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Full Length Research Paper

Characterization of actinobacteria from the semiarid region, and their antagonistic effect on strains of rhizobia

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The actinobacteria are the most abundant bacterial group in the soil, where they present different characteristics and antagonistic effects on other microorganisms. The objectives of this study were to characterize and evaluate the antagonistic effect of actinobacteria from the semiarid region on rhizobia from the same climatic region. Tests were performed to observe chromogenic and micro-morphological characteristics, tolerance to pH levels of melanin production, and use of carbon sources. This occurred due to the use of modified CDA culture media and basal medium supplemented with specific carbon sources, as well as the use of Ral color chat to observe the colonies. The *in vitro* antagonism on rhizobia was evaluated in yeast malt agar (YMA) culture medium for the observation of inhibition halo by actinobacteria. We observed different characteristics in relation to the color of aerial mycelium and reverse pigments; tolerance to media with acid and alkaline pH; and production of melanoid pigment occurred only in three strains. The morphological characteristics were described for genera *Streptomyces* and *Saccharothrix*, later confirmed by molecular sequencing. These genera were generalists in use of carbon sources, showing their physiological versatility. Regarding antagonism, nine strains of the genus *Streptomyces* were able to inhibit *in vitro* strains of *Rhizobium tropici* and *Bradyrhizobium yuanmingense* also coming from semiarid soils. The antagonism between actinobacteria and rhizobia can directly affect the symbiosis between nitrogen-fixing bacteria and leguminous plants. These results are pioneering in observing antagonism of these species of rhizobia under the climate condition of the semiarid region, and may contribute to agricultural biotechnology.

Key words: *Streptomyces*, soil, microbe interactions, Brazil, agricultural biotechnology, actinobacteria, antagonistic effect.

INTRODUCTION

Several mechanisms occur in the soil that influence the structure and composition of the soil communities, as well as intra and inter-specific interactions, for example, competition. The competitor microbial species often secrete compounds that affect the fitness of other species. This fact is characterized by antibiosis (Morris

and Blackwood, 2007). The actinobacteria are an example of organisms that antagonize microorganisms by means of secondary metabolites (Karlovsky, 2008; Tarkka and Hampp, 2008) and/or secretion of enzymes that inhibit the growth of other organisms in the soil (Suneetha and Zaved, 2011).

These filamentous microorganisms are gram-positive bacteria, and have a high content of guanine and cytosine in their DNA (Monciardini et al., 2002; Flärdh and Buttner, 2009). They are abundant and dispersed in different ecosystems; soil is their main habitat, where they perform important functions in soil fertility (El-Tarabily and Sivasithamparam, 2006; Jayasinghe and Parkinson, 2008). The genera *Streptomyces*, *Nocardia*, *Micromonospora*, *Actinoplanes* and *Streptosporangium* stand out in the edaphic environment (Anandan et al., 2016). The actinobacteria have crucial role in the decomposition of organic matter in ecosystems and thereby influence the cycling of nutrients in the soil (Mabrouk and Saleh, 2014), making them an excellent indicator of biological activity of the soil (Arifuzzaman et al., 2010). On the other hand, the literature contains records of the antagonistic effect of actinobacteria, *in vitro* and/or *in vivo* on other microorganisms (Parmar and Dufresne, 2011), for example, when they interact with rhizobia (Gregor et al., 2003; Mingma et al., 2014).

Rhizobia are bacteria responsible for biological nitrogen fixation (BNF) through the established symbiosis with leguminous plants (Zilli et al., 2010). These bacteria are of great importance for maintenance and restoration of ecosystems, being useful for the recovery of areas degraded by different impacts (Pontes et al., 2012). Soils with high population density of actinobacteria can inhibit the growth of rhizobia, negatively affecting plant nodulation and consequently reducing nitrogen fixation and plant productivity (Pereira et al., 1999; Parmar and Dufresne, 2011).

There are few studies that address the relationship of actinobacteria versus rhizobia, especially in isolates from semiarid regions. Thus, the aim of this study was to characterize the actinobacteria obtained in the Brazilian semiarid region and test their antagonistic effect *in vitro* on rhizobia strains from semiarid soils.

MATERIALS AND METHODS

Microorganisms

Fourteen strains of actinobacteria (Lima et al., 2014) obtained from samples of soil (AC 46, AC 49, AC 50 and AC 56) and leaf litter ((AC 3, AC 5, AC 10, AC 12, AC 13, AC 14, AC16, AC 38, AC 42 and AC43) at the Aiuaba Ecological Station (6°40' S & 40°10' W), located in the southwestern part of the municipality of Aiuaba, Ceará state, and five strains of rhizobia (L 4, L 7, L 9, L 16, L 18 and L 22) originating from the rhizosphere of leguminous plants (*Mimosa hostilis, Mimosa caesalpiniaefolia, Poincianella bracteosa* and *Erythrina verna*) (Pinheiro et al., 2014) in areas of Ceará (4°58' S and 39°1' W and 4°7' S and 38°14' W), and the state of Rio Grande do Norte (5°39' S to 35°58' W and 5°57' S to 36°39' W) were used in this study. All microorganism collection sites are located in the semiarid region of northeastern Brazil with prevailing vegetation of Caatinga, shallow soils rich in minerals, and an average annual rainfall of 400 to 800 mm. These microorganisms belong to the collection of the Environmental Microbiology Laboratory of Micro-organisms (LAMAB) of Federal University of Ceará, Department of Biology.

Morphological characterization

The actinobacteria strains were cultured in Casein Dextrose Agar (CDA) medium (0.5 g of K_2HPO_4, 0.2 g of $MgSO_4.7H_2O$, 0.01 g of $FeSO_4.7H_2O$, 2.0 g of dextrose, 0.2 g of casein, 15 g of agar, 1000 mL of distilled water, and pH 6.5 to 6.6) (Clark, 1965), for seven days at 28°C in a biochemical oxygen demand (BOD) chamber. The chromogenic characterization was performed by observing the aerial and reverse mycelium after growth of strains in Petri dishes, according to Wink (2012), with the use of a RAL color chart. The micro-morphological characterization was carried out by microcultivation according to Kern and Blevins (2003), with modifications. A blade and two pieces of cotton moistened with distilled water (sterile) were placed in sterilized Petri dishes. A cube of CDA culture medium measuring approximately 1 cm^3 (Clark, 1965) was placed on the blade. The strain was inoculated on the cube sides, covered with a sterile coverslip, while the dish was closed and incubated in a BOD chamber for 7 to 14 days at 28°C. After this time, the coverslip was removed and placed on a clean slide containing a drop of cotton blue stain. The slides were observed under a Zeiss Axioplan optical microscope (Leica DM750 M, Heerbrugg, Switzerland) with a 100x magnification to visualize the actinobacteria's characteristics (Miyadoh, 1997; Goodfellow et al., 2012).

Utilization of carbon sources

The use of carbon sources was analyzed according to Shirling and Gottlieb (1966), using mineral salts agar as basal medium. A solution was prepared by 10% of each carbon source (arabinose, sucrose, xylose, inositol, mannitol, fructose, rhamnose, raffinose and cellulose), with glucose (positive control) and water (negative control). After this, each dish with medium was sterilized and cooled to about 60°C, a concentration of 1% of each carbon source was added. These carbon sources were sterilized by a Millipore filter (0.45 µm). Finally, the strains were transferred to Petri dishes (triplicates) and incubated in a BOD chamber at 28°C for 10 days. The use of the sources was analyzed by the observation of increase (+) or no growth (-) in Petri dishes with medium.

Production of melanoid pigment

Strains were grown in tyrosine agar medium (Shinobu, 1958) with

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Table 1. ERIC and PCR primers used in this study.

S/N	Resistant gene	Oligos sequence	Amplicon size (bp)	Reference
1	ERIC1	5'-ATGTAAGCTCCCTGGGGATTCAC-3'	100- 5000	Tian-Xing (2011)
2	ERIC2	5'-AAGTAAGTGACTGGGGTGAGCG-3'		
3	P027F	5'-GAGAGTTTGATCCTGGCTCAG-3'	1400	Weisburg et al. (1991)
4	1492R	5'-ACGGTTACCTTGTTACGACTT -3'		
5	StrepB	5'-ACAAGCCCTGGAAACGGGGT-3'	519	Rintala et al. (2001)
6	StrepE	5'- CACCAGGAATTCCGATCT-3'		

and without tyrosine. The Petri dishes were incubated at 28°C in a biochemical oxygen demand (BOD) chamber for 10 days. The formation of melanoid pigment was considered (formation of dark-colored pigment) after growth in a Petri dishes with tyrosine, this is because the pigment formation in both media does not reflect the production of melanin.

Tolerance to different pH values

The test for growth of actinobacteria at different pH was carried out according to Kishore et al. (2012), modified by using the CDA culture medium (Clark, 1965). The medium was adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0 or 9.0. The strains were transferred to Petri dishes (triplicates) with a solid medium and incubated at 28°C for 10 days. The growth of the strains will indicate tolerance of the tested pH. The soil from which the actinobacteria had been collected was analyzed to determine the pH according to Donagema et al. (2011).

Genomic DNA extraction and analysis of genetic variability and amplification of 16S rRNA

Total genomic DNA was obtained from actinobacteria and rhizobia belonging to the microbiological collection with the Wizard® genomic DNA purification kit (Promega, Madison, WI, USA), following the manufacturer's instructions. The Wizard® genomic DNA kit is based on a four-step process.

The first step is the purification procedure, lysing of the cell and the nucleus; an RNase digestion step was included at this time. The cellular proteins are then removed by a salt-precipitation step, which precipitates the proteins but leave the high molecular weight genomic DNA in solution. Finally, the genomic DNA was concentrated and desalted by isopropanol precipitation. The oligonucleotides (Tabe 1), enterobacterial repetitive intergenic consensus (ERIC) were used to evaluate the genetic diversity of strains (Tian-Xing, 2011).

The polymerase reaction (PCR) was performed with a final volume of 25 µL, using 60 ng of DNA; 2.5 µL of a reaction buffer 10x; 0.75 µL of $MgCl_2$ (50 mM); 2 µL of dNTP (2.5 mM); 1 µL of forward oligonucleotide, and 1 µL of reverse oligonucleotide (both in concentration of 10 pmol); 0.25 µL of Taq polymerase enzyme (5 U/µL) and ultrapure q.s.p. water to 25 µL. Amplification was performed using the following steps: initial denaturation at 94°C for 2 min followed by 30 cycles at 94°C for 1 min, 50°C for 1.5 min and 68°C for 4 min, and final extension at 68°C for 10 min. The ERIC-PCR product was subjected to electrophoresis on 1.2% agarose gel in triton extraction buffer (TEB) buffer 0.5x stained with ethidium bromide and was visualized under ultraviolet light. For amplification of 16S rRNA, the primers 3 and 4 (Table 1) were for the rhizobia (Weisburg et al., 1991), and the oligos 5 and 6 (Table 1) for the actinobacteria (Rintala et al., 2001).

The final volume of the PC reaction was 25 µL and amplification conditions are presented in Table 2. The reaction was carried out in an AmpliTherm thermal cycler. The amplicons generated by the PC reactions were purified using ExoSAP (exonuclease I, shrimp alkaline phosphatase). Sequencing was performed by the Sanger method with the BigDye Kit ABI3100 and an Applied Biosystem sequencer. The genetic diversity of the isolates was assessed by ERIC-PCR markers. The sequences were compared with existing sequences in the GenBank database using the BLAST program available at the site National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov).

Antagonistic test *in vitro* between actinobacteria and rhizobia

The antagonistic effect of actinobacteria on the rhizobia was evaluated according to the methods of Gregor et al. (2003). The strains of rhizobia were grown in glass tubes with liquid (without agar) YMA medium (10 g of mannitol, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4.7H_2O$, 0.1 g of NaCl, 0.5 g of yeast extract, 5 mL of bromothymol blue, 15 g of agar, 1000 mL of distilled water, and pH 6.8) (Vincent, 1970), and shaken (150 rpm) on a shaker orbital for about seven days. The actinobacteria were also grown in glass tubes, but with liquid CDA medium on a shaker orbital at the same speed and time period as foe rhizobia. The inhibition test was performed in Petri dishes (90 mm in diameter), divided into four quadrants with solid (with agar) YMA medium, plus 100 uL of YMA broth spread with rhizobia with a Drigalski handle. Then, 5 uL of CDA broth with actinobacteria was added. The Petri dishes (triplicate) were incubated for 5 days at 28°C in a BOD chamber. After this period, the plates were evaluated by observing the presence of inhibition zones, which were measured (cm) using a caliper rule.

Data analysis

The number of strains with and without growth at the different pH levels were subjected to the chi-square test with $p \leq 0.05$, based at pH 7. The radii of the inhibition halos were measured and then the inhibition area (πr^2) was calculated. These area values were log-transformed. The Shapiro-Wilk test was applied to test for normality, and then the data were subjected to analysis of variance (ANOVA) at $p \leq 0.05$ with the agricolae package software R®.

Table 2. PCR thermal cycle performed in this study.

Oligos	Step	Temperature (°C)	Time (min)	Number of cycle
ERIC1/ERIC2	Initial denaturation	94	2	30
	Denaturation	94	1	
	Annealing	50	1	
	Extension	68	4	
P027F/1492R	Initial denaturation	94	5	30
	Denaturation	94	0.40	
	Annealing	58	0.35	
	Extension	72	1.20	
StrepB/StrepE	Initial denaturation	98	5	30
	Denaturation	95	1	
	Annealing	54	1	
	Extension	72	2	

Table 3. Coloration of the colonies and spore morphologies of strains of actinobacteria from soil.

Strain	Color		Morphology
	Aerial Mycelium	Reverse Pigment	
AC 03*	Grey (RAL 9007)	Yellow (RAL 1002)	Spore chain hooked to looped
AC 05*	Brown (RAL 8024)	Brown (RAL 7030)	Spore chain straight to flexuous
AC 10**	White (RAL 9003)	Cream (RAL 9001)	Long chains of smooth spores
AC 12*	Green (RAL 7009)	Yellow (RAL 1012)	Spore chain hooked to looped
AC 13*	Brown (RAL 8007)	Brown (RAL 8012)	Spiral spore chain
AC 14*	Green (RAL 7002)	Yellow (RAL 7034)	Spiral spore chain
AC 16*	Green (RAL 7013)	Green (RAL 7013)	Spore chain straight to flexuous
AC 38*	Brown (RAL 8007)	Brown (RAL 4009)	Spore chain straight to flexuous
AC 42*	Brown (RAL 8008)	Brown (RAL 4009)	Spore chain to spiral
AC 43*	Cream (RAL 9001)	Cream (RAL 9001)	Spore chain hooked to looped
AC 46*	Beige (RAL 1015)	Beige (RAL 1015)	Smooth spore surface
AC 49*	Green (RAL 7006)	Green (RAL 7000)	Spore chain to spiral
AC 50*	Brown (RAL 8007)	Brown (RAL 8008)	Spore chain to spiral
AC 56*	Beige (RAL 1011)	Green (RAL 6013)	Spore chain straight to flexuous

*Genus *Streptomyces*; ** Genus *Saccharothrix.*

RESULTS

Chromogenic and morphological characteristics

The strains from the semiarid region had different characteristics (Table 3), in relation to color from aerial mycelium and reverse pigment. The predominant colors in the air mass were brown (5), green (4), beige (2), and only 1 each of grey, white and cream. The pigmentations of the reverse mycelium were brown (5), green (3), yellow (3), cream (2) and beige (1). After the microcultivation, it was observed that the actinobacteria had the following morphological characteristics: spore chain hooked to looped, spore chain straight to flexuous, long chains of smooth spores, spiral spore chain and smooth spore surface. These morphological traits are similar and

Table 4. Tolerance of the strains of actinobacteria to different pH ranges and melanoid pigment production.

Genera	Strain	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	Melanin pigment
Streptomyces	AC 03	+	-	-	+	+	-	+
Streptomyces	AC 05	+	+	+	+	+	+	-
Streptomyces	AC 12	+	+	+	+	+	+	-
Streptomyces	AC 13	+	+	+	+	+	+	-
Streptomyces	AC 14	+	+	+	+	+	+	-
Streptomyces	AC 16	+	+	+	+	+	+	-
Streptomyces	AC 38	+	+	+	+	+	+	-
Streptomyces	AC 42	+	+	+	+	+	+	-
Streptomyces	AC 43	-	-	-	+	+	-	-
Streptomyces	AC 46	+	+	+	+	+	+	-
Streptomyces	AC 49	-	-	+	+	+	-	+
Streptomyces	AC 50	+	+	+	+	+	+	-
Streptomyces	AC 56	+	+	+	+	+	+	-
Saccharothrix	AC 10	-	-	-	+	+	-	+

described for genera *Streptomyces* and *Saccharothrix*.

pH tolerance and melanin production

Table 4 shows the growth characteristics of the strains at different pH and the production of melanin. Regarding tolerance to extreme pH levels, the strains of the two genera had different behavior, except at pH 7.0 and 8.0, where all were able to grow in CDA medium with modified pH. The pH of the soil samples from which the actinobacteria were obtained had acid characteristics ranging from 4.5 to 6.3. Finally, melanoid pigment production occurred only in three strains (AC 3, AC 10 and AC 49) of actinobacteria.

Use of carbon sources

The strains of actinobacteria had wide use of carbon sources (Figure 1), with glucose being used by all strains tested. The strains of the genus *Saccharothrix,* used all the carbon sources except sucrose. However, the use of these sources by strains of the genus *Streptomyces* varied, with 85% for cellulose, 92% for sucrose and mannitol, and 100% for the other sources.

Molecular identification of microorganisms

The amplification profile ranged from 1 to 9 amplicons with sizes of 500 to 1400 bp for actinobacteria and 4 to 12 amplicons with sizes from 300 to 1400 bp for strains of rhizobia. After the molecular sequencing of actinobacteria and rhizobia, we observed the presence of actinobacteria of the genera: *Saccharothrix* and *Streptomyces* (Table 5). Regarding rhizobia, we identified two genera: *Bradyrhizobium* and *Rhizobium*.

In vitro inhibition of rhizobia

Only the actinobacteria strains of the genus *Streptomyces* had an antagonistic effect on *in vitro* rhizobial strains tested (Table 6). Of the 14 tested actinobacteria, nine presented antibiosis, and of five strains of rhizobia used, only two were inhibited. There was no statistical difference between the inhibition of actinobacteria ($F = 1.55$ and $P = 0.14$) and between the rhizobia strains inhibited ($F = 1.29$ and $P = 0.28$). However, *Rhizobium tropici* was inhibited by six strains of *Streptomyces*, particularly *Streptomyces graminisoli*, with 9.4 cm² inhibition halo. *Bradyrhizobium yuanmingense* was antagonized by seven strains of *Streptomyces*, particularly *Streptomyces* sp. with 6.8 cm² inhibition area.

DISCUSSION

Observation of the colors of the aerial mycelium mass and reverse side is one of the first methods used to distinguish isolates.

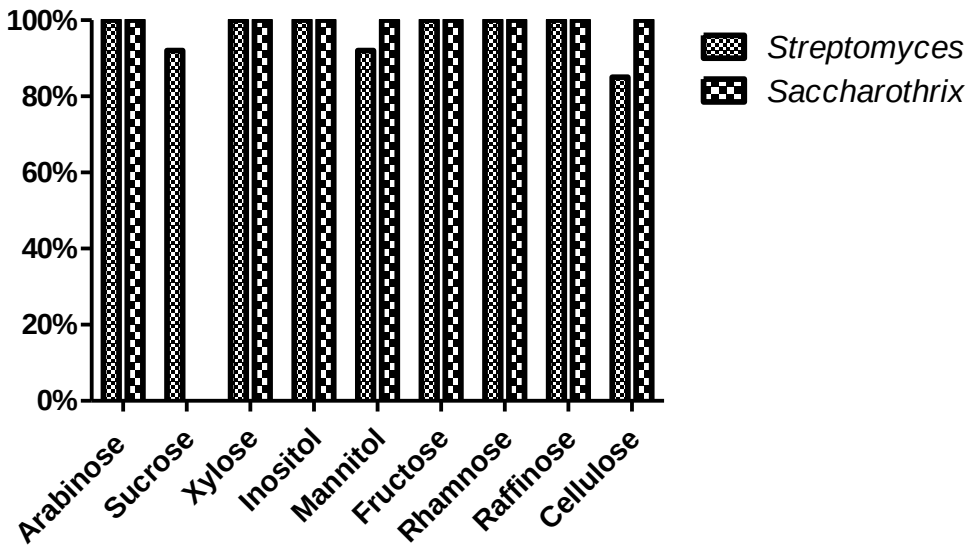

Figure 1. Percentage of use pattern of carbon sources for genera of actinobacteria identified.

