and further scanned under SEM (Model: JOEL-JSM 5600) at RUSKA Lab, Rajendranagar, Hyderabad, Telangana, India.

Table 1. Effect of the seven *Streptomyces* spp. on the morphological observations of sorghum under greenhouse conditions at 30 days after sowing.

Strains	Plant height (cm)	Leaf area (m^{-2} cm)	Root length (m $plant^{-1}$)	Root weight (g $plant^{-1}$)	Shoot weight (g $plant^{-1}$)	Leaf weight (g $plant^{-1}$)
BCA-546	85.0	589	54.2	0.57	1.08	2.35
BCA-659	82.7	462	43.2	0.46	1.60	1.81
BCA-667	82.0	479	46.8	0.50	1.53	2.00
BCA-689	84.3	569	52.5	0.70	1.58	2.12
BCA-698	77.7	473	45.0	0.48	1.75	1.73
CAI-8	90.0	569	45.0	0.46	1.52	1.76
CAI-133	78.7	481	49.2	0.46	1.65	1.70
Control	77.3	454	42.3	0.45	1.49	1.68
LSD (5%)	6.12	76.5	7.21	0.057	0.152	0.413
CV%	4	9	9	6	5	12

The presented data are the averages of three replications; LSD= least significant difference; CV= coefficient of variation.

Gene expression studies

All the seven *Streptomyces* spp. were grown in SCB broth and incubated at 28 ±2°C for five days. At the end of the incubation, the cultures were centrifuged at 10000 g, cell pellet was collected (500 mg) and RNA extracted using conventional Trizol method (Chomczynski and Mackey, 1995). The purity of extracted RNA was checked on agarose gel electrophoresis while the quality and quantity of RNA was estimated by Nanodrop (Thermo Scientific, Wilmington, USA) and RNA integrity by 2100 Bioanalyzer (Agilent, Redwood City, CA, USA). The RNA was diluted to 200 ng and cDNA was constructed. The quality and quantity of the cDNA was checked using Nanodrop and the concentrations were adjusted accordingly. Quantitative real-time polymerized chain reaction (qRT-PCR) was performed as per the manufacturer's instructions using Applied Biosystems 7500 Real Time PCR System with the SYBR green chemistry (Applied Biosystems, Foster City, CA, USA). IAA, siderophore and β-1,3-glucanase gene-specific primers for qRT-PCR were designed using primer 3 software (Rosen and Skaletsky, 2000). Genes relating to IAA (F: GTCACCGGGATCTTCTTCAAC; R: GATGTCGGTGTTCTTGTCCAG), siderophore (F: ATCCTCAACACCCTGGTCTG; R: TCCTTGTACTGGTACGGGACTT) and β-1,3-glucanase (F: CCGAACACCACCTACTCCAC; R: CCAGGTTGAGGATCAGGAAG) production were collected from UniprotKB database (http://www.uniprot.org/uniprot) as described in Gopalakrishnan et al. (2015a). PCR reactions and data analysis were done as described by Gopalakrishnan et al. (2015a).

Statistical analysis

Data were analysed by using Analysis of Variance (ANOVA) technique (Genstat 10.1version) to evaluate the different treatments and mean separations were done with LSD at significant levels of 1 and 5%.

RESULTS

Greenhouse studies

When the seven *Streptomyces* strains were evaluated for their PGP traits under greenhouse conditions, considerable enhancement in the growth and yield parameters were observed. At 30 DAS, all the strains resulted in enhanced plant height (up to 14%), leaf area (up to 23%), root length (up to 22%), root weight (up to 36%), shoot weight (up to 17%) and leaf weight (up to 28%) than the un-inoculated control (Table 1). Similarly, treatments with *Streptomyces* led to growth enhancements than the un-inoculated control at 60 DAS, although the rate of increase was relatively lower. Among the seven tested strains of *Streptomyces*, BCA-546 and BCA-689 significantly enhanced most of the PGP traits including plant height, leaf area, leaf weight, root length, root weight, shoot weight, panicle weight and seed weight (Table 2).

Field studies

When the PGP potentials of the seven *Streptomyces* strains were evaluated under field conditions, considerable enhancement in growth and yield parameters were observed in sorghum. At 60 DAS, the *Streptomyces* strains showed increased leaf area (up to 18%), leaf weight (up to 17%), stem weight (up to 11%) and root weight (up to 29%) while at final harvest, panicle length (up to 19%), 1000 seed weight (up to 7%), grain yield (up to 17%) and stover yield (up to 20%) than the un-inoculated control (Table 3). The soil mineral parameters including soil organic C (up to 12%), available P (up to 6%) and total N (up to 12%) were also found to be enhanced at both flowering and final harvest stages than the un-inoculated control (Table 4). Among the tested strains, three strains (BCA-546, BCA-659 and BCA-689) were found to consistently and significantly enhance growth parameters, grain and stover yields.

Colonization studies

All the seven strains of *Streptomyces* showed extensive

Table 2. Effect of the seven *Streptomyces* spp. on the morphological and yield observations of sorghum under greenhouse conditions at 60 days after sowing and final harvest

Strains	60 days after sowing					At final harvest			
	Plant height (cm)	Leaf area (m^{-2} cm)	Root weight (g $plant^{-1}$)	Shoot weight (g $plant^{-1}$)	Leaf weight (g $plant^{-1}$)	Panicle weight (g $plant^{-1}$)	Seed weight (g $plant^{-1}$)	Shoot weight (g $plant^{-1}$)	Root weight (g $plant^{-1}$)
BCA-546	146.0	2856	5.00	33.20	25.59	50.25	45.88	48.66	13.59
BCA-659	150.3	2781	4.85	33.06	25.68	50.98	46.16	47.88	14.88
BCA-667	148.3	2851	4.78	29.66	25.10	53.58	46.55	47.88	12.90
BCA-689	138.3	2712	5.53	29.96	25.39	57.65	50.11	49.27	14.84
BCA-698	149.3	2726	4.84	29.46	25.12	50.04	46.86	48.89	14.92
CAI-8	143.0	2671	4.88	29.06	25.17	49.87	45.45	47.89	12.00
CAI-133	144.3	2693	4.80	29.08	25.00	50.04	46.21	47.75	12.00
Control	133.7	2652	4.76	28.81	24.31	49.70	44.80	47.69	11.76
LSD (5%)	8.75	124.1	0.230	1.547	0.551	1.954	2.59	0.784	2.121
CV%	4	3	3	3	1	2	3	1	9

The presented data are the averages of three replications; LSD = least significant difference; CV = coefficient of variation.

Table 3. Effect of the seven *Streptomyces* spp. on the morphological and yield observations of sorghum under field conditions at 60 days after sowing and final harvest.

Strains	60 days after sowing					At final harvest				
	Plant height (m)	Leaf area (m^{-2} cm)	Root weigh t (g $plant^{-1}$)	Shoot weight (g $plant^{-1}$)	Leaf (g weight $plant^{-1}$)	Plant height (m)	Panicle length (cm)	1000 seed weight (g)	Grain yield (t ha^{-1})	Stover yield (t ha^{-1})
BCA-546	1.96	2759	7.79	26.46	14.17	2.17	16.1	39.6	3.81	11.96
BCA-659	1.92	3331	10.91	25.08	16.55	2.08	17.2	40.3	4.11	13.75
BCA-667	1.95	2930	7.88	23.98	14.67	2.13	16.4	40.8	3.43	11.95
BCA-689	1.93	2936	7.80	26.04	16.45	2.16	15.6	40.6	3.52	11.20
BCA-698	1.91	2972	7.79	25.87	16.21	2.16	16.1	39.6	4.00	13.85
CAI-8	1.86	2798	7.82	24.34	15.20	2.29	16.7	40.4	3.74	12.50
CAI-133	1.89	2839	7.78	23.72	14.45	2.13	14.4	40.7	3.50	11.18
Control	1.88	2718	7.78	23.49	13.81	2.07	13.9	38.1	3.41	11.13
LSD (5%)	0.036	192.3	1.073	1.715	1.556	0.047	1.16	0.55	0.300	0.355
CV%	1	4	8	4	6	1	4	1	5	2

The presented data are the averages of three replications; LSD = least significant difference; CV = coefficient of variation.

colonization on the roots of sorghum. However, the extent of colonization was found to be most pronounced with BCA-546. Extensive mycelial growth penetrating the outer layer of the root was noticed and also sporulation was observed in all the isolates, as compared to the un-inoculated

Table 4. Effect of seven *Streptomyces* spp. on the soil mineral properties of sorghum grown under field conditions at flowering and final harvest.

Strains	At flowering stage			At harvest stage		
	Total N (ppm)	Available P (ppm)	OC (%)	Total N (ppm)	Available P (ppm)	OC (%)
BCA-546	666	6.0	0.50	583	6.3	0.46
BCA-659	679	5.8	0.51	585	7.1	0.45
BCA-667	610	5.9	0.50	552	6.0	0.46
BCA-689	614	5.9	0.51	575	5.9	0.46
BCA-698	616	6.5	0.50	584	9.7	0.46
CAI-8	615	6.3	0.55	570	6.0	0.44
CAI-133	643	8.1	0.50	551	5.9	0.46
Control	605	5.8	0.49	535	5.9	0.44
LSD (5%)	39.9	0.37	0.022	21.6	0.41	0.009
CV%	3	3	2	2	3	1

The presented data are the averages of three replications; N = nitrogen; P = phosphorus; OC = organic carbon; LSD = least significant difference; CV = coefficient of variation.

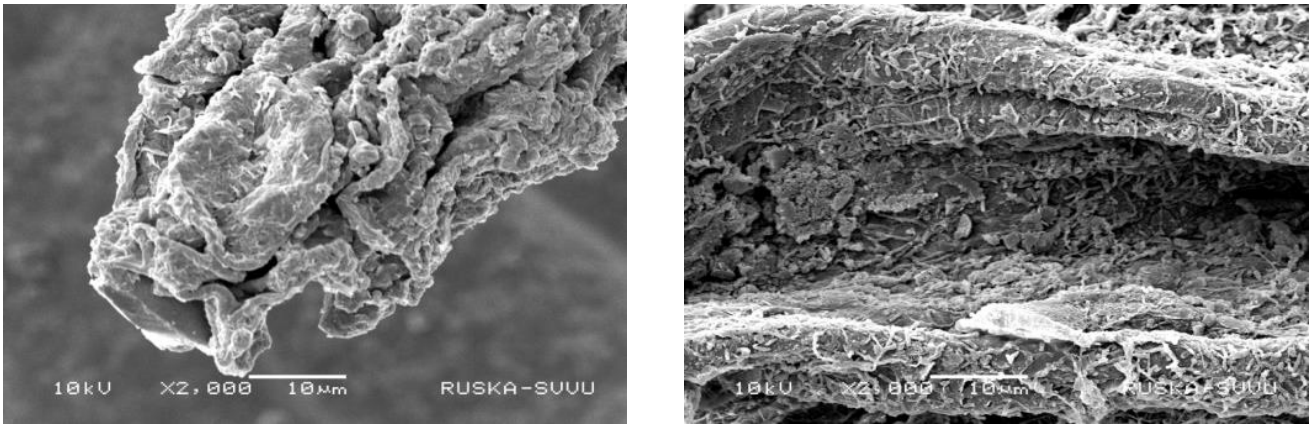

Figure 1. SEM photograph of BCA-546 strain showing extensive colonization on the roots of sorghum.

control (Figure 1).