Table 5. Molecular identification of strains of actinobacteria and rhizobia from semiarid soil by sequencing of 16S rDNA.

Bacteria	Strain	Number of Access GenBank	Species identity	Similarity %
Actinobacteria	AC 03	KY412816	*Streptomyces misionensis*	99%
	AC 05	KY412817	*Streptomyces lucensis*	99%
	AC 10	KY412821	*Saccharothrix* sp.	98%
	AC 12	KY412822	*Streptomyces graminisoli*	99%
	AC 13	KY412823	*Streptomyces sp.*	99%
	AC 14	KY412824	*Streptomyces misionensis*	98%
	AC 16	KY412825	*Streptomyces misionensis*	98%
	AC 38	KY412831	*Streptomyces griseoaurantiacus*	97%
	AC 42	KY412832	*Streptomyces corchorusii*	97%
	AC 43	KY412833	*Streptomyces sp*	97%
	AC 46	KY412835	*Streptomyces sp*	97%
	AC 49	KY412836	*Streptomyces* sp	94%
	AC 50	KY412837	*Streptomyces sp.*	97%
	AC 56	KY412839	*Streptomyces sp*	97%
Rhizobia	L 07	KY412842	*Bradyrhizobium elkanii*	98%
	L 09	KY412843	*Rhizobium tropici*	98%
	L 16	KY412844	*Bradyrhizobium japonicum*	97%
	L 18	KY412845	*Bradyrhizobium sp.*	97%
	L 22	KY412846	*Bradyrhizobium yuanmingense*	98%

In this study, brown was the predominant color, in contrast to those observed by Ramos et al. (2015) and Silva et al. (2015), who in characterizing actinobacteria strains coming from the semiarid region by cultures noted the predominance of gray, cream and white. This color variation is widely reported in the literature in actinobacteria strains originating from different regions, such as the Himalayas (Duraipandiyan et al., 2010), India (Kumar et al., 2010; Kumar et al., 2012; Das et al., 2014; Amsaveni et al., 2015), China (Yu et al., 2015), Egypt (Mabrouk e Saleh, 2014) and Iraq (Jaralla et al., 2014).

The color differences in the strains can be related to

Table 6. Inhibition zone area (cm) in vitro of antagonistic effect of actinobacteria on strains of rhizobia.

Strains	Actinobacteria	Inhibition area (cm^2) * of strains of rhizobia	
		Rhizobium tropici	***Bradyrhizobium yuanmingense***
AC 03	*Streptomyces misionensis*	6.4	NI
AC 05	*Streptomyces lucensis*	4.5	6.1
AC 12	*Streptomyces graminisoli*	9.4	NI
AC 13	*Streptomyces* sp.	5.1	6.8
AC 14	*Streptomyces misionensis*	6.5	5.2
AC 38	*Streptomyces griseoaurantiacus*	NI	2.2
AC 42	*Streptomyces corchorusii*	4.4	6.7
AC 43	*Streptomyces* sp.	NI	6.6
AC 50	*Streptomyces* sp.	NI	6.3
Coefficient of variation (CV) (%)		17.5	23.9

*Inhibition area calculated by πr^2; * NI, No inhibition.

factors such as temperature, soil type, pH and carbon sources in the environment (Amal et al., 2011). However, since the pigmentation of the colonies is similar among the different genera of actinobacteria, this feature would not be decisive for the strains classification.

Through microcultivation it was possible to distinguish two genera. This result of the spore chain through microcultivation corroborates the molecular identification analysis. This number was lower than that reported by Brito et al. (2015), who observed a similar genus (*Streptomyces*) to those found for this study. The genus *Streptomyces* was previously reported by Silva et al. (2013) in soils from the Brazilian Cerrado which is the most common in several regions (Anandan et al., 2016), with reports in the literature describing its occurrence in Atacama desert soils in Chile (Okoro et al., 2009), India (Kumar et al., 2012; Das et al., 2014) and Egypt (Mabrouk and Saleh, 2014).

Most actinobacteria strains did not produce melanoid pigments, but two (one of each genus), were able to produce melanin. Ramos et al. (2015) also observed this lower number of melanin-producing strains in strains coming from the semiarid region. Strains of *Streptomyces* derived from soil in India (Dastager et al., 2006) and Egypt (Mabrouk and Saleh, 2014) were characterized as not producing this pigment.

In mangrove sediments, Janaki et al. (2014) found a similar percentage of isolates capable of producing melanin as observed for us. This pigment is synthesized by fermentative oxidation, and has properties that can protect microorganisms against gamma and ultraviolet radiation (Amal et al., 2011; Manivasagan et al., 2013; Ahmed et al., 2014); this mechanism is against environmental stresses (Zhu et al., 2007; Manivasagan et al., 2013). However in our work, this mechanism was only used by some isolated actinobacteria from litter leaf (AC 3 and AC10), perhaps due to direct exposure to sunlight.

Regarding tolerance to pH, there was greater growth range of *Streptomyces* strains in media with pH 4.0, 7.0 and 8.0, but some strains were able to grow with other levels. However, the *Saccharothrix* strain only grew at pH 7.0 and 8.0. In soils of Colorado (USA), Lauber et al. (2009) found similar behavior to that of actinobacteria strains from the semiarid region of Brazil, an increase in pH from < 4.0 to > 8.0, while in coastal sediments in India, Ramesh and Mathivanan (2009) observed strains at pH 7.0 to 8.5, with most isolates between pH 8.1 to 8.5. But the variation in pH observed in the literature was different from that found in the soil from which actinobacteria were isolated. Actinobacteria are distributed in soils of different pH levels and are generally sensitive to extreme pH.

However, some genera can grow in acidophilus habitats (Anandan et al., 2016), with the most abundant microbial communities being found at pH < 4.1 (Rousk et al., 2010), and in alkaline soils (Meena et al., 2013). This allows actinobacteria to adapt and colonize different types of environments (Shivlata and Satyanarayana, 2015).

The carbon sources were used by both genera, except for sucrose by *Saccharothrix*. This general use has been reported in actinobacteria from marine sediments (Augustine et al., 2013; Meena et al., 2013) and soil of various habitats (Das et al., 2014; Jaralla et al., 2014.). Species within the genus are diversified regarding the use of sources, based on what the environment offers (Amal et al., 2011; Goodfellow et al., 2012.). This may explain the variation in some strains of the genus *Streptomyces*. Thus, the usage profile of sugars and alcohol (inositol) suggests potential generalist ability of the strains, and these differences in use may be due to the availability of carbon sources and adjustment of the isolates to various niches (Augustine et al., 2013).

The actinobacteria of the genus *Streptomyces* inhibited

in vitro rhizobia strains, forming inhibition zones ranging from 2.2 cm² to 9.4 cm², with antagonistic interaction with the species *Rhizobium tropici* and *Bradyrhizobium yuanmingense*. These two species of rhizobia, is known to nodulate some legume genera (*Phaseolus*, *Leucaena* and *Lespedeza*), vary in some traits, such as growth time (faster in *R. tropici* and slower in *B. yuanmingense*) and reaction in YMA culture medium (acid for *R. tropici* and alkaline for *B. yuanmingense*) (Martinez-Romero et al., 1991; Yau et al., 2002). The first record of antagonistic activity of actinobacteria against rhizobia was reported by Landerkin and Lochhead (1948), which test actinobacteria strains isolated from soil of the genus *Rhizobium*, and Van Schreven (1964) that compared actinobacteria of the genera *Streptomyces*, *Actinomyces* and *Nocardia* over *Rhizobium* strains. In *Bradyrhizobium japonicum*, Gregor et al. (2003) observed inhibition of these bacteria in *in vitro* growth by actinobacteria of the genera *Streptomyces* and *Amycolatopsis*. Mingma et al. (2014) isolated actinobacteria from roots and rhizosphere of leguminous plants which showed that, strains of the genus *Streptomyces* have negative effects on the growth of strains of *Rhizobium* sp. and *Rhizobium japonicum*.

There are reports in literature of such inhibition of rhizobia *B. japonicum* and *B. elkanii*, used as inoculants for soybean by actinobacteria of Brazilian Cerrado soils (Pereira et al., 1999). This work is the first reported observation of an antagonistic relationship, between actinobacteria and rhizobia coming from semiarid region soils from Brazil. The antagonistic inhibition occurs in the rhizosphere host with the release of secondary metabolites, enzymes and antibiotics (Karlovsky, 2008; Tarkka and Hampp, 2008; Suneetha and Zaved, 2011). The acquisition of nitrogen in some plants such as legumes often occurs by symbiosis with rhizobia, and the effect of inhibiting actinobacteria on rhizobia in the soil can be the cause of failure of nodulation process, and hence stunted plant growth (Tarkka and Hampp, 2008; Parmar and Dufresne, 2011).

Conclusion

Finally, results obtained expand knowledge of actinobacteria of the semiarid region of Brazil, and show the occurrence of an antagonistic effect exerted by these microorganisms on the rhizobia *R. tropici* and *B. yuanmingense*. This information can serve as a base for future studies, aiming to confirm this relationship and the *in vivo* effect which may contribute to agricultural biotechnology.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Post-treatment of municipal sewer in shallow polishing ponds

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This work aims to investigate the influence of polishing ponds (PP) depth on the post-treatment of sanitary sewers. Two treatment systems were designed and monitored. One system was an upflow anaerobic sludge blanket (UASB) rector and intermittent flow sand filter; followed by a PP of 0.20 m depth with low superficial application rate (24.9 kgBOD_5.ha^{-1}.day^{-1}), high sunlight incidence (597 Wm^{-2}) and enough biological carbon dioxide used to raise the average pH to 9.6, ensure an average removal of 81% orthophosphate and concentration *of Escherichia coli* lower than 10^3UFC/100 ml, which are the meeting requirements for unrestricted irrigation. The other system was a UASB followed by two PPs operated in parallel, at 0.20 and 0.60 m depths. The 0.20 m PP depth removed 80% of total Kjeldahl nitrogen, 53% of total phosphorus and 44% of orthophosphate. The 0.60 m pond depth showed low nutrient removal and a poor *E. coli* removal efficiency, 98.33% equivalent to 2 logs units. The ponds were fed by continuous systems.

Key words: Nutrient removal, polishing ponds, disinfection, sunlight.

INTRODUCTION

The setup of a UASB reactor followed by a polishing pond is highly consolidated, mainly at a tropical climate region and in developing countries like Brazil. The polishing ponds are generally used as post-treatment of UASB reactors effluent, they are used to remove pathogens and nutrients, but do not stabilize the organic matter (Cavalcanti et al., 2002).

The main nitrogen removal mechanisms used for the domestic sewage treatment in stabilization ponds are: Ammonia volatilization, biological nitrogen uptake, nitrification, denitrification, dead biomass sedimentation and sludge layer accumulation (Craggs, 2005). Despite all these nitrogen removal mechanisms in stabilization ponds, there are a general understanding among researchers that the predominant is ammonia volatilization due to high pH (Craggs, 2005; Park and

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Craggs, 2011; Assunção and Sperling, 2013). However, the operational and environmental conditions in which the removal occurs need more studies (Valero et al., 2010), because there are those who affirm that under favourable conditions for the algae growth, the main nitrogen removal occurs by algae absorption, despite the high pH values (Valero and Mara, 2007).

The residence time in stabilization pond permits various phosphorus removal mechanism, bio-assimilation, adsorption and chemical precipitation in pH values above 8 and high dissolved oxygen concentrations (Sperling and Chernicharo, 2005; Aslan and Kapdan, 2006, Cai et al., 2013; Wang et al., 2014). However, phosphates precipitation with earth alkali metals present in natural waters is considered as the main phosphorus removal (Cavalcanti et al., 2002), as a consequence of the rise in pH that favours phosphate precipitation (Sperling and Chernicharo, 2005). This results in a change in the balance of phosphate species, favoring the insoluble phosphate salts precipitation, e.g. calcium phosphate ($Ca_3(PO_4)_2$, hydroxyapatite ($Ca_{10}(OH)_2(PO_4)_6$ and struvite ($Mg(NH_4)$ (PO_4) (Haandel and Lettinga, 1994). Large phosphorus amounts may be stored as polyphosphate within bacteria and phytoplankton that can be released subsequently (Hupfer et al., 2007).

In this context, the polishing ponds suit a different reality. This is due to the possibility of exercising high photosynthetic rates, enabling high pH values providing a significant nutrients removal (Sperling and Chernicharo, 2005; Aslan and Kapdan, 2006; Cai et al., 2013; Wang et al., 2014; Gonçalves et al., 2016). When the pH reaches a value exceeding 9, the ammoniacal nitrogen removal is mainly attributed to ammonia volatilization and organic nitrogen decays due to sedimentation of algal materials (Mayo, 2013; Leite et al., 2011).

The solar irradiance greatly contributes to the algal biomass production, which also collaborates with bacterial decay, high hydraulic retention time (R_h), low carbon dioxide levels and the bactericidal action of toxins originated from cyanobacteria and algae itself (Beran and Kargi, 2005; Moreira et al., 2009). When this solar energy goes directly into pond systems, it favours the decay of pathogens through the direct action of ultraviolet light-A (UVA) and ultraviolet light-B (UVB) in the destruction of DNA, RNA and other cell constituents of organisms, as well as joint activity of solar radiation, dissolved oxygen and high pH (Bolton et al., 2011). Algae and macrophyte organisms through photosynthesis make significant dissolved oxygen levels increase and ensure a diurnal pH changes, by consumption and remotion of CO_2.

The secondary objective of this research is to investigate the influence of the pond depth and the solar radiation, as primary energy source, on the polishing pond systems, using nutrients and *E. coli* removal indicators as base parameters. The main aim of this work is to complement previous ponds studies and their variables, e.g. depth, sunlight irradiance and nutrient removal.

MATERIALS AND METHODS

System description

The experiment was performed at the Experimental Station of Biological Sewage Sanitary Treatment (EXTRABES - Estação Experimental de Tratamentos Biológicos de Esgotos Sanitários), part of the Universidade Estadual da Paraíba (UEPB). At EXTRABES is located the east interceptor of the water and sewage company of the state (CAGEPA - Companhia de Água e Esgoto do Estado da Paraíba) in Campina Grande - PB, Brazil, whose altitude is 550 meters. All experiments with sewage in this laboratory are developed with the east interceptor, from where the sewage is collected by a motor-pump setup. It is routed to a vertical sand column followed by a 1000 liter tank, which operates as an equalization tank. However, before the systems are fed, the sewage is homogenized by an automated shaker pump.

Two experimental treatment systems were built to treat the raw sewage (RS) from the CAGEPA's interceptor: the first system was a compact station (UASB reactor coupled to a decanter followed by an intermittent flow sand filter); and the second system was the combination of UASB reactor and decanter. The effluents of these two systems were post-treated in polishing ponds.

Operation condition

Each polishing pond area was 10 m^2 (1 m wide and 10 m long). Ponds 1 and 2 (PP1 and PP2) were 0.20 m deep and operated with an hydraulic retention time (R_h) of 6 days, while the polishing ponds 3 (PP3) with depth of 0.60 m were operated with R_h of 12 days. In Figure 1, a scheme of the system is presented.

PP1 was supplied by a compact station (CS) made up with fiberglass. The CS is composed of UASB in the center followed by a coupled decanter. Its effluent was pumped to feed the intermittent flow sand filter that fed the PP1 by gravity. To ensure intermittence in the sand filter of the CS, the pond was fed at intervals of 4 h (6 ciclos.day^{-1}) with 55L pumped from the decanter to the filter, comprising 330 L.day^{-1}. In Table 1 the physical and operating system configuration is presented for systems 1.

System 2 consists of a fibre UASB reactor with "Y" geometry coupled to a secondary sedimentation tank followed by two shallow polishing ponds operated in parallel. PP2 and PP3 were fed with 330 and 500 L.day^{-1} continuous flow, respectively. The physical and operating system configurations for system 2, with ponds 2 and 3 (PP2 and PP3) are presented in Table 2.

Analyses

The dissolved oxygen and oxygen production rate (OPR) analysis was performed using the semi-continuous method from Beluga S32c software. The whole procedure was carried out *in local*, seeking to maintain the actual conditions. The respirometer received signals provided by the dissolved oxygen (YSI 5718) and temperature electrodes, sending them to a computer running S32c software.

The alkalinity determinations were performed using Kapp method (Buchauer, 1998). The *Escherichia coli* determination and the analysis of the variables of interest were made regarding the recommendations of the Standard Methods for the Examination of Water and Wastewater (APHA, 2012). The variables are COD, BOD_5, total phosphorus, total kjeldahl nitrogen, ammonia nitrogen, total solids, settleable solids, suspended solids, volatile solids and

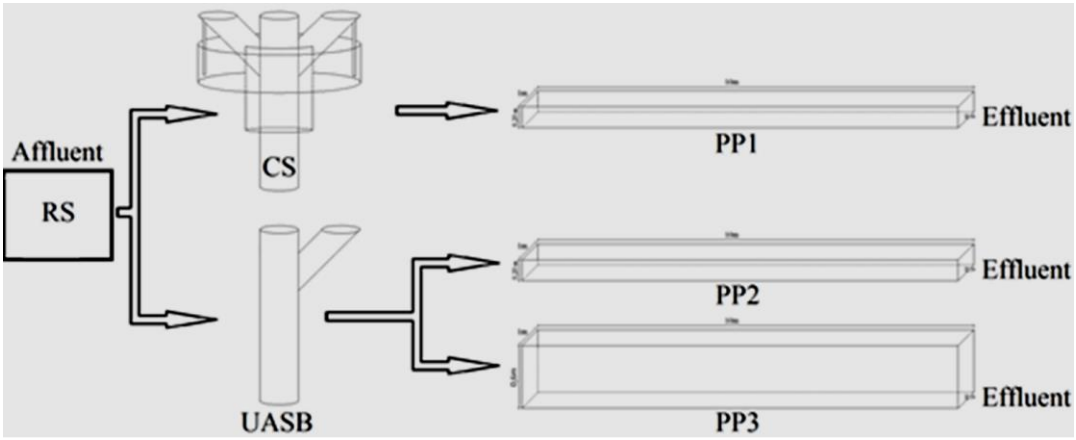

Figure 1. Scheme of the treatment systems used during for the research.

Table 1. System 1 operating parameters.

Characteristics	UASB	Sand filter	PP1
Operation mode	Continuous	Batch	Continuous
Height (m)	1.8	0.9	0.20
Area (m^2)	0.126	0.26	10
Volume (m^3)	0.227	0.234	2.0
R_h (days)	0.68	0.02	6.0
Flow ($L.day^{-1}$)	330	330	330

R_h, Hydraulic retention time; PP1, polishing pond post-treatment for sand filter effluent.

Table 2. System 2 operating parameters.

Characteristics	UASB	PP2	PP3
Operation mode	Continuous	Continuous	Continuous
Height (m)	1.9	0.20	0.60
Area (m^2)	0.237	10	10
Volume (m^3)	0.45	2.0	6.0
R_h (days)	0.29	6.0	12
Flow ($L.day^{-1}$)	1550	330	500

R_h, Hydraulic retention time; PP2, polishing pond 0.20 m deep treating effluent from UASB; PP3, polishing pond 0.60 m deep treating effluent from UASB.

dissolved solids, and they were measured at the entry and exit points of the pond. Temperature measurements and alkalinity and pH analyses were performed daily.

To determine the mass balance of nitrogen matter at the ponds, Equation 1 was used. With it was possible to determine the total affluent nitrogen load daily applied (MN_{ti}). The other fractions were determined using equations 1 to 4, according to Haandel and Van Der Lubbe (2012) and Mayo (2013).

$$MN_{ti} = MN_{te} + MN_x + MN_{lost} \quad (1)$$

$$MN_{ti} = Q_i(NTK_i + N_{oq}) \quad (2)$$

$$MN_{te} = Q_i(NTK_e + N_{oe}) \quad (3)$$

$$MN_p = MN_{ti} - (MN_{te} + MN_x) \quad (4)$$

MN_{lost}: Nitrogenous matter lost (volatilization + denitrification); MN_{ti}: flux of nitrogenous matter in the affluent ($mgN.d^{-1}$); MN_{te}: flux of nitrogenous matter in the effluent ($mgN.d^{-1}$); MN_x: nitrogenous matter in algae sludge ($mgN.d^{-1}$); NTK_a: total Kjeldahl nitrogen affluent ($mg.L^{-1}$); NTK_e: total Kjeldahl nitrogen effluent ($mg.L^{-1}$);N_{oi}: oxidized nitrogen affluent ($mg.L^{-1}$);N_{oe}: oxidized nitrogen effluent ($mg.L^{-1}$).

Statistical inference methods with variance analysis (ANOVA) were applied to test the sample variable averages of the treatment systems at 5% significance level.

RESULTS AND DISCUSSION

The average values of the parameters of interest for RS, effluents from CS, UASB reactor and the three ponds: PP1, PP2, and PP3 are presented in Table 3. Remember that RS was the affluent for both CS and UASB. The parameters of interest are: COD, BOD_5, orthophosphate, total phosphorus, total Kjeldahl nitrogen, ammonia, nitrite, nitrate, solids and its fractions, and *E. coli* as pathogen indicators. These parameters were obtained during 9 months.

Organic matter removal

It is observed in Table 3 that the CS and UASB reactor produced effluents with average COD 136 and 189 $mg0_2.L^{-1}$, BOD_5 83 $mgO_2.L^{-1}$ and 120 $mg.L^{-1}$ and volatile suspended solids of 59 and 74 $mgTSS.L^{-1}$, respectively. An ANOVA statistical analyses results show that at a 5%

Table 3. Average values and standard deviation of the raw sewage and treated effluents during the experimental phase.

Parameter	Affluent		Effluents			
	RS ($\bar{x}\pm\delta$)	CS ($\bar{x}\pm\delta$)	UASB ($\bar{x}\pm\delta$)	PP1 ($\bar{x}\pm\delta$)	PP2 ($\bar{x}\pm\delta$)	PP3 ($\bar{x}\pm\delta$)
COD ($mgO_2.L^{-1}$)	489±26	136±34	189±26	140±57	185±50	147±37
BOD_5 ($mgO_2.L^{-1}$)	200±29	83±25	120±27	87±32	115±41	73±23
Phosphorus total ($mgP.L^{-1}$)	8.06±1.15	4.73±0.88	7.22±1.14	1.46±0.74	3.38±1.20	7.14±0.68
Orthophosphate (mg $P\text{-}PO_4^-.L^{-1}$)	5.38±1.52	3.49±0.75	4.83±1.01	0.65±0.62	2.69±0.71	3.75±0.64
T.K.N (mg $N\text{-}TKN.L^{-1}$)	48.62±3.74	11.11±1.07	40.72±4.28	6.97±0.73	9.54±1.28	12.97±3.58
Ammonia nitrogen (mg $N\text{-}NH_4^+.L^{-1}$)	39.03±3.81	8.36±0.61	38.27±5.47	2.02±0.33	3.78±1.34	7.63±3.88
Nitrite (mg $N\text{-}NO_2^-.L^{-1}$)	-	0.47±0.42	-	0.84±0.23	0.06±0.18	0.05±0.10
Nitrate (mg $N\text{-}NO_3^-.L^{-1}$)	-	29.63±4.27	0.30±0.02	0.47±0.32	1.30±0.83	2.90±0.50
Total solids ($mg.L^{-1}$)	1112±154	1138±221	1068±155	1278±98	1217±110	1158±89
Volatile solids ($mg.L^{-1}$)	436±120	216±113	283±150	232±89	301±139	303±102
Total suspended solids ($mg.L^{-1}$)	248±44	59±17	74±14	27±10	48±28	44±10
Volatile suspended solids ($mg.L^{-1}$)	196±40	49±6	57±29	19±10	47±22	42±14
pH	7.56±0.27	6.58±0.65	7.41±0.25	9.61±0.69	8.59±0.34	8.09±0.20
Alkalinity (mg $CaCO_3.L^{-1}$)	360±42	137±30	373±21	159±50	270±30	426±48
Escherichia coli (UFC/100 mL)	$5.25x\ 10^6$	$7.13x\ 10^4$	$3.10\ x\ 10^6$	$7.88x\ 10^2$	$9.40\ x10^3$	$8.65x\ 10^4$

RS, Raw sewage; UASB, effluent from UASB reactor; CS, compact station; PP1, effluent polishing pond coming from the compact station; PP2; PP3, effluent polishing pond coming from the UASB reactor.

significance level there was a significant difference ($p < 0.05$) between the average COD values of the two produced effluents.

The low organic matter concentration provides a higher light penetration (Sperling, 2002), which results in a higher photosynthesis rate (Sigee, 2004). Therefore, it is expected, for these effluents, a high pH and dissolved oxygen rates that will be discussed further below. Bastos et al. (2011), studying polishing ponds, obtained high pH for low depth, which is in line with this work.

Nitrogen removal

PP1 is fed with an already nitrified affluent from the CS, as shown in Table 3, so that, the low concentrations of total Kjeldahl nitrogen (N-TKN 6.97 $mg.L^{-1}$) and ammonia (2.02 $mgN\text{-}NH_4^+.L^{-1}$) were expected. The high organic nitrogen concentration (71%) is associated with the particulate matter present in the algae (Mayo, 2013).

PP2, with a depth of 0.20 m, was fed UASB reactor effluent and showed a considerable nitrogen removal. As can be seen in Table 3, the concentration of the final effluent from PP2 was 9.54 mg N-NTK. L^{-1} and 3.78 mg $N\text{-}NH_4^+.L^{-1}$, so, about 60% of the N-NTK corresponds to N-organic, which was probably incorporated into the algal mass.

According to the mass balance of nitrogenous matter presented in Figure 2 and Table 4, the affluent of PP2 (effluent from UASB reactor) had an average flux of 13.54 $gN.day^{-1}$, while the PP2 effluent had an average flux of 3.60 $gN.day^{-1}$ (3.15 $gN\text{-}NTK.day^{-1}$ + 0.02 $gN\text{-}NO_2.day^{-1}$ + 0,43 $gN\text{-}NO_3.day^{-1}$), resulting in a removal efficiency of 73.41%. The algal biomass consists of 52.4% carbon, 9.2% nitrogen and 1.3% phosphorus by weight (Park and Craggs, 2011). Also, there is a particulate nitrogenous matter that is settled at the sludge. It was not considered. Observing the values presented in Figure 2, it is noted that the organic nitrogen present at the effluent is 14% (1.90/13.54) $gN.day^{-1}$ of the affluent nitrogen. This percentage corresponds to the algal biomass produced at the polishing pond (Mayo, 2013). On the other hand, the fraction lost by ammonia gas desorption was 59.38% (8.04/13.54 $gN.day^{-1}$), disregarding the denitrification at night. The desorption process can be considered as main mechanisms of nitrogen removal at PP2 (0.20 m deep and fed by UASB reactor effluent). The nitrified fraction was only 3%, even at good conditions such as: high dissolved oxygen concentration (Figure 4), considerable algal sludge layer settled at the pond with temperature higher than 30°C and average pH of 8.59 (Table 3). This finding is corroborated by Park and Carggs (2011) who ensure that in high rates ponds, major ammonia nitrogen transformation processes are nitrification and assimilation.

Zimmo et al. (2004) confirmed experimentally that 25% of nitrogen removal in shallow ponds (0.90 m) happens in biological processes of nitrification and denitrification. Leite et al. (2011), treating landfill leachate in a series of shallow ponds (0.50 m), was able to remove 99.5% of ammonia nitrogen and attributed this efficiency to the process of desorption of ammonia. Valero and Mara (2007) conducted experiments in a maturation pond on a pilot scale at United Kingdom and obtained low removal

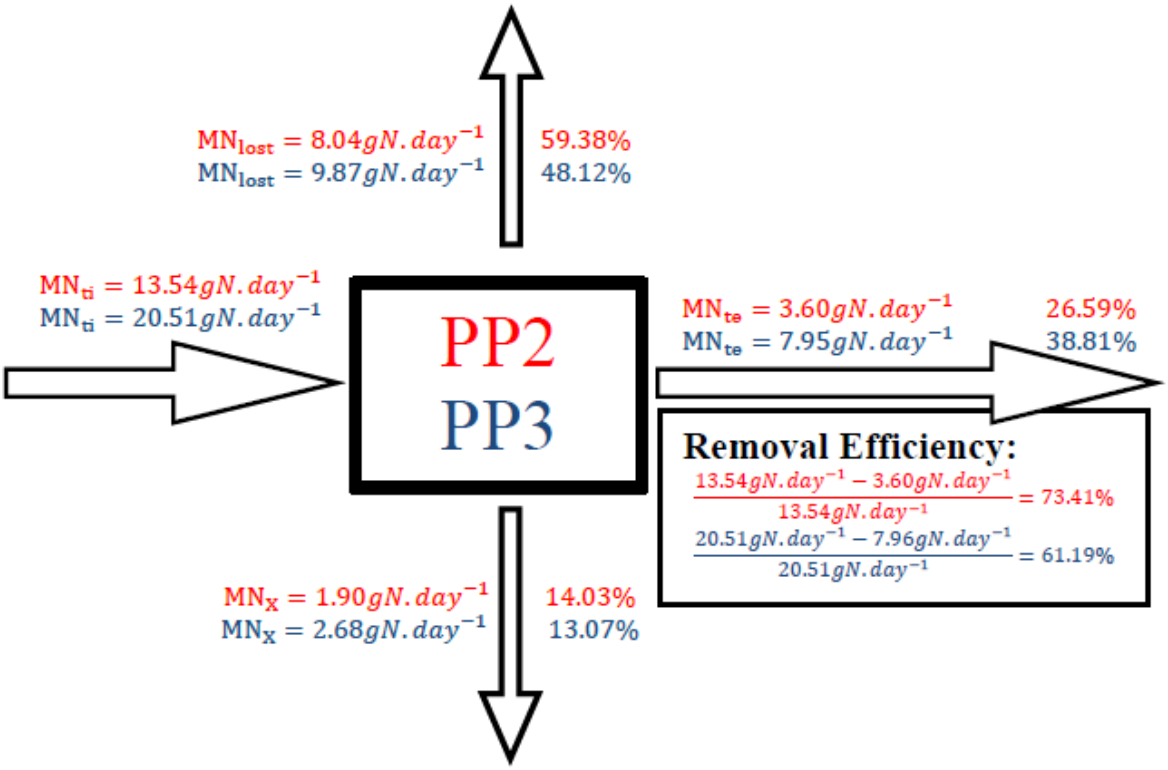

Figure 2. Nitrogenous mass balance for PP2 and PP3.

Table 4. Average values of nitrogenous affluent and effluent fractions.

PPP	Affluent					Effluent				
	N-NTK ($g.day^{-1}$)	N-N_{Org} ($g.day^{-1}$)	N-NH_4^+ ($g.day^{-1}$)	N-NO_x ($g.day^{-1}$)	MN_{ti} ($g.day^{-1}$)	N-NTK ($g.day^{-1}$)	N-N_{Org} ($g.day^{-1}$)	N-NH_4^+ ($g.day^{-1}$)	N-NO_x ($g.day^{-1}$)	MN_{te} ($g.day^{-1}$)
PP2	13.44	0.81	12.63	0.10	13.54	3.15	1.90	1.25	0.45	3.60
PP3	20.36	1.22	19.14	0.15	20.51	6.49	2.68	3.81	1.47	7.96

MN_{ti}, Flux of nitrogenous matter in the affluent; MN_{te}, flux of nitrogenous matter in effluent; N-NO_x, oxidated forms of nitrogen.

of ammonia volatilization (15 gN-NH_3.ha^{-1}.day^{-1}).

The average values of total nitrogen and its fractions for the RS and the effluents from CS, UASB reactor and the polishing ponds (PP1, PP2 and PP3) are shown in Table 4. In Figure 2, in the 0.6 m depth pond (PP3), the ammonia gas desorption was only 48.12 and 13% at the algal sludge.

The nitrogen mass balance (Figure 2) was used to calculate the average nitrogen removal variation from 61.19 to 73.41%. The associated mechanisms were ammonia volatilization, settle and biological absorption of nitrogen.

In Table 3, the pH was increased due to the biological use of CO_2 during intense algal metabolism (Shilton et al., 2008; Formagini et al., 2014). The rise of pH in the pond is a consequence of algal activity, which in the specific case of this work, contributed little to the gas ammonia volatilization, a conclusion corroborated by Valero and Mara (2010).

It is important to note that the comparisons of organic nitrogen concentration between all ponds show that there are increases in its fraction among inflow and outflow. But while the PP3 and PP2 received a poor affluent concentration of 2.45 gN-Organic (25%) and produced effluent with 5.34 gN-Organic (41%) and 5.76 gN-Organic (60%), respectively, the PP1 with almost the same affluent concentration of 2.75 gN-Organic (25%) was responsible for major organic effluent concentration of 4.95 gN-Organic (71%). And these results make sense with the apparent behaviours (Figure 3). Algae production was high in PP1, which blocked the sunlight across the pond.

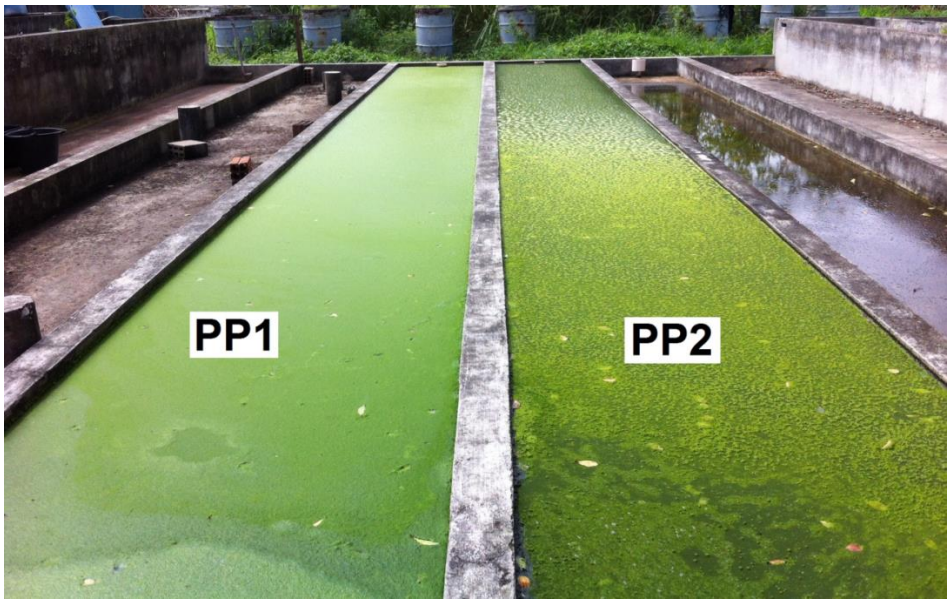

Figure 3. A picture of PP1 and PP2 from the operational period.

Phosphorus removal

The average concentrations of orthophosphate and total phosphorus for raw sewage were 5.4 and 8.0 $mg.L^{-1}$, respectively. These are typical values of an average sewage (Metcalf and Eddy, 2003). These same concentrations for PP1 affluent were 3.49 mg $P\text{-}PO_4^{-3}.L^{-1}$ and 4.73 mg $P.L^{-1}$, and for PP1 effluent they were 0.65 mg $P\text{-}PO_4^{-3}.L^{-1}$ and 1.46 mg $P.L^{-1}$ (Table 3), providing an efficiency of 81 and 69 %, for orthophosphate and total phosphorus respectively.

The phosphate removal is primarily due to pH increase, which increases because the CO_2 consumption is greater than its production rate. The extent of the pH increase depends on the buffering capacity which in turn depends on the alkalinity. Low alkalinity leads to a greater pH increase as the results of Table 3 with PP1 (pH 9.6 for 159 mg $CaCO_3.L^{-1}$) and PP2 (8.6 for 270 mg $CaCO_3.L^{-1}$) show.

This total phosphorus removal efficiency (69%) was due to the low concentration of organic matter expressed as BOD_5 (83 $mgO_2.L^{-1}$) and 59 $mgTSS.L^{-1}$, as shown in Table 3. There are sufficient conditions that make the photosynthesis average rate greater than the organic matter oxidation rate in the polishing pond (Figure 4); they ensure a 9.6 pH (Table 3), favouring the phosphate salts precipitation (Mara et al., 1992; Haandel and Lettinga, 1994; Mara and Pearson, 1998; Sperling et al., 2010). The low depth associated with a low organic matter concentration promotes greater light penetration (Sperling, 2002), which results in a higher photosynthesis rate (Sigee, 2004). Much lower results were obtained in South-Eastern Brazil in no similar experimental conditions: Average removal of total phosphorus was of 23% in a pond with a depth of 0.3 m and HRT of 2.3 days (Bastos et al., 2007).

PP1 and PP2 produced effluents with average values of significant statistical differences ($p < 0.05$). These ponds had the same dimensions and were operated in parallel, having similar temperature and solar radiance. Their only difference was the affluent. PP1 was fed by the CS and PP2 fed by UASB reactor. A quick analysis of UASB reactor effluent data from Table 3 revealed a high BOD_5 average concentration of 120 $mgO_2.L^{-1}$ and total suspended solids concentration of 74 $mgTSS.L^{-1}$. These are organic substances that hinder solar light penetration into the pond, limiting the pH to 8.6. This resulted in an overall phosphorus removal of 53%, producing effluent of 3.4 mg $P.L^{-1}$ (Table 3).

With respect to phosphorus removal in PP3 of 0.60 m depth, there was no significant total phosphorus removal (efficiency <2%), as shown in Table 3. It can be explained that 8.3 pH in this pond does not favour phosphate precipitation, and it is known that phosphorus removal in ponds is associated with insoluble phosphate salts precipitation and depends on a pH greater than nine.

Godos et al. (2009) confirm phosphorus removal efficiency less than 10% for stabilization ponds pH (~ 8.0). These authors ensure that pond effluent usually keeps high buffering capacity negatively interfering with the abiotic precipitation of phosphate ion.