Gene expression studies

The gene expression profiles of β-1,3-glucanase, IAA and siderophore genes for all the strains (except CAI-133) showed up-regulation. Among the seven strains, β-1,3-glucanase was up-regulated up to 10 fold, IAA by 11 fold and siderophore by 15 fold for CAI-8, BCA-689 and CAI-8, respectively (Figure 2).

DISCUSSION

The major reasons for the lower yield in sorghum includes fungal pathogens and unavailability of essential nutrients and iron to the plants (Davis and Bockus, 2001; Igual et al., 2001). PGP microbes including actinomycetes can play a vital role in enhancing the yields of sorghum. Most of the actinomycetes in soil belong to the genus *Streptomyces* and are reported to have potentials for PGP and biocontrol in many crops. It is reported that 60% of the biologically active compounds in agriculture such as antifungal, antibacterial and PGP substances are produced by *Streptomyces* spp. (Suzuki et al., 2000; Ilic et al., 2007; Khamna et al., 2010). In the present study, seven strains of *Streptomyces* having potential to produce PGP and biocontrol traits such as IAA, siderophores, lipase, cellulase, protease, β-1,3-glucanase, chitinase and hydrocyanic acid (Alekhya and Gopalakrishnan, 2016) were further studied for their PGP

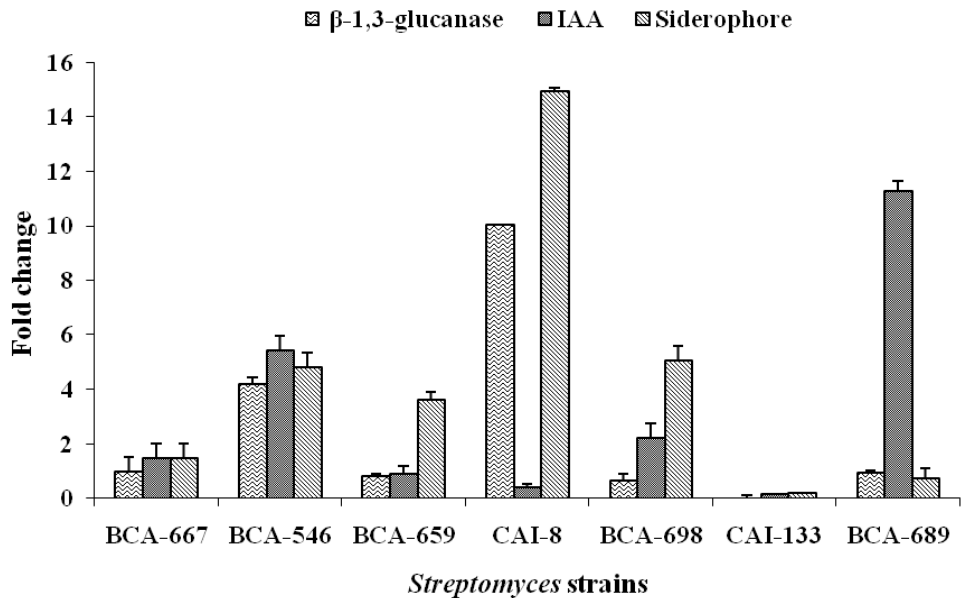

Figure 2. Gene expression profiling of PGP genes of the seven *Streptomyces* strains.

traits in sorghum under greenhouse and field conditions. The results showed that all the *Streptomyces* strains enhanced the growth and yield parameters under both greenhouse and field conditions than the un-inoculated control plots when applied as seed coatings and soil inoculations. Among the seven *Streptomyces* strains studied, BCA-689, BCA-698, BCA-546, BCA-659 were found to be the best sorghum growth and yield promoting strains. They were also found to be the best strain which enhanced the soil mineral parameters including total N, available P and organic C as compared to the other *Streptomyces* strains. Hence, these *Streptomyces* strains may be promoted as inoculants for growth and yield enhancement in sorghum.