Pathogenic organisms' removal

The polishing ponds are designed shallow to facilitate sunlight penetration through the whole water body,

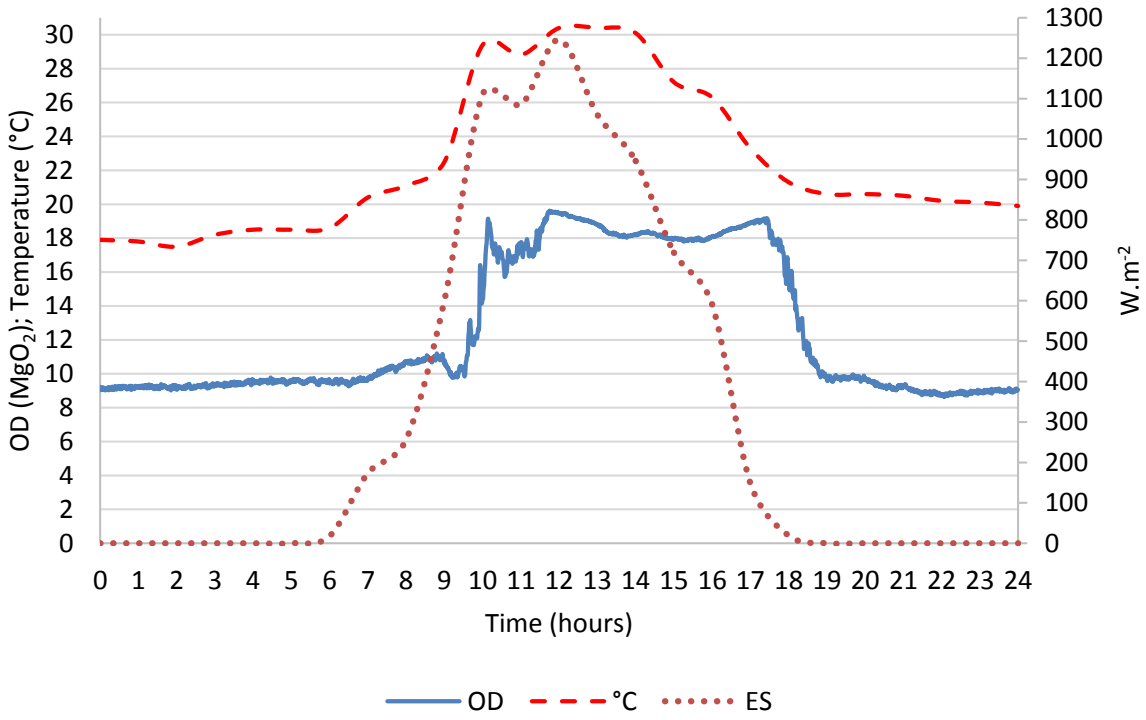

Figure 4. Dissolved oxygen behaviour (mg.L^{-1}), temperature and irradiance held *in loco* in the pond of 0.20 m deep.

optimizing the performance of the mechanisms of pathogen inactivation by sunlight (Shilton and Walmsley 2005; Verbyla and Mihelcic, 2015). *E. coli* bacteria are organisms that have a higher survival in treatment systems, so that these *E. coli* are obvious indicators of hygienic quality, regarding the removal of pathogens.

According to the data presented in Table 3, the geometric average of *E. coli* present at raw sewage was 4.65×10^{6} CFU/100 mL. These values are within the range of 10^{6} to 10^{9} CFU/100 mL established by Metcalf and Eddy (2003) as typical for wastewater.

The effluent from PP1, with a 6 days hydraulic detention time (R_h), had an *E. coli* concentration under 10^{3} CFU/100mL, which fits microbiological values suggested by the World Health Organization WHO (2006) in treated sewage for unrestricted irrigation. Note that PP1 was fed with CS effluent, an *E. coli* concentration of 7.13 × 10^{4} CFU/100 mL only. PP1 results are close to those of other studies (Sousa et al., 2005, Leite et al., 2009), wherein they suggest the application for agriculture reuse, favorable in semi-arid agriculture in Northeast Brazil.

Furthermore, PP2, whose affluent had an *E. coli* average concentration of 3.10 × 10^{6} CFU/100 mL, produced an average concentration effluent above 10^{3} CFU/100 mL; therefore it did not suit the requirements for sanitary quality suggested by the World Health Organization, WHO (2006).

As can be shown in Figure 4, the solar irradiance remained on the average value of 591 W.m^{-2}, assuring intense photosynthetic process by algae. It resulted in an oxygen average concentration of 18.0 mgO$_2$.L^{-1}, that ensures an average rate of oxygen production of 2.00 mgO$_2$.L^{-1}.h^{-1} over 9 h per day, as shown in respirogram in Figure 4. In previous study, Sweeney et al. (2007) reported for the summer a dissolved oxygen level over than 30 mgO$_2$.L^{-1} in the upper area of the stabilization pond. Nevertheless, *E. coli* removal efficiency was only 3 log units.

According to the authors (Ouali et al., 2014; Beutel and Larson, 2015), *E. coli* decay increases in direct proportion with the dissolved oxygen and increased pH. The high dissolved oxygen concentrations imply a reactive oxygen formation, such as singlet and superoxide that contribute to the pathogenic organisms decay (EI Hamouri et al., 1994; Bolton et al., 2010; Bolton et al., 2011). These cases were observed during the experiment. Figure 4 and Table 3 showed that PP2 with a dissolved oxygen close to 18 mgO$_2$.L^{-1} at midday and average 8.6 pH had the highest nutrient and pathogens removal efficiency among all the ponds treated effluent from UASB reactor. Beutel and Larson (2015) obtained similar behaviour for dissolved oxygen and temperature relation.

The polishing pond PP2 (0.20 m), operated with full R_h of 6 days, produced an effluent with *E. coli* average concentration of 9.1×10^{3} CFU/100 mL. This concentration is of the same magnitude order with this parameter measured for the effluent from PP3 (0.60 m ; 8.6 × 10^{4}

CFU/100 mL). This pond was operated at R_h of 12 days. Both ponds had an *E. coli* removal, not achieving the requirements recommended by the World Health Organization WHO (2006). Therefore, these effluents are fit for cereals and other fertigation application. Sperling et al. (2010) obtained similar results in operating polishing pond depth of 0.60 m and TDH for 12 days.

Conclusion

The compact station effluent favored PP1 performance (R_h: 6 days, high sunlight incidence: 597W.m^{-2} and temperature ranging from 20 to 32°C), achieving a CO_2 biological consumption sufficient to raise the pH to 9.6, ensuring an phosphorus average removal of 69 and 81% of orthophosphate and *E. coli* concentration less than 10^3CFU/100mL. This effluent can be used for unrestricted reuse. On the other hand, PP2 with similar conditions (0.20 m deep and same R_h) but different affluent removed only 73.42% of N-TKN, 53% of phosphorus and 44% of orthophosphate. The difference was due to the better affluent of PP1 (10^4-10^5 CFU/100mL, BOD_5 83 mgO_2.L^{-1} and 59 mg.TSS.L^{-1}).

The present study provided relevant contributions to pond research. The short hydraulic retention time for shallow pond ensured a higher algae production at PP1, allowing a new perspective for the next studies on algae production for power generation and an efficient combination of anaerobic and aerobic treatment with potential effluent for reuse.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effect of two different habitats on some primary and secondary phytochemicals of Miswak (*Salvadora persica* L.)

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***Salvadora persica* is a popular medicinal plant in the Middle East countries. It is well adapted to a wide range of habitats. In this study we have compared the effect of two different habitats (Al-ahsabah valley** and **Shada Mountain) in Al-Baha City, south west of Saudi Arabia on the content of some primary and secondary metabolites in *S. persica*. The results show that soil texture, soil moisture and organic matter contents varied in the studied regions. This variation was accompanied by differences in total count and distribution of Mycorrhizal spores in both studied habitats. Soil total count of mycorrhizal spores and root colonization percentage in Shada Mountain was significantly higher than that in Al-Ahsabah valley. Moisture content, carbohydrates, proteins and amino acids concentrations in leaves and roots of plants collected from Al-ahsabah valley were significantly higher than those collected from Shada Mountain. Gas chromatography mass spectrometry (GC-MS) analysis showed that benzene, (isothiocyanatomethyl) was the most abundant analyt in both extracts. There was a slight variation in the secondary metabolites pattern, where benzene, 1-isocyano-2-methyl- in extract of *S. persica* roots collected from Al-Ahsabah valley substituted with Benzyl nitrile in *S. persica* roots of Shada Mountain. Taken together we could conclude that, different habitats in the studied regions affect markedly the concentration of its primary metabolites and to less extent the secondary metabolites. Isolation of the active phytochemical constituents from plants of different habitats and studding of its biological activity will definitely give fruitful results.**

Key words: Miswak, soil factors, mycorrhiza, carbohydrates, protein, amino acids, gas chromatography mass spectrometry (GC-MS).

INTRODUCTION

Salvadora persica L. (family Salvadoraceae) is an evergreen shrub, 4 to 6 m tall with a short trunk, white bark, fleshy leaves and drupes, 3 mm diameter smooth fruits (Sher et al., 2010; Wasimuzzama et al., 2010). It is

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commonly called Arak, Miswak or toothbrush tree where its roots and branches are used to prepare chewing sticks in many third world countries (Elvin-Lewis, 1982; Almas and Al-Lafi, 1995). The tree is well adapted to different edaphic and topographical factors and widespread in desert areas of Africa and South Asia. It has been found growing both in the plains and the hills. It could be found in dry water courses and rocky depressions; however, it occurs more widely in wet sites including riverbanks, perimeters of waterholes, and drainage lines in arid zones where the ground water is near the surface (Iyenger et al., 1992; Khafagi et al., 2006). Therefore, it can tolerate high rainfall, low humidity, extreme drought, and temperature range from -3 to +48°C (Iyengar et al., 1992). It thrives on a variety of soils including sandy loam, clayey loam, gravelly, shallow, calcareous and sand dunes; tolerates a degree of salinity or alkalinity with pH of 6.5 to 8.5. Thus, it is distributed in many countries in the Middle East (Al-Samh and Al-Nazhan, 1997; Zodape and Indusekhar, 1997).

Distribution behavior of *S. persica* varies, to some extent, in different countries probably due to the changes in water resources, climatic and edaphic factors and anthropogenic demands (Hassan et al., 2010). It is distributed in India, Sri Lanka, Egypt, Pakistan, Sudan, Ethiopia, Senegal and Gulf including Saudi Arabia (Iyenger et al., 1992; Sher et al., 2010). In Saudi Arabia, *S. persica* has wide distribution pattern from 0 to 1500 m altitude in the whole arid and semi-arid ecosystem. It mostly grows on relatively open south facing slopes in Saudi Arabia and has a preference to the moist habitat (Al-Yemeni and Zayed, 1999; Sher et al., 2010).

S. persica is one of the most popular medicinal plants in the Muslim world. Several studies identified a variety of biologically active constituents in *S. persica* extracts. These chemical compounds are classified as volatile oils, flavonoids, alkaloids, steroids, terpenoids, saponins, carbohydrates, vitamins, and salts mostly as chlorides (Rajesh et al., 2009), in addition to organic sulphur compounds (Daxenbichler et al., 1991) and lignin glycosides (Kamel et al., 1992) defined in almost every part of the plant including leaves, roots and stem bark and have a pharmaceutical importance (Kumar et al., 2012).

Many reviews presented data on high number of different phytochemicals like benzylisothio-cyanate, benzylamides, trimethylamine, salvadourea, salvadorine, salvadoricine, rutin quercetin, 1-8-cineole, α-tocopherol, α- lonone, α-caryophellene, β-pinene, and β-sisterol (Attar, 1979; Al-Lafi and Ababneh, 1995; Sofrata et al., 2011). Consequently, the plant shows strong antibacterial (Sofrata et al., 2008), antifungal (Al-Mohaya et al., 2002; Hamza et al*.,* 2006), antiulcer (Sanogo et al., 1999), antiparasitic, and antiviral (Ali et al*.,* 2002) characteristics. It is also utilized in most dental treatments and cleansers (Almas, 2002; Al-Otaibi et al*.,* 2004; Almas et al., 2005; Darmani et al., 2006). The World Health Organization (WHO, 1987) and foreign direct investment (FDI, 2000) have recommended and encouraged the use of Meswak as chewing sticks after examination of its effectiveness as an oral hygiene aid. The *Salvadora* species have other medicinal applications in which, all plant parts could be used (Almas et al., 2005; Darmani et al*.,* 2006). The roots are used for chest diseases while the latex is used for treating sores, leaves, root bark, fruits and seeds are used for cough, fever and asthma treatment and as purgative (Mahar and Malik, 2001; Savithramma et al., 2007). Recently, Farag et al. (2017) provided a complete profile of volatiles, sugars, and organic acids in *S. persica* organs.

In addition to its great medicinal value, *S. persica* holds several other potentialities. The tree is suitable for growth in the shelter belts and as wind breaks. The fruits are used as food and the fresh leaves, rich in minerals, are eaten as salad (Sujata, 2015). Leaves also make a good fodder where it is readily consumed by goats and cattle (Abou-Zaid et al., 2015). Resin used during manufacturing stains. Oils of *S. persica*seeds are used for soap and detergent industries (Kumar et al., 2012).

As mentioned previously, *S. persica* is well adapted to different natural habitats. In spite of the tremendous studies that are available regarding the importance of *S. persica* and its bioactive compounds, nothing in the literature, as far as we know, is available to compare the effect of different habitats on its chemical constituents and consequently, its medical value. In the present investigation, a comparative study was conducted to explore the effect of two different habitats (Al-Ahsabah valley and Shada Mountain) in Al-Baha City, south west of Saudi Arabia on the concentrations of some primary and secondary metabolites in *S. persica* extracts.

MATERIALS AND METHODS

Collection of soil and plant samples

Soil samples and *S. persica* plants were collected from Al-Ahsabah valley and Shada Mountain, Al-Baha City, south west of Saudi Arabia at latitude of 19° 20' N and longitude 41° 42' E. Soil samples were used for characterization of soil physiochemical properties and isolation and identification of Mycorrhizal spores. The plants were gently washed with distilled water, dried between two paper towels and brought into the laboratory. Freshly collected plants were used for the determination of moisture and organic matter contents in plants leaves and roots in addition to arbuscular mycorrhizal fungi studies in plant roots. Other plants were separated into leaves and roots, shade dried and then ground separately with the help of electronic grinder to fine powder. Water extracts of the powdered tissues were used for primary metabolites estimation. Some secondary metabolites in crude root extract were analyzed using GC-mass spectroscopy.

Characterization of soil physiochemical properties

Soil texture was determined according to the method of Al Yamani

Table 1. Soil texture of Al-Ahsabah valley and Shada Mountain.

Site	Coarse sand (%)	Fine sand (%)	Silt (%)	Clay (%)
Al-ahsabah valley	37.69 ± 0.53[b]	13.83 ± 0.48[b]	37.16 ± 0.89[a]	11.31 ± 1.90[a]
Shada mountain	72.32 ± 1.79[a]	18.28 ± 1.13[a]	6.20 ± 1.38[b]	3.20 ± 0.46[b]

and Al-Desoki (2006). 100 g of soil samples were weighed and sieved with variable sizes sieves (mesh sizes 0.5 to 0.005 mm). Soil particle size was determined depending on the United States Department of Agriculture classification as follows: clay<0.002 mm, silt<0.05 mm, fine sand<0.25 mm and coarse sand<0.5 mm. The weighed soil samples were put separately into the top sieve, and the sieves were shaken for about 1 h by a sieve shaker. After 1 h, each sieve was individually weighed and the relative percentage of clay, silt, fine sand and coarse sand was calculated.

Moisture and organic matter percentages in soil and plant samples were determined using procedures previously described by other investigators (Wilde et al., 1972; Yousef, 1999; Conklin, 2005).

The following formula was used to determine the percentage of organic matter in the soil and plant samples:

$$\left(\frac{\text{Dry sample (g) - incinerated sample (g)}}{\text{Dry sample (g)}}\right) \times 100$$

The percentage of water in the soil and plant samples was determined using the following formula:

$$\left(\frac{\text{Wet sample (g) - Dry sample (g)}}{\text{Wet sample (g)}}\right) \times 100$$

Isolation and identification of mycorrhizal spores in soil samples

The method described by Brundrett et al. (1996) was modified and used to isolate the mycorrhizal spores. Approximately, 50 g of soil were placed into a container and mixed with 1 L of water to obtain a suspension, which was sieved using sieve sizes of 250, 100 and 50 µm. The sieved soil was then rinsed with water several times until clear water seeped through the 50 µm sieve. The sieved water was collected and centrifuged twice. Spores were isolated from denser soil components by carefully disposing of the supernatant and floating debris. Then, the sample was re-suspended in 1.17 M of sucrose until a volume of 30 mL was reached. Next, the suspension was mixed again and centrifuged at 2000 RPM for 10 min. The resulting materials were further sieved by utilizing a 50 µm-sized sieve, and trapped spores were rinsed with water and filtered through a filter paper prior to their transfer to a Petri dish.

Based on morphological characteristics, spores were stained with a mixture of Melzer's reagent and polyvinyl alcohol-lactoglycerol (Koske and Tessier, 1983; Morton and Benny, 1990). The spores were immobilized with a cover slip and examined under a stereomicroscope (CX 41 RF, Olympus Corporation, Philippines) in order to classify them by genus level (Morton and Benny, 1990).

Estimation of colonization and spore density in plant roots

Roots were removed from the lactoglycerol solution and placed on a Petri dish. A piece of paper with grid sizes of 1 cm × 1 cm was placed under the Petri dish. The number of arbuscular mycorrhizal fungi (AMF) was counted under a microscope. The estimated density of AMF was then calculated according to the following equation (Brundrett et al., 1996):

$$\left(\frac{\text{Number of cells with AMF present}}{\text{Total cells}}\right) \times 100$$

Analysis of primary metabolites

The anthrone sulphuric acid method (Fales, 1951; Schlegel, 1956) was used for soluble carbohydrates determination in plant leaves and roots. Soluble proteins content was determined in the plant extracts using Folin reagent as followed by Lowry et al. (1951). The free amino acids were determined depending on the method of Moore and Stein (1948) in the same water extract of proteins and carbohydrates.

GC-mass spectroscopy analysis of secondary metabolites

Crude roots extracts of *S. Persica* were analyzed by GC-Mass spectroscopy (GC/MS (7890A/5975B)) and column (GD-5MS) in the Analytical Chemistry Unit (ACAL), Faculty of Science, Assiut University, Assiut, Egypt. The results were used to determine the analyst composition and their percentage in the samples.

Statistical analyses

All data obtained were subjected to a one-way analysis of variance (ANOVA), using the SPSS statistical package. For comparison of the means, the Duncan's multiple range tests ($p< 0.05$) were used.

RESULTS

The results in Table 1 shows considerable differences in soil texture between the two studied regions. Al-ahsabah valley soil consisted mainly of coarse sand and silt in comparable values (around 37%). However, coarse sand represented the most dominant soil fraction (72.32%) in Shada Mountain. The percentages of silt and clay soil in Al-Ahsabah valley were significantly higher than those in Shada Mountain, while the percentages of coarse and fine sands in Shada Mountain were significantly higher than those in Al-Ahsabah valley.

There were differences also recorded in soil chemical prosperities (Table 2). Al-Ahsabah valley soil had significantly higher soil moisture and organic matter

Table 2. Soil chemical properties of Al-Ahsabah valley and Shada Mountain.

Site	Soil moisture (%)	Soil organic matter (%)	Soil pH
Al-ahsabah valley	47.36 ± 1.79[a]	48.72 ± 0.49[a]	7.84 ± 0.07[a]
Shada mountain	39.20 ± 0.91[b]	36.24 ± 1.05[b]	7.35 ± 0.06[b]

Table 3. Distribution of some arbuscular mycorrhizal fungi as affected by the studied habitats.

Site	Spore count (per 100 g dry soil)	Root colonization (%)	Most dominant spp.
Al-Ahsabah valley	2733.33 ± 305.51[b]	65.67 ± 3.21[b]	*G. fasiculatum* *G. mosseae* *G. macrocarpus* *S. clavispora*
Shada mountain	3066.67 ± 152.75[a]	78.67 ± 3.51[a]	*G. fasiculatum* *G. mosseae* *G. macrocarpus*

contents and pH value compared to Shada Mountain.

As presented in Table 3, soil total count of mycorrhizal spores in Shada Mountain (3066.67 spore/100 g dry soil) was significantly higher than that in Al-Ahsabah valley (2733.33 spore/100 g dry soil). The spores found in the soil samples of both regions belonged to families Glomaceae and Acaulosporaceae. The spores of four mycorrhizal species could be identified in Al-Ahsabah valley namely, *Glomus fasiculatum*, *Glomus mosseae, Glomus macrocarpusand* and *Sclarocystis clavispora*. In Shada mountain, the spores of only three mycorrhizal species were recorded*; Glomus fasiculatum*, *Glomus mosseae* and *Glomus macrocarpus* (Table 3). In addition, it was observed that, the percentage of roots mycorrhizal colonization in Shada Mountain (78.67%) was significantly higher than that in Al-Ahsabah valley (65.67 %). The identified mycorrhizal spores is Figure 1.

Moisture and organic matter percentages in leaves and roots of *S. persica* collected from the studied regions were estimated and presented in Figure 2. Moisture percentage in plant leaves collected from Al-ahsabah valley was significantly higher than that collected from Shada Mountain (Figure 2a). Similar trend was observed for root moisture percentage (Figure 2b). However, no significant differences were recorded in organic matter content of *S. persica* leaves and roots collected from both regions (Figure 2).

Different habitats in the studied regions had marked effects on primary metabolites concentrations as shown in Figure 3. Carbohydrates, proteins and amino acids concentrations in leaves of *S. persica* collected from Al-ahsabah valley were significantly higher than those collected from Shada mountain (Figure 3a). *S. persica* roots exhibited similar attitude where carbohydrates, proteins and amino acids concentrations in plants collected from Al-ahsabah valley were significantly higher than those collected from Shada Mountain (Figure 3b). Figure 3 displayed also that, amino acids represented the leaves main constituent while proteins represented the roots main constituent in plants collected from both studied regions.

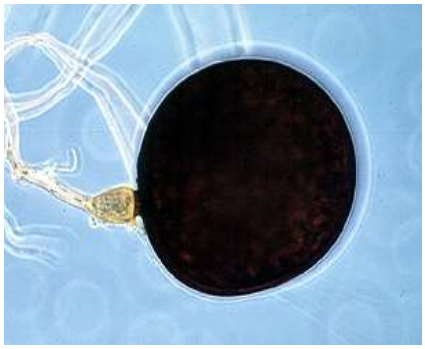
The effect of the studied habitats on some secondary metabolites composition of *S. persica* roots crude extracts, retention time in minutes, molecular weight and relative peak area of identified compounds were summarized in Table 4. These obtained results are presented in their elution order on GD-5MS column. GC-MS chromatogram is presented in Figure 4. Four compounds were identified in *S. persica* roots (Figures 5 to 8).

In crude extract of *S. persica* roots collected from Al-Ahsabah valley, three compounds were identified, namely (benzene, 1-isocyano-2-methyl-), benzene, isothiocyanatomethyl)-), and (butylated hydroxytoluene) (Table 4). In extract of *S. persica* roots collected from Shada mountain, the same compounds were identified, however Benzene, 1-isocyano-2-methyl- was substituted by Benzyl nitrile. In both extracts, the most abundant compound was benzene, (isothiocyanatomethyl), its peak area was about 99% (Table 4).

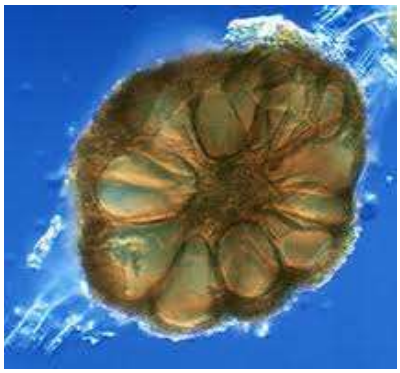
DISCUSSION

Meswak is frequently used for brushing teeth in the Middle East, including Saudi Arabia (Ahmad and

Glomus macrocarpus



Glomus mossaea



Sclerocystis clavispora

Glomus fasiculatum

Figure 1. The most dominant mycorrhizal spores identified in both habitats.

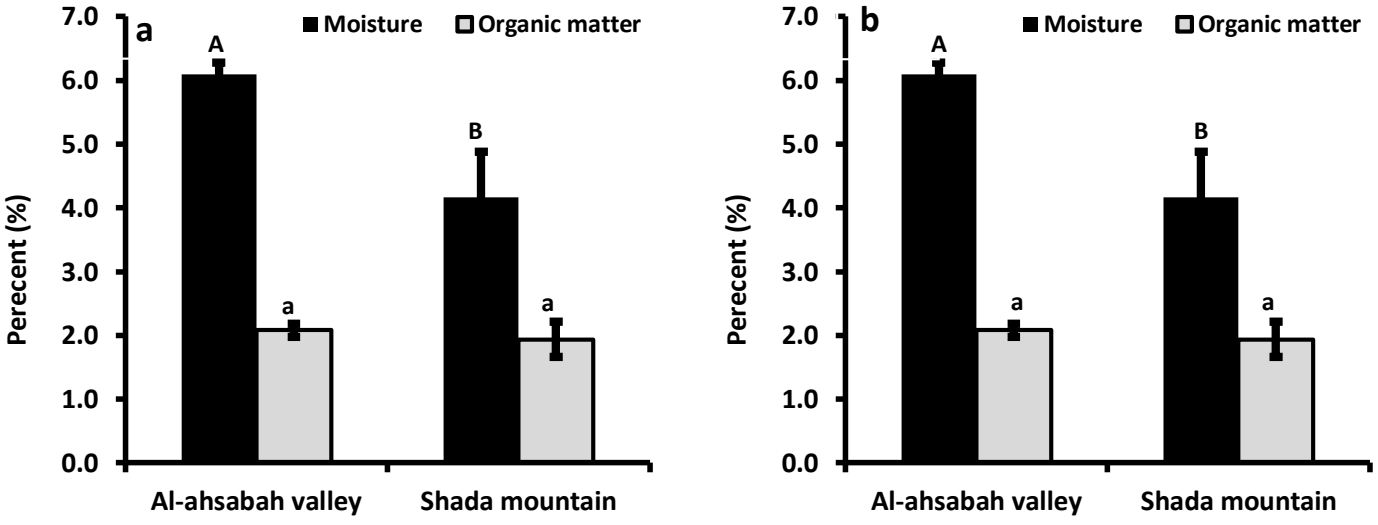


Figure 2. Moisture and organic matter percentage (%) of *S. persica* (I) leaves and (II) roots as affected by the studied habitats. Each point is a mean of three replicates ± SE. The letters A and B represent the statistical significance of each treatment on moisture percentage at P<0.05; a and b similarly represent those of organic matter percentage.

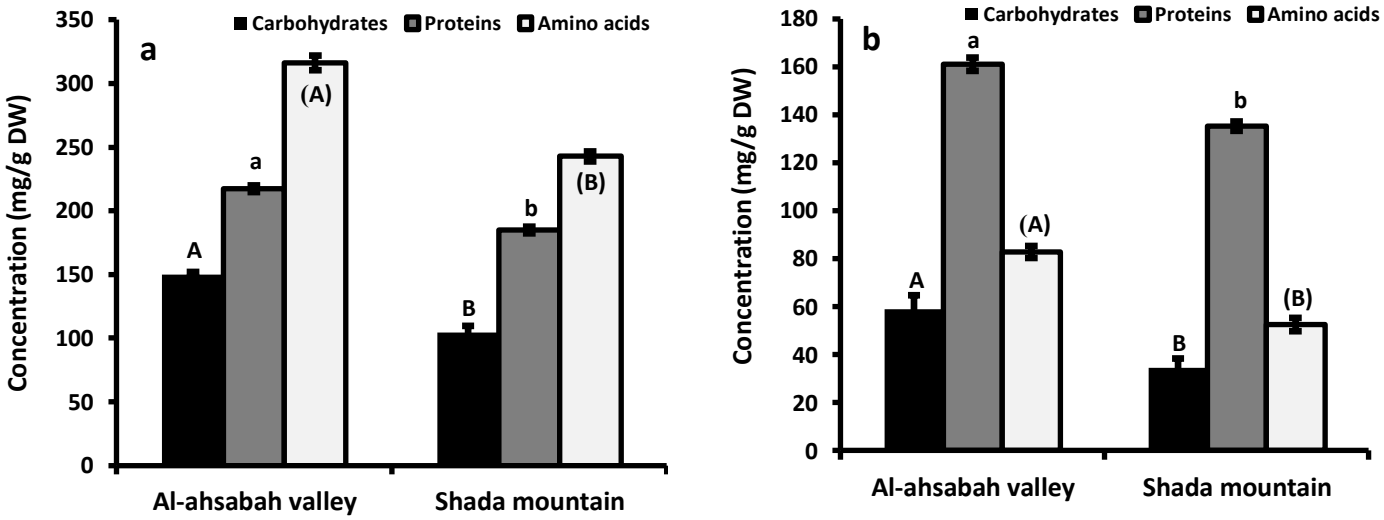


Figure 3. Soluble carbohydrates, proteins and amino acids contents (mg/g DW) of *S. persica*(I) leaves and (II) roots as affected by the studied habitats. The letters A and B represent the statistical significance of each treatment on carbohydrates content at P<0.05; a and b similarly represent those of proteins content, while (A) and (B) represent those of amino acids content.

Table 4. Some secondary metabolites identified in *S. persica* roots crude extract by GC-MS analysis.

Compound	Retention time (minute)	Molecular weight (g)	Peak area (%)	
			Al-Ahsabah valley	Shada mountain
Benzyl nitrile	12.48	117	-	0.72
Benzene, 1-isocyano-2-methyl-	13.13	117	0.14	-
Benzene, (isothiocyanatomethyl)-	21.72	149	99.79	99.04
Butylated hydroxytoluene	26.82	220	0.07	0.24

Rajagopal, 2014). The plant is also found indifferent habitats including valleys, termite mounds and dunes and at an altitude up to 1800 m (Kumar et al., 2012). The studied regions showed different edaphic factors. Al-ahsabah valley soil consists mainly of coarse sand and silt in comparable values. Coarse sand represents the most dominant soil fraction in Shada Mountain. The percentage of silt and clay soil in Al-ahsabah valley are significantly higher than that in Shada mountain, while coarse and fine sands in Shada mountain are significantly higher than that in Al-ahsabah valley. Al-Ahsabah valley soil has significantly higher soil moisture and organic matter contents compared to Shada Mountain, while both of them showed slightly alkaline soils with pH values 7.83 to 7.53. These results supported by the finding of Kumar et al. (2012), who reported that *S. persica* found on clays, loam, black soils and sand. Sujata (2015) concluded that, *S. persica* is adapted to alkaline, non-saline or very saline soils.

Arbuscular mycorrhizal fungi can strongly influence the metabolism of their host plant (Fontana et al., 2009). These are fungal symbionts that are well known to improve plant nutritional status by enhancing the uptake of essential nutrients and by improving the water supply through an increase in root surface area (Smith and Read, 1997). It also enhances secondary metabolites content (Lu et al., 2015). Both habitats showed variation also in the total count and distribution of arbuscular mycorrhizal fungi. Soil total count of mycorrhizal spores and intensity of root colonization in Shada mountain were significantly higher than those in Al-ahsabah valley. In Al-ahsabah valley four AMR species were defined, while in Shada Mountain only three species were defied. This variation could attribute to the differences in soil moisture and organic matter contents of both regions and could influence the growth and chemical constituents of *S. persica* collected from these regions. Fitter et al. (2000) and Jeffries et al. (2003) concluded that, AMF are found everywhere in land ecosystems and in a variety of climate and soil. Soil pH, mineral constitution and soil

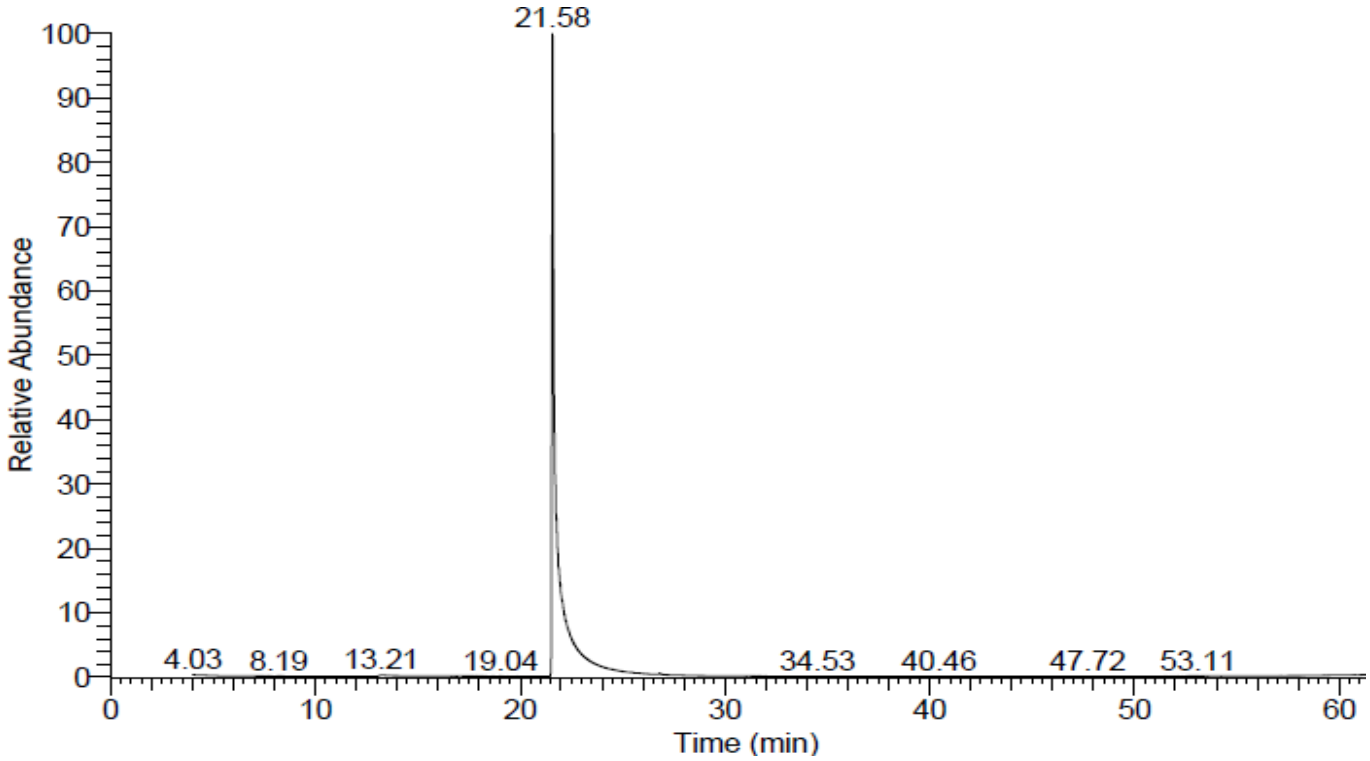


Figure 4. GC-MS chromatogram of *S. persica* root crude extract.

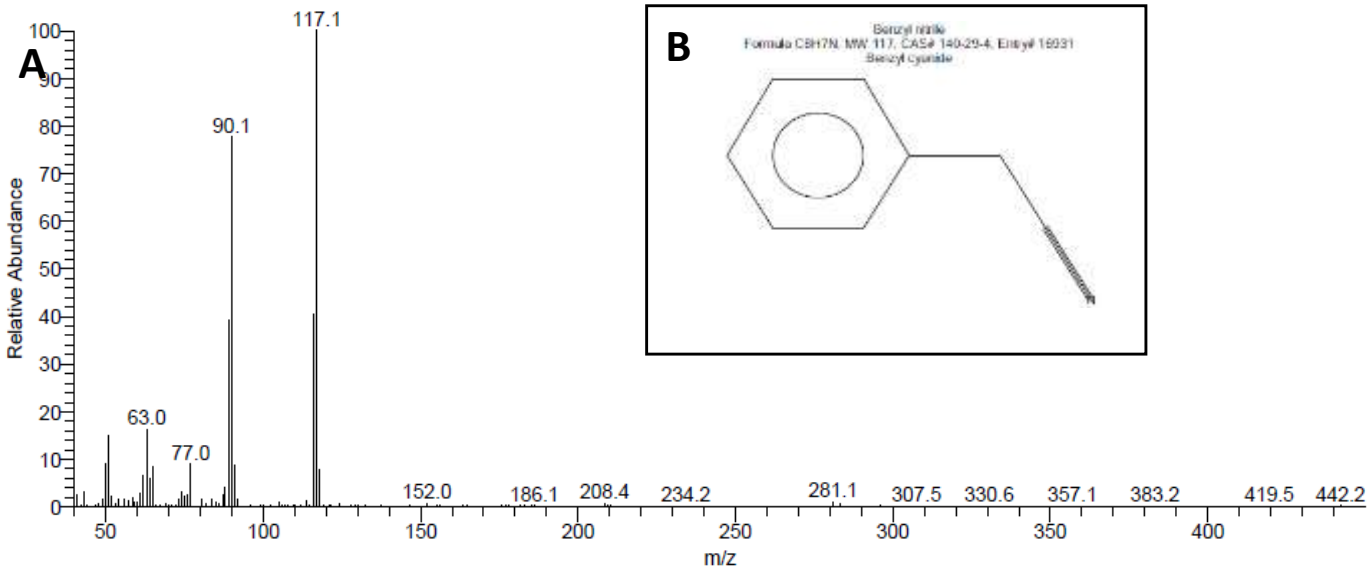


Figure 5. Benzyl nitrile (A) mass spectra, (B) formula.

type has been reported to affect the degree of specialization exhibited by AMF (Klironomos et al.,1993) as well as their growth and colonization (Abler, 2004). Many reports have shown that AMF play an important role in helping plants withstand water deficiency (Augé, 2001; Augé, 2004; Aroca et al., 2008). In general, soil characteristics of such as its texture, pH, presence of microaggregates, main cations and organic matter, can

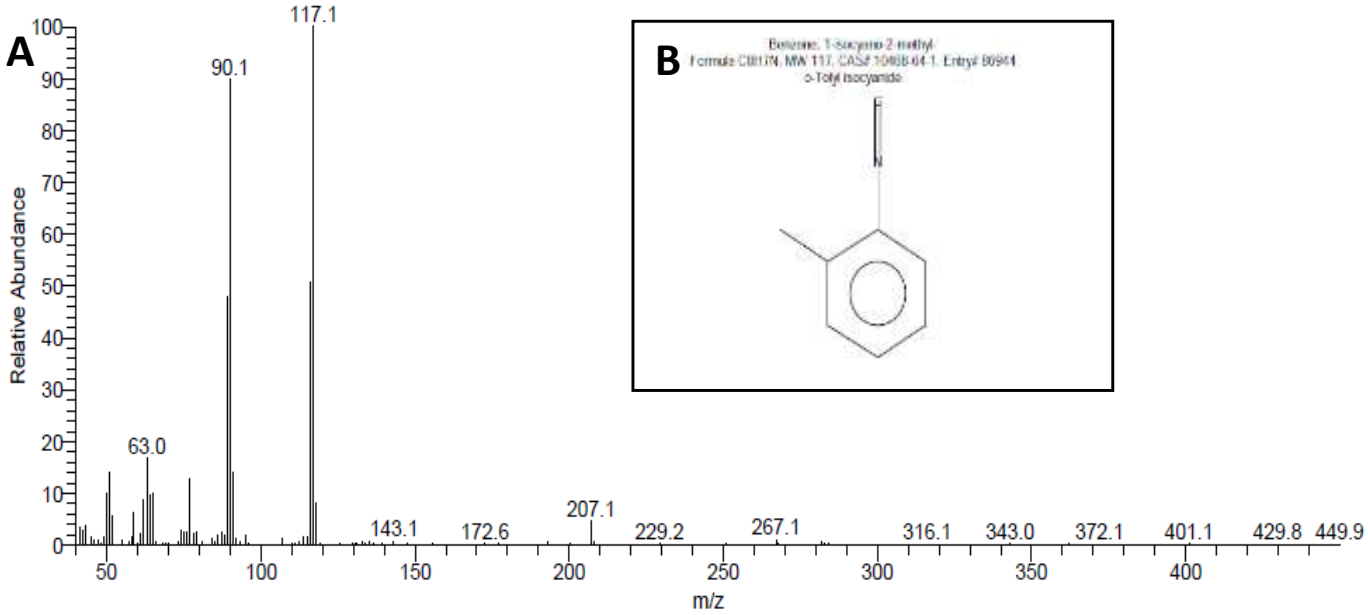


Figure 6. Benzene, 1-isocyano-2-methyl (A) mass spectra, (B) formula.