In the present study, under greenhouse, all the *Streptomyces* strains consistently enhanced root length and weights of sorghum (Table 1). The enhanced root length and mass will help the sorghum plants to absorb moisture and nutrients from the deeper zone of soil. This could be one of the reason why the yield and shoot and root biomass were found more in *Streptomyces* treated plots as compared to the control plots. The production of growth-promoting substances by PGP strains causes modifications in the morphology of roots, influencing nutrient and water absorption, and consequently promoting plant growth (Bashan and Holgium, 1997; Carla et al., 2008). Colonization observed in the present study adds further evidence to the effect of PGP microbes on root modifications. In the authors' previous study, these seven *Streptomyces* strains were also reported to be capable of producing several direct PGP traits including IAA and siderophore and indirect PGP traits including β-1,3-glucanase, chitinase and hydrocyanic acid (Alekhya and Gopalakrishnan, 2016). Hence, these direct and indirect PGP traits of these strains could also be one of the reasons for the yield as well as shoot and root biomass enhancement of sorghum. PGP microorganisms enhance the plant growth directly by synthesis of phytohormones (Xie and Pasternak, 1996) or indirectly by preventing deleterious effect of pathogenic microorganisms, mostly due to the synthesis of antibiotics (Sivan and Chet, 1992). Actinomycetes are reported to promote plant growth by producing IAA which enhance the root growth or produce siderophores which enhance the nutrient uptake (Khamna et al., 2009). Actinomycetes including *Streptomyces* were previously reported for the control of plant fungal diseases and also enhance plant growth in cucumber, guava and tomato (El-Tarabily and Sivasithamparam, 2006; El-Tarabily et al., 2010; Shimizu, 2011; Mohandas et al., 2013; Sreeja and Surendra, 2013; Talwinder et al., 2013). PGP was also reported in sorghum using *Streptomyces* spp. (Alekhya and Gopalakrishnan, 2014; Gopalakrishnan et al., 2013a) and bacteria using *Pseudomonas fluorescens* and *Bacillus subtilis* under greenhouse conditions (Prathibha and Siddalingeshwara, 2013). In addition to their ability to inhibit plant pathogens, some actinomycetes are also known to form close associations with plants, colonize their internal tissues without causing disease symptoms, and promote their growth (Kunoh, 2002). The use of *Streptomyces* spp. for PGP in sorghum at field level has not been reported before, which makes the present study a novel approach for PGP in sorghum.

It is accepted that microorganisms effective as biocontrol and PGP agents must have good rhizosphere

competence, that is, have ability to colonize root of the host plant (Buell et al., 1991; Chiarini et al., 1998). In the present study, based on the SEM analysis, it was found that all the strains colonized the roots of sorghum. Beneficial actinomycetes were reported to colonize many host plants (Cao et al., 2005; Shi et al., 2009; Ruanpanun et al., 2010). *Streptomyces* spp. has been previously described as rhizosphere-colonizing bacteria (Miller et al., 1990a, b; Tokala et al., 2002). Hence, it is concluded that the selected strains of *Streptomyces* exhibited extensive colonization which correlates with their PGP properties.

In the present study, when the seven strains were evaluated for their gene expression profile, all strains (except CAI-133) up regulated β-1,3-glucanase, IAA and siderophore genes. The reason for selecting only β-1,3-glucanase, IAA and siderophore traits for expression profiles is that these three traits are directly linked to growth promotion of the plants. Similar results were also reported by Gopalakrishnan et al. (2013b, 2015a, b) which support the PGP by *Streptomyces* strains.

Conclusion

In the present study, the seven selected *Streptomyces* spp. were found to enhance the growth of sorghum under both greenhouse as well as field conditions. These isolates were also found to have strong colonizing capability for the root surface of the sorghum plant and expressed PGP genes. Hence these isolates can be best employed for the PGP in sorghum. Further, the PGP and biocontrol potentials of the seven strains can be evaluated in other crops.

Conflict of Interests

All the authors declare that they have no financial/commercial conflicts of interest.

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Nutritional and antimicrobial evaluation of *Saccharum officinarum* consumed in Calabar, Nigeria

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Sugarcane (*Saccharum officinarum*) stem pulp is widely consumed in Nigeria as a snack mainly for sugar content and is believed to possess some remedy against infectious diseases. This study therefore quantitatively evaluated the nutritional and chemical composition of the sugarcane stem pulp with a view to validating this claim. The results of proximate composition indicate relatively high amount of moisture (71.91 ± 0.05%) and a low level of dry matter (28.09%) consisting of carbohydrate (58.55 ± 0.04 g/100 g), fibre (29.88 g/100 g), ash (6.69 g/100 g) and some mineral elements, implying an active role in nutrient supplementation. Generous amounts of phytochemical compounds such as alkaloids (8.07 ± 0.04 µg/100 g), saponins (5.57 ± 0.01 µg/100 g) and flavonoids (1.52 ± 0.02 µg/100 g); and mineral elements including magnesium (1.596 mg/100 g), potassium (0.639 mg/100 g), calcium (0.318 mg/100 g) and others in trace amounts were also obtained. .Antimicrobial results revealed that the sugarcane extract showed the highest growth inhibition against *Staphylococcus aureus* (8.67 - 24.00 mm) among the bacterial isolates and *C. albicans* (6.00 – 14.00 mm) for the fungal isolates studied. Data from the study suggest that sugarcane stem pulp could be suitable for use in rehydration and as a functional food plant. Also, the plant possesses some antimicrobial qualities which could be beneficial to both pharmaceutical and food industries.

Key words: *Saccharum officinarum*, sugarcane, proximate analysis, mineral, phytochemical, antimicrobial.

INTRODUCTION

Diet plays a key role in disease prevention and therapy. Plant foods make up a larger percentage of foods consumed in developing nations including Nigeria because animal foods are relatively expensive hence not easily affordable. It is believed that nutrients in plant foods do more than just prevent deficiency diseases like beriberi or rickets with the most publicized findings indicating notable chemicals like vitamin C, beta-carotene, and polyphenols as powerful antioxidants (Palmer, 2005; Martinez and Martinez, 2007; Mates et al., 2011), which help to prevent molecular damage caused by oxidation. This protection is known to fend off many diseases including cancer, cardiovascular diseases and muscular degeneration (Islam et al., 2002; Mates et al., 2011). This

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has resulted in an increased demand for foods that provide additional health benefits, popularly known as functional foods (Kim et al., 2009).