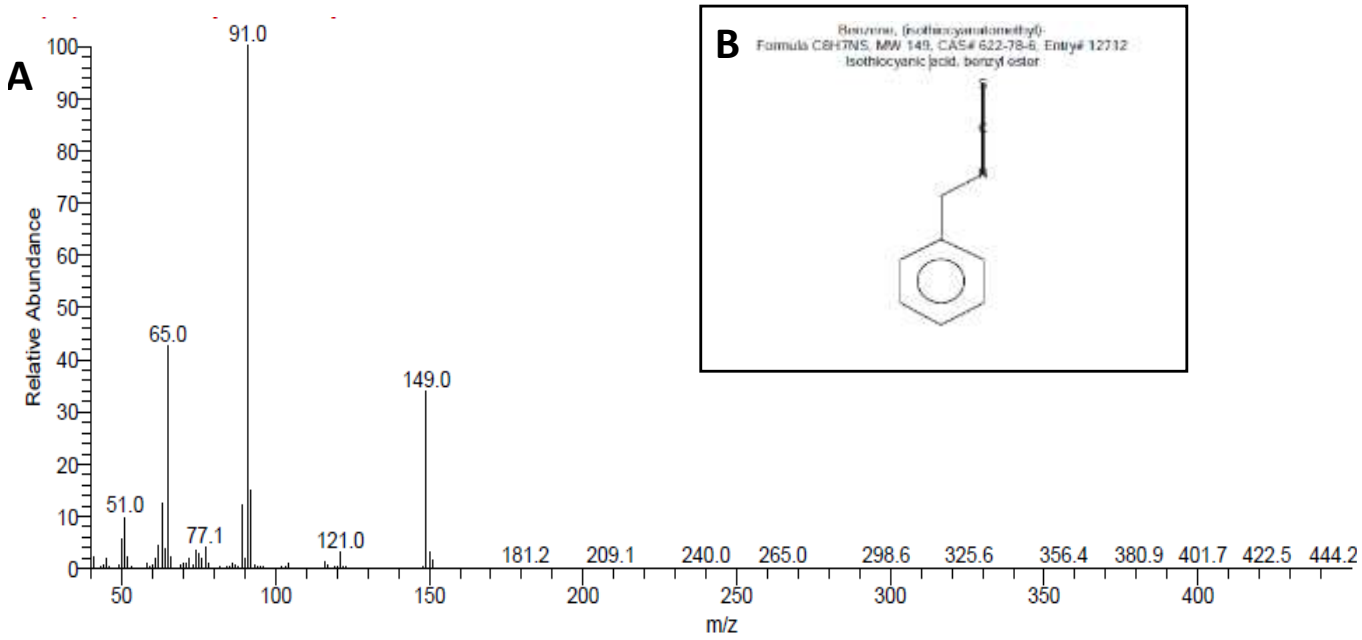


Figure 7. Benzene, (isothiocyanatomethyl) (A) mass spectra, (B) formula.

directly or indirectly influence rhizospheric community (Garbeva et al., 2004).

Different habitats in the studied regions have marked effects on leaves and root moisture content and primary metabolites concentrations. Moisture percentage and carbohydrates, proteins and amino acids concentrations in leaves and roots of plants collected from Al-ahsabah valley in significantly higher than those collected from Shada Mountain. The results of the present study displayed also that leaves body is composed of amino acids while root body is composed mainly of proteins in plants collected from both studied regions. In accordance

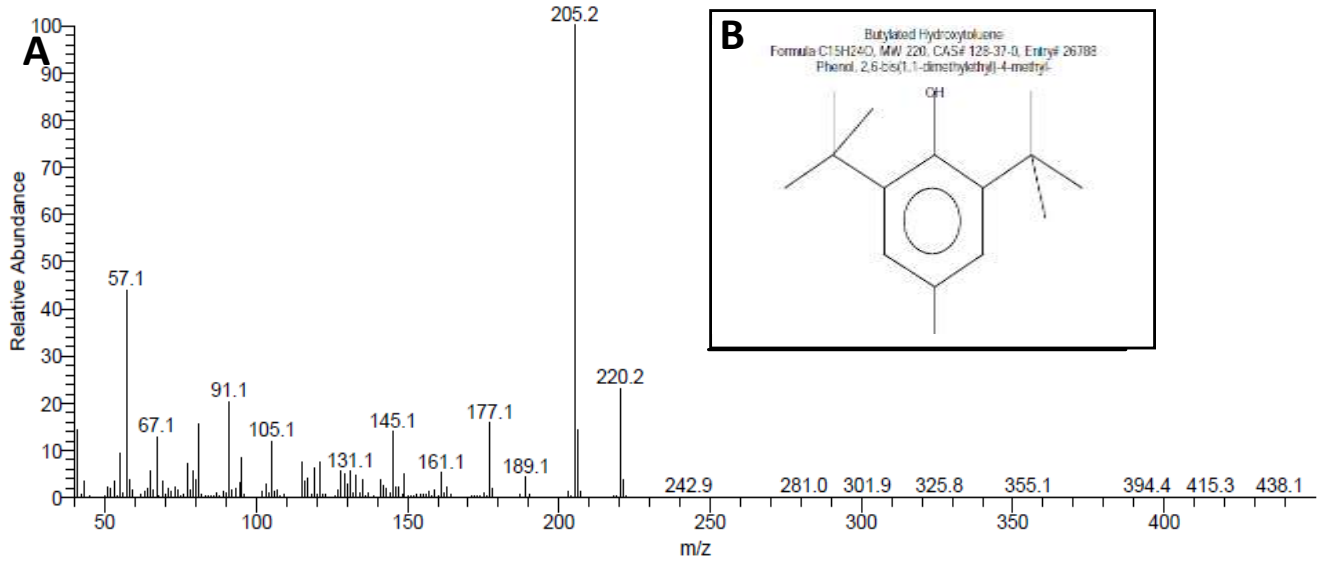


Figure 8. Butylated hydroxytoluene (A) mass spectra, and (B) formula.

with our results, other studies (Bharucha and Rangnekar, 1957; Chaturvedi and Maheshwari, 1980; Joshi et al., 1993) demonstrated that *S. persica* leaves contain high level of different amino acids including glutamic acid, asparagine, valine, aspartic acid, tyrosine, phenylalanine, serine, alanine and threonine.

Traditionally all parts of *S. persica* could be used for many medicinal purposes because of its high content of different secondary metabolites that could be used in the treatment against bacterial and fungal infections and have wide range of health care properties (Arora and Gupta, 2011; Sher et al., 2011; Mohamed and Khan, 2013; Gupta et al., 2015; Kumari and Parida, 2016). In the current study, the studied habitats affect the secondary metabolites composition of *S. persica* roots extract. In crude extract of *S. persica* roots collected from Al-ahsabah valley, Benzene, 1-isocyano-2-methyl- and benzene, (isothiocyanatomethyl), and butylated hydroxytoluene were recorded. In extract of *S. persica* roots collected from Shada mountain, benzyl nitrile was added while benzene, 1-isocyano-2-methyl- disappeared. In both extracts, principal compound was benzene, (isothiocyanatomethyl). benzylisothionate was the main constituent of *S. persica* root extract in many other studies (Christy et al., 2001; Arora and Gupta, 2011).

Benzyl isothiocyanate suggested acting as chemo-preventive agents (Al-Dosari et al., 1992), prevent genotoxic compounds (Attar, 1979), has exhibited broad-spectrum bactericidal activity (Al-Lafi and Ababneh, 1995). Benzylisothio-cyanate and related compound from *S. persica* was reported also to have inhibition of carcinogenic effects of polycylic hydrocarbons (Wasimuzzama et al., 2010), and also shows virucidal activity against Herpes simplex virus-1 (Al-Bagieh, 1992). Butylated Hydroxytoluene is a phenol derivative that is useful for its antioxidant properties (Babu and Wu, 2008; Yehye et al., 2015).

Conclusion

Soil total count of mycorrhizal spores and root colonization percentage in Shada Mountain was significantly higher than that in Al-Ahsabah valley. Moisture content, carbohydrates, proteins and amino acids concentrations in leaves and roots of plants collected from Al-ahsabah valley was significantly higher than that collected from Shada Mountain. Benzene, (isothiocyanatomethyl) was the most abundant analyt in root extract of *S. persica* collected from both regions. Different habitats in the studied regions affect the concentration of its primary metabolites more than secondary compounds. Further study to evaluate the differences in biochemical activity of phytochemicals isolated from plants collected in both regions is needed.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Nested polymerase chain reaction (nPCR) based diagnosis of bovine leukemia virus in Panama

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The bovine leukemia virus is an exogenous retrovirus that causes enzootic bovine leukosis. The aim of this study was to apply and compare a diagnostic test in an outbreak of bovine leukemia virus by nested polymerase chain reaction (PCR) in a core conservation of native cattle Guaymí. From the results obtained by the three techniques used, the agar gel immunodiffusion (AGID) test detected 33 positive animals. The nested polymerase chain reaction (nPCR) tested blood and enzyme-linked immunosorbent assay (ELISA) detected more positive animals than AGID with 17 and 30%, respectively. Animals positive to the ELISA and AGID test but negative to nPCR could be attributed to the existence of animals with genotypes of BoLA-DRB3.2 of major histocompatibility complex class II alleles with favorable resistant to bovine leukaemia virus (BLV). The possibility of further studies on resistance against BLV can be done. It is concluded that the ELISA and nPCR are the diagnostic tests of option for BLV.

Key words: Biotechnology, bovine enzootic leukemia, electrophoresis, Guaymi.

INTRODUCTION

The bovine leukemia virus (BLV; family *Retroviridae*; subfamily *Orthoretrovirinae*, genus *Deltaretrovirus*) is an exogenous retrovirus that causes enzootic bovine leukosis (EBL), the most common malignancy of cattle worldwide (Schwartz et al., 1994; Dequiedt et al., 1999; Beyer et al., 2002; Moratorio et al., 2013). This virus is related to the human *T-lymphotropic* virus types 1, 2 and *3* (HTLV-1, -2 and -3) and to the primate *T-lymphotropic* virus types 1, 2 and 3 (PTLV-1, -2 and -3) (Gelmann et al., 1983; Tanaka et al., 1990; Heneeman et al., 2012). Horizontal transmission of the infection occurs through the transfer of infected cells by direct contact, milk ingestion and possibly by hematophagous insects (Ferrer et al., 1979; Gillet et al., 2007). Vertical transmission (mother-child) via the uterus has also been demonstrated (Ferrer et al., 1979; Van der Maaten et al., 1981; Romero

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et al., 1983; Lassauzet et al., 1991). Dairy herds are more prone to infection due to the constant management of the animals and the frequent iatrogenic transmission caused by fomites such as milking equipment, surgery, equipment, needles and rectal palpation (Birgel Junior et al., 1995). Although the B lymphocyte is known to be the primary target of the virus, research has shown that monocytes, granulocytes, T cells and $CD2^+$, $CD3^+$, $CD4^+$, $CD8^+$and γ/δ cells are also target cells of the virus (Williams et al., 1988; Stott et al., 1991; Schwartz et al., 1994; Mirsky et al., 1996). Another study by Panei et al. (2013) showed that B lymphocytes, $CD5^+$cells and IgM^+ cells maintain the highest BLV proviral load, but in subclinical states, T lymphocytes, $CD4^+$cells and $CD8^+$ cells are also observed as primary targets. BLV infection may remain clinically silent in the so-called aleukemic (AL) stage. However, in 30% of the infected animals, infection can be presented as a persistent lymphocytosis (PL), particularly with an increase in B lymphocytes. Between 1 and 5% of cases can be presented as B-cell lymphoma after a long latency period (Panei et al., 2013) caused by virus suppression (Kettmann et al., 1980), which is a possible strategy to evade the immune response and allow tumor development (Merimi et al., 2007). Certainly, the provirus-carrying B lymphocytes do not produce detectable levels of viral RNA or proteins (Lagarias and Radke, 1989; Jimba et al., 2012). Bovine leukemia virus (BLV) infection is associated with the natural emergence of B-cell tumors in bovine cattle and can be experimentally induced in sheep. Nevertheless, the complete understanding of how BLV induces tumorigenesis is still enigmatic, mainly because the majority of these tumor cells are positive for an integrated proviral genome of BLV, but they lack an abundant expression of transcriptomes or proteins encoded by RNA polymerase II (pol II) (Kettmann et al., 1985; Gaynor et al., 1996; Gillet et al., 2007).

According to Kettman et al. (1980), the lymphocytosis stage and tumor stage generally represent early and late disease stages, but these two pathological conditions do not necessarily affect the same herd, with separate responses as a result. This result suggests some genetic condition related to the response to the disease. Therefore, polymerase chain reaction (PCR), in addition to routine diagnostic tests such as agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA),is important for the diagnosis because PCR enables detection of the proviral genome integrated into the host genome (Panei et al., 2013). The three serological tests commonly used in the diagnosis of the disease are radioimmunoassay (RIA), AGID and ELISA. Dot Blot and PCR have been less widely used (Cockerell et al., 1992). Although AGID has been an indicator of BLV infection for decades and remains gold standard method in the Republic of Panama for the transport of animals inside the country, it has now been shown to be an extremely insensitive test compared to newer techniques (Buehring et al., 2003). During the 1990s, many cattle that were negative for anti-BLV antibodies when tested by AGID were shown by immunoblot or ELISA to be positive (Walker et al., 1987; Have and Hoff, 1991; Grover and Guillemin, 1992). Have and Hoff (1991) found that ELISA was 50 to 100% more sensitive than AGID. In addition, Walker et al. (1987) and Grover and Guillemin (1992) showed that AGID failed in 53 and 70% of cases, respectively, in the detection of anti-BLV antibodies in immunoblot-positive cattle. Although RIA and ELISA are more sensitive than AGID, the latter has the advantage of being less expensive and easier to perform (Have and Hoff, 1991); however, the detection failure of AGID would be catastrophic, particularly for the bovine dairy industry, when animals are transported from areas with high prevalence of BLV to disease-free areas. ELISA is superior when there is a need to analyze many milk samples because it has the ability to detect low levels of anti-BLV antibodies (Nguyen and Maes, 1993). AGID cannot differentiate passively acquired antibodies (colostral) from those acquired by natural infection (Hugh-Jones, 1992). Another disadvantage of these techniques is that they cannot detect infected young animals or animals in the early stages of infection. PCR has been used for the early detection of EBL in animals less than six months old (Agresti et al., 1993; Kelly, 1993), thus avoiding the false positive reactions caused by passive immunoglobulin transfer through colostrum. Another advantage of PCR is its ability to detect the virus in immune-tolerant animals (Fechner et al., 1997). The BLV is one of the diseases reported by official authorities in most of their epidemiological reports (Real, 2008). However, there is no program to control and eradicate this disease. The aim of this study was to apply and compare nested PCR (nPCR) with serological tests ELISA and AGID in the diagnosis of an EBL outbreak of Guaymí creole cattle breed. This comparison would allow the selection of the test or diagnostic tests to be used in herds where the conservation of germplasm *in vivo* is the priority.

MATERIALS AND METHODS

The study was carried out in the laboratory of Agro-biotechnology at the Instituto de Investigación Agropecuaria de Panamá, Carretera Panamericana, km 214, Divisa, Provincia de Herrera. Five milliliter blood samples were drawn and placed into ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes. These samples were retrieved from a leukosis outbreak in five conservation nuclei of Guaymí creole bovine breeds. The samples were taken from 33 females and seven bulls that were positive with AGID test, the gold standard method of the Ministry of Agriculture laboratory, for the disease out of 96 animals that make up the original nucleus. ELISA, AGID and nPCR techniques were used for the analysis. For the AGID test, the PORQUIER® IDG kit for Bovine Leukosis was used, and the IDEXX Leukosis *Blocking* anti-gp51 ELISA test was used in the animal health laboratory of the

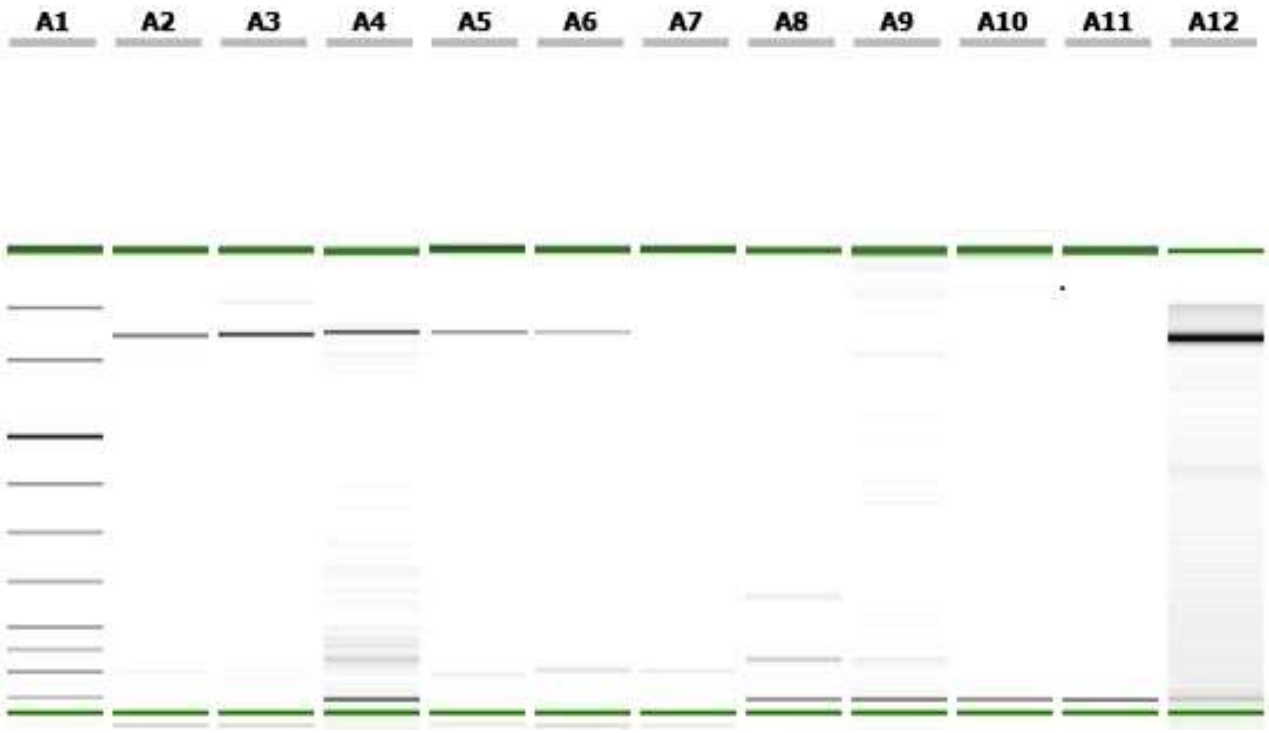

Figure 1. Capillary electrophoresis of Guaymi cattle (A1, marker; A2-A6, positive animals; A7-A10, negative animals; A11, negative control; A12, positive control).