Sugarcane (*Saccharum* sp.) is one such functional foods, described as one of the world's most efficient living collectors of solar energy, stored in the form of fibre and fermentable sugars (FAO, 1988). It is a giant, thick, perennial grass belonging to the family Poaceae, and constitutes the main crop cultivated in Brazil, India, China, Thailand, Mexico and Pakistan, where it plays a vital role in the economy and provides employment opportunities (OECD, 2011; Chandel et al., 2012). There are six confirmed species of *Saccharum*, two of which are wild while four are cultivated (Bakker, 1999). All modern cultivated varieties of sugarcane are hybrids derived from breeding between these local species (OECD, 2011), and supply about 75% of the world edible sugar (Dillon et al., 2007). Sugarcane and its hybrids are grown for the production of sugar, ethanol and other industrial uses. For instance, Brazil the world leading producer of sugarcane is also the largest exporter of sugar and the second largest exporter of ethanol after the United States (Antunes et al., 2014; FAOSTAT, 2014; Licht, 2015).

Nigeria is among the 106 countries of the world categorized as minor sugarcane producers (Tiamiyu et al., 2013). From the coastal region where it was first introduced by European sailors in the 15th century, the crop has spread to other parts of the country (Busari, 2004). However, much of the sugarcane production in Nigeria is in the northern region (Tiamiyu et al., 2013; Sulaiman et al., 2015). Currently, Nigeria is the second largest producer of sugarcane in West Africa after Ivory Coast and the 19th in Africa (FAOSTAT, 2012; Sulaiman et al., 2015). However, the country depends largely on raw sugar import to meet domestic requirements (Gourichon, 2013; Sulaiman et al., 2015). According to GAIN (2014) report, Nigeria is Africa's largest sugar importer. The yearly average share of imports of raw sugar in the country's domestic supply is about 96% (Gourichon, 2013). Brazil is the largest raw sugar supplier to Nigeria (GAIN, 2015). In 2013/2014, Brazil harvested about 652 million metric tons of sugarcane while Nigeria's domestic sugar production in the same year was 70,000 tons (GAIN, 2014).

In Nigeria, particularly in the northern part, *Saccharum officinarum,* popularly known as sugarcane is consumed as a snack, by chewing the stem pulp to extract its juice, while the bagasse is thrown away. It is probable that sugarcane may also supply some other vital nutrients alongside its renowned sugar content, since it is eaten in its natural form. Hence, the suggestion that the sugarcane intended for human consumption should be analysed for proximate constituents (OECD, 2011).

Also, it is believed that in the process of chewing sugarcane to extract the juice from the pulp, large amounts of bioactive compounds may not be fully utilized (Deng et al., 2012). According to research reports, these compounds are proven to have important biological and medicinal properties that may make sugarcane a valuable functional food plant (Iacopini et al., 2008). Additionally, the use of *S. officinarum* in traditional medicine in Nigeria and some parts of Asia especially India for the treatment of diseases such as jaundice and liver-related disorders, dyspepsia, haemorrhoids, menorrhagia, dysentery, agalactia, phthisis and general debility (Kadam et al., 2008; Suresh-Kumar et al., 2010), suggest inherent medicinal phytochemicals. Yet scientific information on the chemical composition in relation to its medicinal properties is scanty and largely uncollated. Moreover, edaphic and climatic factors in general are known to influence the chemical and nutrient composition of plants, underscoring the imperative to do a nutrient and chemical screening of the sugarcane consumed in Calabar, Cross River State, Nigeria.

Therefore, the study evaluated the nutritional and phytochemical composition of *S. officinarum* as well as its antimicrobial properties. Data generated from such studies as this, will contribute to the nutrient composition database useful in the assessment of dietary intake of individuals/ population groups, a major prerequisite for solving the problem of malnutrition in developing countries including Nigeria. The study will also serve to provide some baseline data necessary for further investigation into the functional properties of sugarcane.

MATERIALS AND METHODS

Sample collection

Sugarcane (*S. officinarum*) stems were purchased from a vendor in Watt Market in Calabar, who brought them from Kano in Northern Nigeria, and transported to the Department of Botany, University of Calabar where they were properly identified. Thereafter, the stems were taken to the research laboratory in the Department of Biochemistry, where they were washed thoroughly and allowed to drain.

Sample preparation

The outer woody layer of each sugarcane stem was peeled off, while the inner sugary pulp was cut into small cubes using a kitchen knife and further ground to a fine consistency using a homogenizer. From this, 700 g portion was weighed out and used for the study.

Proximate analysis

The moisture, fat, crude protein, ash and fibre contents of the sample were determined by the methods of the Association of Official Analytical Chemists (AOAC, 2005). In brief, moisture content was determined by drying a 20 g portion of the raw homogenized pulp to a constant weight, using a vacuum oven (Astell- Hearson) at 100°C for about 5 h. The moisture content was taken as the difference in weight between the raw and the constantly dried sample. Fat content was determined by exhaustively extracting 20 g of the sample with petroleum ether (B.P. 40 to 60^{o}C) using a Soxhlet apparatus (Corning, England). The crude protein content was determined by the micro-Kjeldahl

digestion apparatus. The method estimated the amount of nitrogen in the sample which was subsequently used to calculate the protein content by multiplying with the factor of 6.25. The crude fibre content was estimated by boiling 20 g of the sample in 1.25% (w/v) sulphuric acid and afterwards with 1.25% (w/v) sodium hydroxide. The residue was then incinerated completely at 550°C. The loss in weight represented the crude fibre content of the sample. Total ash was determined from the residue left after incinerating a 20 g portion of the sample in a muffle furnace at 550°C, whereas carbohydrate content was obtained by difference, that is, by subtracting the protein, fat, ash and moisture contents from the total dry matter and expressed in percentage.