Agricultural and Cattle Research Institute (IDIAP) Division. To apply the nPCR technique, a separation protocol of mononuclear cells (PBMCs) was first performed by adding three milliliters of Histopaque® 1077 Sigma-Aldrich in three millilitersof venous blood to an equal volume of phosphate-buffered saline (PBS), followed by centrifugation at 3500 *g* for 30 min. To extract the largest amount of white cells within each sample, a protocol was applied where two milliliters of the PBMC suspension was extracted and centrifuged at 3500 *g* for 10 min. The supernatant was discarded, and the pellet was stored in the bottom of the microtube for further processing. DNA extraction was performed using the commercial reagent QuickExtract™ from Epicenter and applying it to the PBMC isolate. This process consisted of the application of 500 µl of the product, vortexing for 15 s and heating at 65°C for 6 min; then, the sample was vortexed again and the product was heated at 98°C for 2 mins. With this procedure, an average concentration of 218 ng/µl of genomic DNA was obtained.

nPCR was performed on the extracted DNA, in which a highly conserved region of the *env*elope (*env*) gene coding for the *gp51* capsid protein was amplified. The protocol used was a modification of that proposed by Beier et al. (2001). The first reaction was performed using a final volume of 30 µl, which included 70 to 100 ng of DNA, 0.5 Mm each of the Forward-*env*5032 (5'-TCTGTGCCAAGTCTCCCAGATA-3') and Reverse-*env*5608r (5'-AACAACAACCTCTGGGGAGGGT-3') primers, 0.2 mM of each dNTP, 1X PCR buffer, 1.5 mM $MgCl_2$ and 1 U of Taq DNA polymerase. In the second reaction, three microliters of the PCR product from the first amplification was used as the DNA template, along with the same concentrations of the other reagents and the Forward-*env*5099 (5'CCCACAAGGGCGGCGCCGGTTT-3') and Reverse-*env*5521r (5'GCGAGGCCGGGTCCAGAGCTGG-3') primers. The thermal profile included an initial denaturation step at 94°C for 9 min, followed by 35 cycles at 95°C for 30 s, 62°C for 30 s and 72°C for 1 min and ending with a final extension at 72°C for 4 min. In the second reaction, the amplification conditions were the same, except that the annealing temperature was increased to 70°C (Licursi et al., 2003). Identification of the animals that were positive for the provirus was performed using a QIAgen® capillary electrophoresis analyzer of DNA fragments. The presence of a band of 444 base pairs indicates that the provirus is present in the animal, using a QX DNA Size Marker 25 to 500 bp (50 µl) v2.0 from QIAgen®.

To determine the parameters of sensitivity, specificity and concordance (*kappa* value) of nPCR, AGID and ELISA contingency tables (2x2) were used with the WinEpi 2.0 computer program (de Blas et al., 2006). All analyses were performed with a 95% confidence level.

RESULTS AND DISCUSSION

The band of 444 base pairs can be observed in the fragment analyzer consistent with the BLV *env* gene. Of the 40 animals tested by nPCR, 32 animals were positive (80%) and eight animals (20%) were negative (Figure 1).

The results observed are similar to those reported by Beyer et al. (2002), who used PBMC separation and the *env* gene to amplify the BLV provirus. Similarly, Alfaro et al. (2012) used nPCR to detect the BLV provirus; however, they used the gene of the long terminal repeat

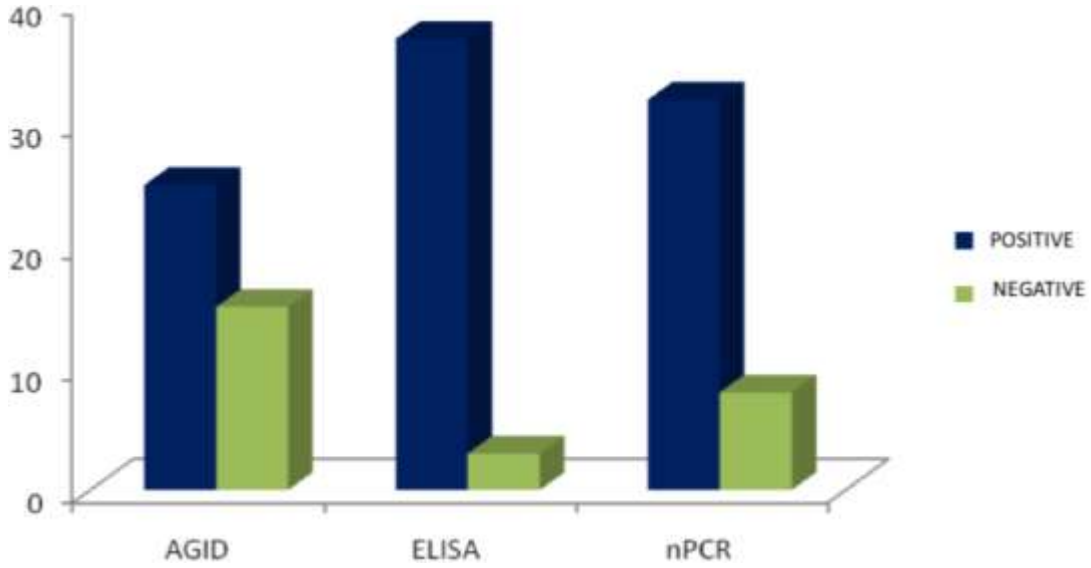

Figure 2. Analysis of the number of positive and negative animals identified with each of the AGID, ELISA and PCR tests in Guaymí cattle.

region (LTR). In the present study, the fragments analyzer was used with a cartridge of 12 capillaries, thus replacing the agarose gels that had been used in the laboratory in recent years. With this change of technology, fewer reagents were used, and less time was spent on the analysis. For each 96-well plate, the equipment use required 48 min, unlike with the agar gel, which required 2 h per run and did not include the time required to add the loading gel to the amplicons and the base pair marker as well as load the samples on the gel. The nPCR technique has also been compared by Lew et al. (2004) and Heenemann et al. (2012) with the real-time PCR technique, with advantage being observed in one methodology over the other in the detection of the provirus, optimized qPCR can detect less than 10 copies. Nevertheless, Jimba et al. (2012) used primers with the Coordination of Common Motif (COCOMO) algorithm to measure the proviral load of new and known variants in BLV-infected animals and were able to detect lower proviral loads than any other currently used PCR techniques. Clearly, real-time PCR has greater advantages relative to nPCR; it is a shorter procedure with a reduced risk of cross-contamination and provides a quantitative assessment of viral load. However, nPCR is still very useful and requires, such as real-time PCR, the use of other immunological techniques such as ELISA since there are variations in the kinetics of the proviral load and in the responses of individuals to the virus. These differences are attributable to the different BoLA-DRB3 genotypes that these animals possess (Jimba et al., 2012). On the other hand, Buerhing et al. (2014) observed a decrease in the gag, *pol* and *env* sequences in BLV-positive samples and in the presence of LTR and Tax sequences, the lack of gag, pol, and *env* sequences in some of the BLV-positive panel samples and the presence of LTR and tax sequences in all of them is consistent with results reported for the closely related HTLV-1, particularly when leukemia progressively increases (Buehring et al., 2014). These deletion mechanisms are postulated as closely related to the virus evasion from the host immune response, so it would be expected that, thereafter, primers of the LTR and Tax sequences should be used for better accuracy in the diagnosis.

From the results obtained from these three techniques, the standard test of the Republic of Panama, AGID, detected a lower number of positive animals than ELISA and nPCR in blood samples (Figure 2).

A comparison of this study with Felmer et al. (2006), Fernandes et al. (2005), Buehring et al. (2003) and Lamas et al. (2012) showed similar results, indicating that AGID has a lower capacity to detect positive animals compared to PCR and ELISA, which would represent a potential risk for the spread of virus within the Republic by allowing the passage of infected animals.

Of the 40 animals analyzed, AGID detected 25 positive animals, equivalent to 63% of the total number of animals (Table 1). On the other hand, nPCR identified 80% of the BLV-positive animals, 17% more than AGID: Four nPCR-positive animals were negative for AGID, and 11 of the 15 AGID-negative animals were positive for nPCR. These results are similar to those observed by Gregory et al. (2004) in Brazil and Felmer et al. (2006) in Chile who reported 63% of BLV-positive animals, having also used

Table 1. Analysis of sensitivity, specificity and concordance of blood PCR in relation to AGID of Guaymí creole.

Test	Result	PCR		Total
		+	-	
AGID	+	21	4	25
	-	11	4	15
Total		32	8	40

Sensitivity: 66%; specificity: 50%; concordance: *kappa* 0.12.

Table 2. Analysis of sensitivity, specificity and concordance of blood PCR in relation to ELISA of Guaymí creole.

Test	Result	PCR		Total
		+	-	
ELISA	+	30	7	37
	-	2	1	3
Total		32	8	40

Sensitivity: 94%; specificity: 13%; concordance: *kappa* 0.082.

AGID, the technique that detected the least number of positive animals. The concordance between the two tests was 63%, whereas the kappa statistic was 0.12, which is considered weak. The diagnostic sensitivity was 66%, whereas the specificity was 50% attributable to AGID.

Of the 40 animals tested, ELISA detected 37 positive animals, equivalent to 93% of the total number of animals (Table 2). On the other hand, ELISA detected 30% more positive animals than AGID and 13% more than nPCR: seven ELISA-positive animals were negative for PCR, and two of the three ELISA-negative animals were positive for nPCR. The concordance between the two tests was 78%, whereas the kappa statistic was 0.082, which was considered weak. The diagnostic sensitivity was 94%, whereas the specificity was 13%. When comparing AGID and ELISA, a kappa value of 0.238 was considered weak but significant, with a sensitivity of 68% and a specificity of 100%.

These results differ from those observed by Felmer et al. (2006) in Chile who reported similar results for nPCR and ELISA in terms of the diagnostic capacity; however, Gregory et al. (2004), in Brazil, reported variable results. ELISA, in the present study, was able to identify 93% positive animals, and the two nPCR tests, which followed the methodology of Ballagi-Pordany et al. (1992) and used the modified test of Beier et al. (2001), were able to identify 13 and 90% of positive animals.

The present study evaluated the use of a modified nPCR method proposed by Beier et al. (2001), as a direct test and compared its results with AGID and ELISA as indirect tests for the detection of BLV in a group of Guaymí creole bovine breeds in a conservation program. According to the results, blood PCR and serum ELISA detected a higher number of positive animals (17 to 30%) than AGID. All serum ELISA-negative samples were also negative for AGID. However, four PCR-negative samples were identified as positive with AGID, and seven nPCR-negative samples were identified as positive with ELISA, which could have several possible explanations. The most common reasons for these discrepancies have been discussed by Eaves et al. (1994), who attributed them to the absence of lymphocytes in blood, and by Marsolais et al. (1994), who attributed them to variations in the nucleotide sequences and a decrease in the sequences of the gag, *env* and *pol* genes as an effect of the evasion of the immune system, which was reported by Buehring et al. (2014) and which, in turn, could prevent the recognition of primers at the time of banding in some viral strains or virus restrictions to the lymphoid organs as reported by Klintevall et al. (1994). However, the amount of PBMC used from the collected blood (2 ml) and the methodology applied increased the availability of lymphocytes; at the same time, nPCR was more effective than other traditional PCR methodologies. The effectiveness of nPCR has been compared with real-time PCR by Lew et al. (2012) and Heenemann et al. (2012). The animals that were positive for nPCR but negative for serological tests indicate the possibility of finding immune-tolerant or low-immune-response animals (Fechner et al., 1997; Jimba et al., 2012), although the virus itself is known to have mechanisms for evading the host immune response (Merimi et al., 2007), which is not surprising. Another striking result in the present study is the low kappa values observed in the three tests analyzed, a result that differs from those observed by Felmer et al. (2006) and Lamas et al. (2012), who found

a moderate statistical correlation between the tests used (Cerda and Villarroel, 2008). However, Jimba et al. (2012), when analyzing the same serological tests (AGID and ELISA) with the PCR tests, found a low correlation among the tests and attributed this result to the differences between humoral and viral kinetics in experimentally infected animals. Likewise, animals that were positive for immunological tests (AGID and ELISA) but negative for nPCR could be the result of animals with BoLA-DRB3.2 genotypes of the major histocompatibility complex type II, which are favorable for BLV resistance (Lewin et al., 1988; Aida 2001; Esteban et al.; 2009; Jimba et al., 2012). There are several author that describe alleles of the BoLA gene that could be associated to the resistance or susceptibility to BLV dissemination; as an example, Nikbakht et al. (2016) in Holstein cattle, Miyasaka et al. (2013) in Black Japanese cattle.

On the other hands, most of the authors are not conclusive about the results, and suggest the presence of some other genetic or epigenetic factors that could influence the viral spread in infected animals (Juliarena et al., 2013; Ohno et al., 2015; Farias et al., 2016).

The breed used in the BLV test in the present study, the Guaymí, is one of the two Panamanian creole breeds, descendants of races brought by the Spaniards beginning in the 15th century and on which genetic diversity studies have previously been conducted (Villalobos et al., 2010; Delgado et al., 2012; Martínez et al., 2012; Ginja et al., 2013). Studies have shown that creole breeds present various disease resistance genes (Mirsky et al., 1998; Martínez et al., 2005), so that it is possible that some of the animals may be carriers of disease-resistance genes and could be included in BLV control and eradication programs through genetic marker-assisted selection (Esteban et al., 2009). Although it has not been demonstrated in this population, the hypothesis is raised that it possesses favorable genes of DRB3.2 that can be used in programs of crossing that includes tolerance to diseases. Therefore, other studies should be carried out where this hypothesis is demonstrated.

Conclusions

The following conclusions are drawn from the study:

1. The use of the AGID technique for the diagnosis of EBL should be discontinued.
2. nPCR and ELISA should be the techniques of choice for the diagnosis of EBL for the conservation of nucleus of Guaymí creole bovine breeds; the use of these techniques in the official laboratories of the Republic of Panama is recommended.
3. Genotyping studies should be carried out to demonstrate the hypothesis that Guaymi creole cattle population possess diseases resistant genes

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Proteins patterns of eight genera of the Asteraceae family

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Asteraceae family grows as wild plants in the eastern province of Saudi Arabia near the Gulf coast with prevalent high humidity coupled with more moderate temperatures. Eight genera were collected during the flowering season to study genetic diversity according to protein patterns for each plant. The protein patterns showed different numbers of bands, concentrations, molecular weight and intensity. Two common bands were observed to ensure the existence of fixed gene and it can also be considered as a marker of the studied genera. Protein profiles revealed genetic diversity among these species for adaptation to environmental factors. Similarity coefficient was high for *Sonchus oleranceus* and *Senecio desfontainei,* while low for *Launaea capitata* and *Osteospermum vaillantii* among the studied plants. The hierarchical cluster analysis, formed two major clusters, which indicates the existence of genetic diversity among the studied genera. The first cluster was divided into two sub-clusters. Sub-cluster A comprised three species, *Scorzonera papposa* , *Senecio desfontainei* , *and Sonchus oleranceus*; and the second sub-cluster B comprised four species *Anthemis melampodina*, *Echinops hussoni*, *Launaea capitata*, and *O. vaillanti*. The second cluster contained only one species *Artemisia inculta,* which suggests that the Asteraceae family may have more than one evolutionary line.

Key word: Asteraceae, genetic diversity, protein patterns.

INTRODUCTION

The eastern province of Saudi Arabia, near the Gulf coast is rich of wild annual plants especially during the cold seasons (January to April). The climate of Saudi Arabia described by Country Profile: Saudi Arabia (2006) differs greatly between the coast and the interior; high humidity coupled with more moderate temperatures is prevalent along the coast, whereas aridity and extreme temperatures characterize the interior.

Asteraceae is one of the largest families of flowering plants with a world-wide distribution and with more than 1,600 genera and over 24,000 species of herbs, shrubs and trees (Funk et al., 2009). Most Asteraceae (Compositae) family are wild plants in Saudi Arabia. Dempewolf et al. (2008) reported that it is one of the largest families of flowering plants in Saudi Arabia. Also, Al-Farhan (1999) reported that Asteraceae is one of the

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major families in Saudi flora with 222 species and the distribution of life form is closely related to topography and landform (Fakhireh et al., 2012). Some of the Asteraceae plants were and still used in folk medicines and many researchers investigated their medical effects and pharmacological characteristics. Results indicate that some plants are pharmacologically important (Tariq et al., 1987; Elsharkawy et al., 2014), for example *Artemisia*, *Launaea*, *Cichorium*, *Anthemis* and *Sonchus* species.

Most of the taxonomist identifies plant species on the basis of phenotype characters of plants like root, stem and leaf structures (Lifante, 1991). New techniques in molecular biology are considered excellent assessment tools to identify differences among plants. Electrophoresis analysis was used to identify varieties and in evaluating genetic diversity (Sharma and Maloo, 2009). This study aimed to investigate the genetic relations and diversity on the basis of proteins patterns of some wild genera of the Asteraceae family, which grows in the eastern province of Saudi Arabia.

MATERIALS AND METHODS

Description of collected plants

Plants samples of eight genera of Asteraceae family were collected during the flowering season from the central and northern areas of the eastern region of Saudi Arabia. Asteraceae family belongs to order: Campanulales that are generally herbs, rarely woody, and often with latex or oil-passages; inflorescence racemose, with a tendency to form heads. Flowers bisexual or unisexual, regular or zygomorphic, pentamerous with reduction in number of carpels and with one whorl of stamens. Anthers laterally united to form a tube into which the pollen is discharged; style developed into a brush by which the pollen is swept out; ovary inferior, plurilocular, with ∞-1 ovules in each locule or unilocuar with 1 ovule.

All plants described were according to the methods of Migahid (1978) and Mossa et al. (1987).

(1) *Anthemis melampodina*: Collected from the North of Eastern province. Small, annual, ash-coloured, densely grey-woolly desert sand herb. Leaves small pinnatifid, few-lobed or parted with mucronate lobes. Peduncles short not thickened. Heads 2 cm broad. Scales of involucre lanceolate to oblong the outer acute, the inner scarious-tipped. Ray florets white, often red-flushed. Achenes with rounded apex, without auricle. Outer involucral scales prominently scarious-margined, more than in the type.
(2) *Scorzonera papposa*: Collected from the North of Eastern province. Woolly, then glabrescent herb. Leaves often wavy margined, the lower oblong, the upper half clasping, linear. Heads 3 to 4 cm long. Scales of involucre white-margined. Flowers lilac. Achenes 1 cm long, muricate.
(3) *Echinops hussoni*: Stem glabrous, glossy, whitish-yellow. Leaves thin, soft, with flattened margins, with few deep, spiny-tipped lobes, densely cobwebby lanate beneath. Spines few, rather weak. Head with horn-like spines.
(4) *Launaea capitata*: Biennial herb, 5 to 15 cm, high with stiff, stout, scape-like, almost naked, simple or 2-forked stems emerging from a dense rosette of lyrate-cleft leaves. Heads short and thick, nearly sessile, densely-clustered towards the summit of the stem. Involucral scales broad, white-margined. Achenes beakless, broad, yellow, winged, flattened, retuse, short.
(5) *Senecio desfontainei*: Annual sweet smelling herbs, 25 cm high. Leaves fleshy, pinnatipartite into linear remotely toothed lobes, the lower leaves tapering into a short petiole, the rest clasping with minute auricles. Heads large, cup-shaped, 1 cm long and broad in loose corymbs with rather long ray florets. Achenes ribbed, hairy.
(6) *Osteospermum vaillantii*: Perennial plants, 40 cm high, glandular-hirsute, richly branched, with simple, entire or few-toothed, half-clasping, oblong-lanceolate leaves, and a few corymbose heads small, peduncled; scales of involucre, linear. Achenes broadly scarious winged.
(7) *Sonchus oleranceus*: Annual, rarely biennial, herbaceous weed. Stems erect, the young branches with glandular hairs, soft, hollow, stem and leaf milky juiced. Leaves alternate, oblong, acute, 10 to 12 cm long, pinnately parted, margins irregularly tooth, the base auriculate and clasping the stem. Yellow flowers in terminal heads; involucre green, 12 to 15 mm long, the involucral bracts in 3 to 4 rows, oblonglanceolate. Flowers all ligulate, the corolla truncate at the apex, 5-toothed, the lower part tubular, with white hairs. Achenes narrow-margined, 3-nerved and 3-striate, 3 mm long, brown, oblanceolate rugose.
(8) *Artemisia inculta*: Collected from the North of Eastern province. Perennial grey-woolly shrub let with narrow lobed, long leaves. Flower heads brownish, ovoid, erect, densely clustered and sessile. Inner involucral scales linear.