Estimation of mineral elements

The mineral elements were determined using a sample digest prepared by digesting completely 5 g of the sample in perchloric and concentrated nitric acids diluted with deionized water in a 50 ml volumetric flask. Sodium (Na), calcium (Ca), magnesium (Mg), potassium (K), phosphorus (P), iron (Fe), copper (Cu) and zinc (Zn) in the digest were measured using the Perkin Elmer Atomic absorption spectrophotometer (Model 306, UK) (AOAC, 1990).

Phytochemical evaluations

The qualitative phytochemical tests were carried out to identify the various constituents using standard procedures described earlier by Harbone (1998), Trease and Evans (2002), and Sofowara (2008). Some of the phytochemical compounds were also quantified using known procedures. Flavonoids, saponins and tannins were determined by the methods of Trease and Evans (1996). The cyanogenic glycosides were assayed by the alkaline picrate calorimeter method (Balagopalan et al., 1988), whereas total alkaloids were determined by the alkaline precipitation gravimetric method (Harbone, 1998).

Antimicrobial studies

Solvent extraction

One hundred (100) g of the sample was extracted with methanol solvent in Soxhlet extractor for 48 h. The solvent extract was concentrated by evaporating to dryness using rotary evaporator. The concentrate obtained was preserved in the refrigerator at - 4°C until further use.

Reconstitution of extract

The stored extract was reconstituted using methanol to obtain a stock solution which was further diluted serially to obtain concentrations of 100, 50, 25, 12.5, 6.25 and 3.13 µg/ml prior to determination of its antimicrobial activity.

Collection and maintenance of test microorganisms

Six clinical microbial isolates namely *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, Aspergillus fumigatus* and *Candida albicans* collected from the Department of Medical Microbiology, University of Calabar Teaching Hospital were used. The bacteria were maintained on nutrient broth at 37°C, while the fungi were maintained on potato dextrose agar at 28°C.

Determination of antimicrobial activity of the extract

The susceptibility of the test microorganisms to sugarcane extract was determined using the Kirby-Bauer Disk Diffusion method; Ampicillin (10 µg/ml) and Amphotericin B (10 µg/ml) were respectively used as antibacterial and antifungal positive control, while methanol served as negative control. Twenty-four petri-plates previously sterilized in a hot-air oven at 100°C for I h were first labeled accordingly, with the first six set of plates carrying labels of the various concentrations of the sugarcane extract, while the next three set of six plates each carried labels of Ampicillin, Amphotericin B, and methanol control respectively. The labels were placed at specific locations around the petri-plates and their respective clinical isolates were also indicated at the back of the plates. That is, plate 1 for instance carried 100, 50, 25, 12.5, 6.25 and 3.13 µg/ml concentration labels placed at specific locations around the plate. The labeling followed a clock-wise direction. Thereafter, 1 ml each of the clinical isolates was placed in the respective plates and later, 20 ml of Mueller-Hinton agar held at 45°C was added to each plate, which was then swirled to allow for thorough mixing. The plates were kept for 30 min for the agar to solidify.

Afterward, filter paper discs impregnated with various concentrations of the crude extract, Ampicillin, Amphotericin B and methanol control were dried at 60°C for 10 min and the dried discs were later transferred to their petri-plates according to their specific concentrations using sterilized forceps. The plates were then incubated at 37°C for 24 h for the bacteria and at 28°C for 48 h for fungi. All tests were performed in triplicates.

After the incubation period, the sensitivity of the clinical isolates to the various extract concentrations was determined by measuring their zone diameters in millimeters (mm) using a transparent ruler. Antimicrobial activity was expressed as the mean diameter of the clear zone. Extracts producing zones of bacterial growth inhibition 13 to 17 mm and fungal growth inhibition 12 to 15 mm were considered effective (Espinel-Ingroff et al., 2007; CLSI, 2013).

Statistical analysis

Data obtained was expressed as the mean ± SD for the measured variables and further subjected to appropriate statistical analysis such as correlation and one-way analysis of variance (ANOVA) in SPSS statistical package. Statistical significance was accepted at 5% probability level or less.

RESULTS

Proximate composition

The results of the proximate composition of *S. officinarum* pulp presented in Figure 1 indicates that over two-third (71.91%) of the edible portion of the sugarcane pulp is water, while the dry matter makes up less than one-third (28.09%). Carbohydrate represents the largest constituent (58.55 g/100 g) of the dry matter. The results of the evaluation also reveal that *S. officinarum* contains relatively high amount of crude fibre (29.88 g/100 g) and ash (6.69 g/100 g), but low fat (1.68 g/100 g) and crude protein (3.20 g/100 g) contents.

Elemental composition

Figure 2 shows the levels of mineral elements in 100 g

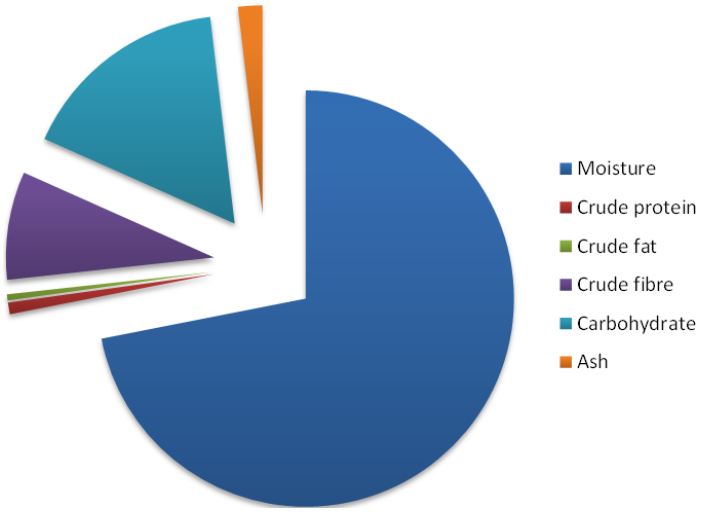

Figure 1. Proximate composition of *S. officinarum*.

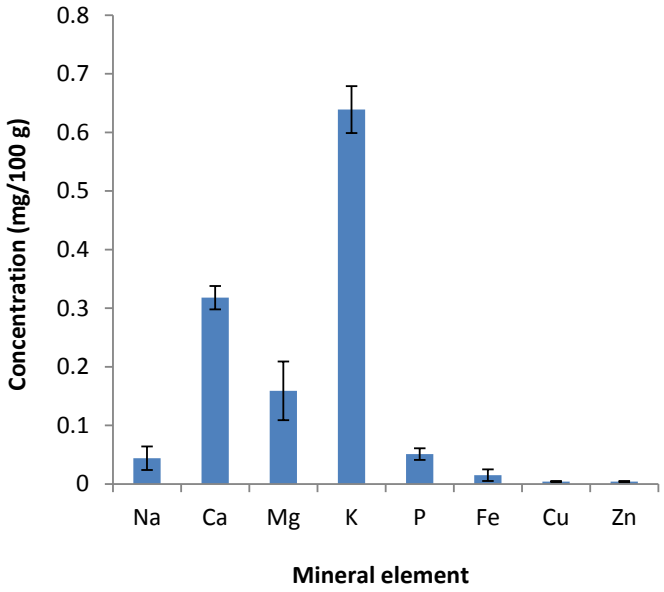

Figure 2. Mineral content of *S. officinarum.*

edible portion of the *S. officinarum* stalk. Potassium had the highest concentration (0.639 ± 0.04 mg/100 g), which was about twice that of calcium (0.318 ± 0.02 mg/ 100 g). Of the trace elements, iron had the highest concentration (0.015 ± 0.01 mg/ 100 g), while copper and zinc had the least concentration (0.004 ± 0.01 mg/ 100 g each) in the sugarcane stalk sample.

Phytochemical constituents

The results of the qualitative and quantitative assay of phytochemicals in the *S. officinarum* sample are presented in Table 1. There was a high correlation (r = 0.92) between the two methods of analysis. Alkaloids were the predominant (8.07 ± 0.04 µg/100 g) phytochemicals, followed by saponins (5.57 ± 0.01 µg/100 g). Flavonoids and cyanogenic glycosides were almost equal in concentration (1.52 ± 0.02 and 1.20 ± 0.01 µg/100 g, respectively), while tannins showed the least concentration (0.25 ± 0.01 µg/100 g) in the sample.

Antimicrobial activity

Table 2 presents the results of the antimicrobial activity of

Table 1. Concentration of phytochemicals in 100 g of *S. officinarum* stem.

Constituents	Qualitative	Quantitative (µg)
Flavonoids	++	1.52 ± 0.02
Saponins	+++	5.57 ± 0.01
Tannins	+	0.25 ± 0.01
Alkaloids	++++	8.07 ± 0.04
Cardiac glycosides	–	ND
Steroids	–	ND
Hydrogen cyanide	++	1.20 ± 0.01
Polyphenols	–	ND

Values are presented as mean of 3 determinations ± SD; ND = not detected; + = present in trace amount; ++ = present in low conc.; +++ = present in medium conc.; present in high conc. r = 0.92; p < 0.05.

Table 2. Antimicrobial activity of methanol extract of *S. officinarum* stem pulp.

Test organism	100	50	25	12.5	6.25	3.12	Methanol control	**Positive control
E. coli	15.33±0.67*	14.00±0.58*	13.33±0.33*	11.00±0.58*	10.33±0.33*	8.67±0.33*	N	19.33±0.88
K. pneumoniae	23.00±0.58	21.00±0.58	20.67±0.33	20.33±0.33	14.33±0.88*	12.33±0.33*	N	19.33±1.45
S. aureus	24.00±0.58*	18.33±0.33*	12.33±0.33	12.00±0.58	10.00±0.58	8.67±0.88*	N	14.33±0.88
P. aeruginosa	14.33±0.33	14.67±0.33	9.33±0.33*	8.33±0.33*	8.00±0.58*	6.00±0.33*	N	14.38±0.87
A. fumigatus	12.00±0.58*	11.33±0.88*	10.33±0.33*	8.00±1.15	6.33±0.33	6.00±0.00	N	7.00±0.58
C. albicans	14.00±0.58*	12.33±0.88*	9.33±0.33	8.33±0.33	6.33±0.88*	6.00±0.58*	N	9.67±0.88

Values are expressed as mean ±SEM of 3 determinants; N = no inhibition; * = significantly different from positive control; p<0.05; ** = Ampicillin was positive control for bacteria; Amphotericin B for fungi.

the *S. officinarum* stem pulp extract against the test microorganisms: *E. coli*, *K. pneumoniae*, *S. aureus*, *P. aeruginosa, A. fumigatus* and *C. albicans*. The extract showed growth inhibition against the various isolates at varying degrees. Among the bacteria, inhibition was highest against *S. aureus* (8.67-24.00 mm) which was even above that of the control antibiotic (Ampicillin, 14.33 mm), while inhibition against *P. aeruginosa* (6.00-14.38mm) was the least. Susceptibility appeared to be concentration-dependent as the mean zones of growth inhibition were generally highest at the 100 µg/ml extract concentration and lowest at the 3.12 µg/ml. For the fungal isolates, the extract showed a stronger growth inhibition against *C. albicans* (6.00-14 mm) than *A. fumigatus* (6.00-12.00 mm). Also, the antimicrobial activity of the extract appeared to be broad spectrum as it was independent of Gram reaction. Methanol which served as negative control showed no activity against any of the test organisms.

DISCUSSION

In Nigeria and other parts of the world, the stem pulp of *S. officinarum* (sugarcane) is consumed as

a snack due largely to its sweet taste occasioned by high sucrose content. In this study, the dry matter was found to be less than one-third of the sugarcane stalk, while over two-third represented the water content, indicating its usefulness as a natural and perhaps a safer source for rehydration compared to carbonated beverages. The moisture content obtained in this study (71.91%) was also found to compare favourably with the range reported in a recent study for four sugarcane varieties (65.72 to 67.29%) cultivated in India (Singh and Singh, 2012). The nutrient with the highest value in the dry matter was carbohydrate (58.55 g/100 g). However, this partly contrasts with the report of Madan et al. (1998) who showed a much higher carbohydrate content (74 to 96%) than that obtained in this study. The observed variation may be attributed to differences in study design. Whereas, carbohydrate evaluation in the present study was based on total dry matter, including the crude fibre content, Madan et al. (1998) based theirs on the total soluble solids (TSS) only, of the cane juice. The variation in study design could also account for differences in other proximal components observed between this study and others (Madan et al., 1998; Singh and Singh, 2012) such as crude protein, fat and ash contents.

Moreover, other factors such as the sugarcane cultivar, degree of maturity, soil type, and other cultivation factors, are also among the many sources of variation in the nutrient content of plant foods (Osagie and Onigbinde, 1998). Changes in the nutritional composition of plant foods caused by age, varietal and climatic/-environmental factors have long been recognized and documented (Bhatnager et al., 2004; Eppendorfer et al., 2006; Marques da Silva and Silva, 2006). Therefore some of the minor data variations observed may be location bound, since this study submits for the first time nutrients and chemical composition of sugarcane consumed in Calabar, Nigeria. Studies on the influence of intrinsic and environmental factors on sugarcane nutrient composition are highly warranted as these could offer more insight into the observed variations.

The present investigation further reveals appreciable quantities of individual elements in the sugarcane stem. It has been reported that the mineral composition of the sugarcane stem is highly dependent on its age and decreases as the internode grows older (Bakker, 1999). Studies by Bakker (1999) and van Dillewijn(1952) showed that the mineral composition of the stem is affected by translocation of some elements especially potassium and nitrogen from the maturing internodes towards the younger ones, while others like magnesium and phosphorus increase towards the bottom of the stalk. However, in this study, whole mature sugarcane stems as commercially available for consumption were used and included both the top and bottom parts. Thus, the mineral content recorded can be said to represent the proximate mineral composition of the sugarcane stem consumed in Calabar.

The study data also indicated the relative presence of alkaloids, saponins, flavonoids, hydrogen cyanide and tannins in the sugarcane. Quantitative data showed a relatively high percentage of alkaloids (8.07 ± 0.04 µg/100 g) and saponins (5.57 ± 0.01 µg/100g), suggesting a validation of its involvement in some pharmacological activities. Alkaloids have been reported to be the active components of numerous medicinal plants and plant-derived drugs with numerous physiological activities (Atangwho et al., 2009). It is plausible that *S. officinarum* owes its reported medicinal properties at least in part to the presence of these alkaloids. The presence of flavonoids in this study agrees with the findings from other studies (Vila et al., 2008; Colombo et al., 2009; Silvia et al., 2009) that have reported the occurrence of several flavonoids in sugarcane. Flavonoids are commonly known for their strong antioxidant activity and have been referred to as “nature’s biological response modifiers” because of strong experimental evidence of their inherent ability to modify the body’s reaction to allergens, viruses, carcinogens, and inflammatory agents (D’Mello et al., 2010; Tiwari and Rao, 2002).

The antimicrobial data obtained indicate that the microorganisms used in this study were susceptible to the *S. officinarum* extract but at varying degrees. Studies have shown for the most part that biological and medicinal properties of plant extracts, including the antimicrobial properties, conferment of disease resistance and reduction in the risk of major degenerative diseases (Liu, 2004) depend largely on their phytochemical composition (Atangwho et al., 2009). It is most likely that the observed antimicrobial activity of *S. officinarum* extract against the select organisms derives from the phyto-compounds (alkaloids, saponins, flavonoids, and tannins) found in the extract studied.

Conclusion

Taken together, data from the present investigation suggest that besides being the major source of sucrose, sugarcane available in Calabar can be exploited as a natural source for rehydration, nutrient supplementation and medicinal or pharmaceutical agents. The study further reveals that apart from eating sugarcane to obtain energy and nutrients, the crop can also be used to prevent or treat intestinal problems of microbial origin.

Conflict of interests

The authors have not declared any conflict of interest.

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