Protein extraction and separation

Leaf samples were used to extract proteins, using Fisher bio-reagents sure-prep RNA/DNA/Protein purification kit. Automated electrophoresis from Bio-Rad with Experion pro260 analysis kit was used for protein separation, scanning and photograph, it is a system that uses a combination of caliper separation technology and sensitive fluorescent sample detection to perform rapid and automated analysis of protein by integrating separation, detection, and data analysis within single platform.

Data analysis

The formula of Nei and Lei (1979) was followed to consider the degree of similarity: $S_{ab}=2N_{ab}/(N_a + N_b)$, where, N_{ab}= number of bands common to both plants, N_a =number of bands in plants a, and N_b = number of bands in plants b. Dendrogram (hierarchical cluster) was used to construct plant samples according to the average linkage (between groups). The data analysis was done using SPSS-16.0 for Windows statistical package.

RESULTS

Table 1 and Figures 1 and 2 show total protein concentration, molecular weight, and number of bands of collected plants. The protein concentrations do not reveal the number of bands for some plants. Although, *A. melampodina* produced 15 protein bands at concentration of 1301.8 kDa, which is higher than that of *E. hussoni*, which produced 17 protein bands with protein concentration 1216.7 kDa, *L. capitata* produced 13 protein bands with protein concentration of 1155.4 kDa; *S. papposa* produced 8 proteins bands with concentration of 1184.7 kDa; *S. desfontainei* produced 11 proteins bands with concentration of 12114 kDa; *S. oleranceus* produced 10 proteins bands with total concentration of 370.9 kDa; *A. inculta* produced 9 proteins bands with

Table 1. Proteins molecular weight, concentrations and number of bands of eight genera of Asteraceae.

Samples	Molecular weight	Concentration	Number of bands
Anthemis melampodina	108.4	1301.8	15
Scorzonera papposa	89.84	1184.7	8
Echinops hussoni	160.1	1216.7	17
Launaea capitata	147.22	1155.4	13
Senecio desfontainei	173.6	1214	11
Osteospermum vaillantii	56.53	103.4	5
Sonchus oleranceus	127.22	370.9	10
Artemisia inculta	154.48	384.8	9

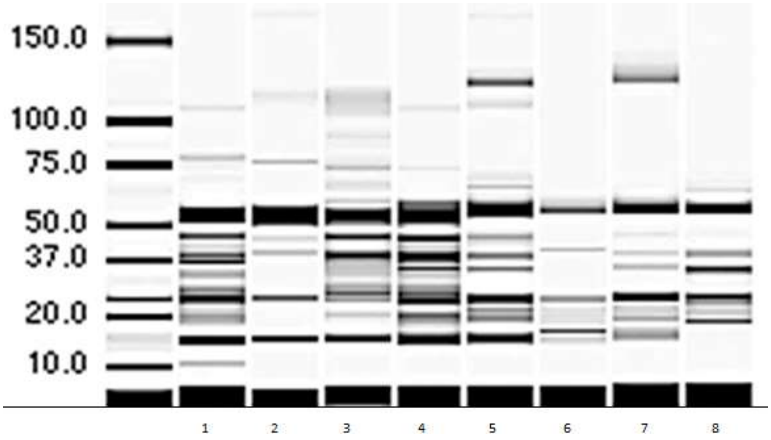

Figure 1. Automated electrophoresis of proteins bands of eight genera of Asteraceae family. 1, *Anthemis melampodina*; 2, *Scorzonera papposa*; 3, *Echinops hussoni*; 4, *Launaea capitata*; 5, *Senecio desfontainei*; 6, *Osteospermum vaillantii*; 7, *Sonchus oleranceus*; 8, *Artemisia inculta.*

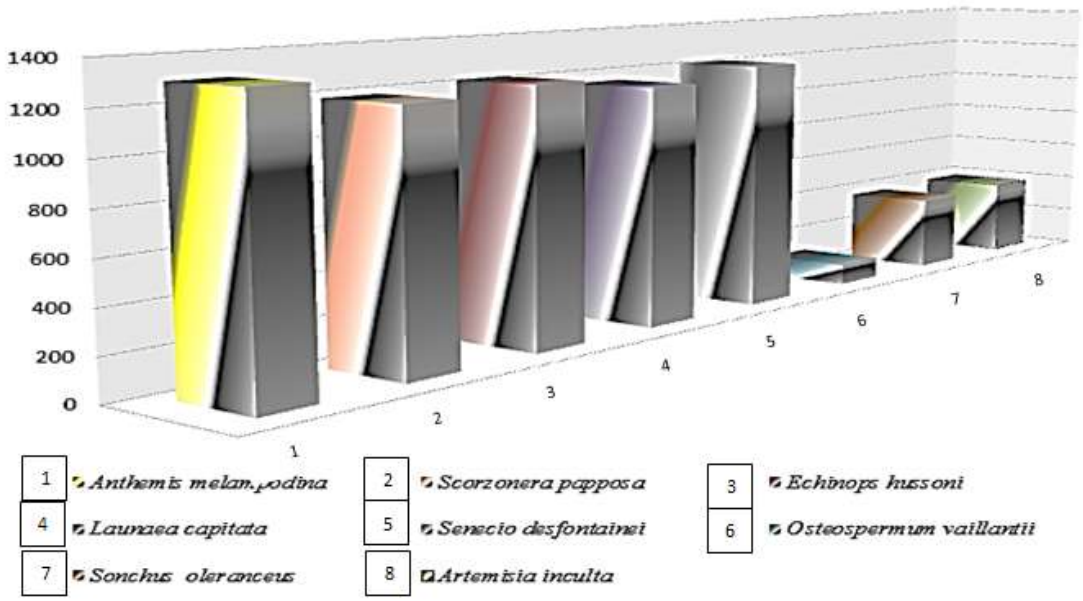

Figure 2. Proteins concentration of some genera of Asteraceae family.

Table 2. Similar degree of eight genera of Asteraceae.

Genera	*Anthemis melampodina*	*Scorzonera papposa*	*Echinops hussoni*	*Launaea capitata*	*Senecio desfontainei*	*Osteospermum vaillantii*	*Sonchus oleranceus*	*Artemisia inculta*
Anthemis melampodina	0							
Scorzonera papposa	0.7	0						
Echinops hussoni	0.94	0.64	0					
Launaea capitata	0.93	0.76	0.87	0				
Senecio desfontainei	0.85	0.84	0.79	0.92	0			
Osteospermum vaillantii	0.5	0.77	0.45	0.56	0.63	0		
Sonchus oleranceus	0.8	0.89	0.74	0.87	0.95	0.67	0	
Artemisia inculta	0.75	0.94	0.69	0.81	0.9	0.71	0.94	0

proteins concentration of 384.8 kDa.

In addition, molecular weight showed similar results to the concentrations. There were some plants with low molecular weight and high proteins concentration: *S. papposa* had 89.84 kDa and protein concentration of 1184.7 kDa; *A. inculta* was distinguished with high molecular weight of 154.48 and low protein concentration of 384.8 kDa. The total number of protein bands was 88 without the marker. Results revealed differences among the studied plants in number and intensity of bands, and only two bands were common in every individual plant. The highest number of bands was observed in *E. hussoni* with 17 protein bands, while *O. vaillantii* with five bands had the lowest number of bands.

Table 2 and Figure 3 show the similarity coefficient degree. Data reveals some genera were closer to each other. The highest similarity value was 0.95 between *S. oleranceus* and *S. desfontainei,* and this indicates a close phylogenetically relation, while the lowest similarity value was 0.5 between *A. melampodina* and *O. vaillantii*. The difference in similarity values indicates the existence of genetic diversity. The hierarchical cluster analysis (dendrogram) shows two major clusters, the first major cluster is subdivided into two sub clusters, sub cluster A contains three genera, *S. papposa, S. desfontainei*, and *S. oleranceus*; the second sub cluster B contains four genera *A. melampodina*, *E. hussoni*, *L. capitata*, and *O. vaillanti*. The arrangement of plant under the same sub cluster indicates close protein patterns and genotype, the second major cluster contains one genera plant *A. inculta* (Figure 3).

Table 3 and Figures 4 to 12 show the automated electrophoresis analysis of proteins patterns for every individual plant, separation times of protein band and molecular weight. All bands appeared with different molecular weight, the highest molecular weight was found in *S. desfontainei* and the lowest found in *O. vaillantii*, the two plants were collected from central area of eastern province. Also, Table 4 shows the studied bands, only consistent protein band between 10.0 and 150.0 kDa were considered; the appearance (+) of bands were 44 and the absence (-) was 36. *S. oleranceus* and *S. desfontainei* showed four common bands out of a total number of 10 bands (the highest) and *A. melampodina* and *O. vaillantii* showed three common bands out of total number of 10 bands; two bands were common in all plants, the first bands appear in all plants with molecular weight range from 25.47 to 25.93 kDa and bands numbers 8, 7, 8, 9, 8, 12, 10, and 11 appear at 28.5 to 28.69 s, the second bands appear at 31.39 to 31.8 s with molecular weight range of 38.82 to 41.07 kDa and bands numbers are 14, 10, 14, 14, 13, 13, 12, and 13 in the order of plants listed in Table 4. Despite of the existing of these two bands in all plants, there were differences in proteins intensity.

DISCUSSION

The total protein concentrations, molecular weight, and intensity revealed variation in gene expression; these variations help plants to adapt to environmental factors. Meena and Shukla (2012) found out that wide range of protein peptides and molecular weight can create additional variability in rice. Automated electrophoresis analysis of protein showed different number of bands, and only for two bands, the first common bands numbers were 8, 7, 8, 9, 8, 12, 10, and 11 and the second bands were 14, 10,

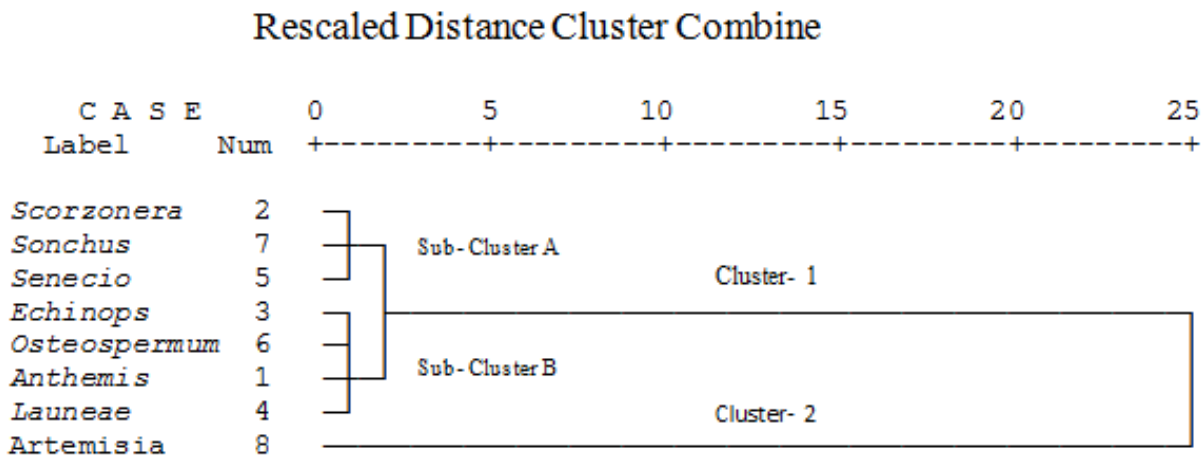

Figure 3. Hierarchical cluster construct for eight plants of Asteraceae. 1, *Anthemis melampodina*; 2, *Scorzonera papposa*; 3, *Echinops hussoni*; 4, *Launaea capitata*; 5, *Senecio desfontainei*; 6, *Osteospermum vaillantii*; 7, *Sonchus oleranceus*; 8, *Artemisia inculta.*

Table 3. Proteins patterns (Number of bands and molecular weight) of eight genera of Asteraceae.

Peak number	Lader		*Anthemis melampodina*		*Scorzonera papposa*		*Echinops hussoni*		*Launaea capitata*		*Senecio desfontainei*		*Osteospermum vaillantii*		Sonchus oleranceus		*Artemisia inculta*	
	Mig. time (s)	Mol. Wt.	Mig. time (s)	Mol. Wt	Mig. time (s)	Mol. Wt	Mig. time (s)	Mol. Wt	Mig. time (s)	Mol. Wt	Mig. time (s)	Mol. Wt	Mig. time (s)	Mol. Wt	Mig. time (s)	Mol. Wt	Mig. time (s)	Mol. Wt
1 *	17.15	1.2	10.85	-	10.89	-	10.99	-	10.91	-	11.08	-	10.92	-	11.07	-	10.49	-
2	21.4	6.66	12.59	-	12.55	-	12.63	-	12.79	-	12.79	-	12.94	-	12.64	-	13.08	-
3	22.1	7.56	15.36	-	15.29	-	15.13	-	15.51	-	15.34	-	15.65	-	15.06	-	15.33	-
4	24	10	16.11	-	16.07	-	16.04	-	16.12	-	16.08	-	16.17	-	15.96	-	16.1	-
5	27.25	20	17.15	1.2	17.15	1.2	17.15	1.2	17.15	1.2	16.59	-	17.15	1.2	17.15	1.2	16.53	-
6	28.5	25	22.06	7.51	21.94	7.36	21.92	7.32	21.19	6.38	17.15	1.2	18.37	2.76	21.81	7.18	17.15	1.2
7	31.05	37	24.29	10.9	25.85	15.7	24.51	11.58	21.7	7.05	21.69	7.03	20.71	5.77	26.13	16.56	18.4	2.8
8	33.45	50	25.88	15.78	28.59	25.42	25.86	15.73	22.41	7.95	25.86	15.73	21.13	6.31	27.18	19.78	21.27	6.49
9	37.35	75	27.76	22.06	30.74	35.55	27.45	20.8	25.78	15.49	27.16	19.72	21.74	7.09	27.99	22.94	21.84	7.23
10	40.3	100	28.61	25.51	31.62	40.09	28.65	25.72	27.33	20.33	27.86	22.42	26.46	17.58	28.7	25.93	27.01	19.28
11	45.7	150	29.2	28.31	32.5	44.86	29.04	27.54	28.6	25.47	28.64	25.67	27.82	22.29	30.69	35.32	28.69	25.9
12*	54.55	260	30.15	32.74	33.92	53.01	30.39	33.88	28.93	27.02	30.54	34.62	28.62	25.56	31.55	39.7	30.56	34.69
13	-	-	31.04	36.95	37.63	77.41	30.72	35.46	30.62	34.97	31.42	39.03	31.8	41.07	32.74	46.14	31.52	39.53
14	-	-	31.39	38.82	39.1	89.84	31.4	38.88	31.37	38.73	34.34	55.73	34.47	56.53	34.59	57.31	32.81	46.53
15	-	-	32.68	45.8	54.55	260	32.12	42.79	32.03	42.28	36.01	66.42	54.55	260	40.96	106.1	34.49	56.64

Table 3. Contd.

16	-	-	33.92	52.98	-	-	32.55	45.14	32.49	44.83	41.43	110.5	-	-	43.24	127.22	35.83	65.23
17	-	-	34.26	55.21	-	-	34	53.51	33.95	53.2	43.01	125.09	-	-	54.55	260	36.78	71.37
18	-	-	36.54	69.84	-	-	35.01	59.98	37.19	73.96	47.6	173.6	-	-	-	-	45.21	145.48
19	-	-	37.29	74.61	-	-	37.22	74.18	38.45	84.36	54.55	260	-	-	-	-	54.55	260
20	-	-	37.88	79.52	-	-	38.91	88.19	42.07	116.37	-	-	-	-	-	-	65.23	-
21	-	-	41.21	108.4	-	-	39.48	93.09	45.4	147.22	-	-	-	-	-	-	-	-
22	-	-	54.55	260	-	-	41.84	114.28	54.55	260	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	46.51	160.09	58.82	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	54.55	260	63.23	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-			64.26	-	-	-	-	-	-	-	-	-

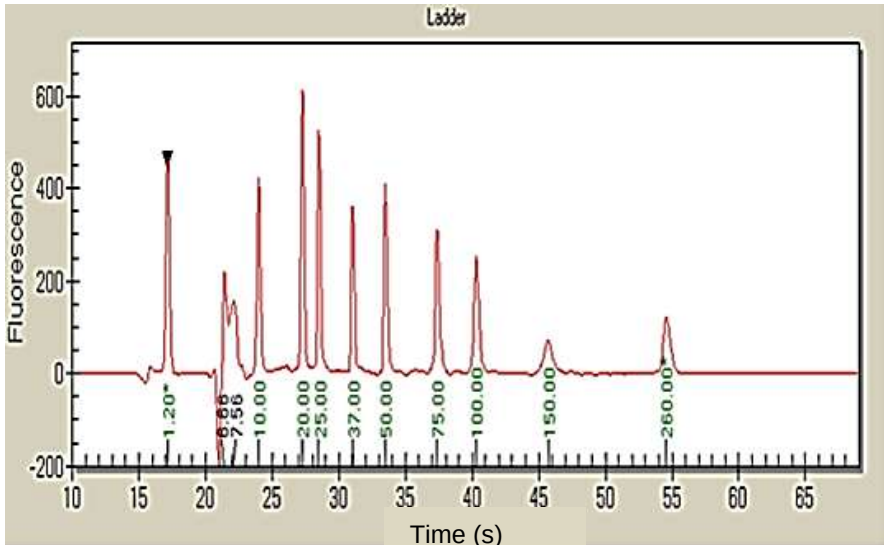

Figure 4. Total proteins bands and molecular weight of the ladder.

14, 14, 13, 13, 12, and 13 among the studied plants. Several researches detected genetic diversity among plants by using electrophoresis technique (Ehsanpour et al., 2010; Sinha et al., 2012; Alege et al., 2014). The presence of two common bands with different intensity in individual plants indicates that the gene expression does not vary and gene coding for the protein band is fixed in Asteraceae family. Akinwusi and IIIoh (1995)

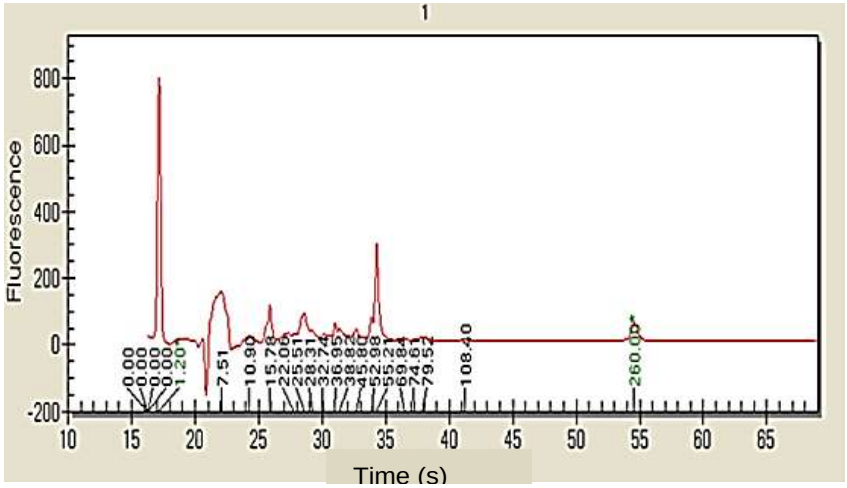

Figure 5. Total proteins bands and molecular weight of *Anthemis melampodina*.

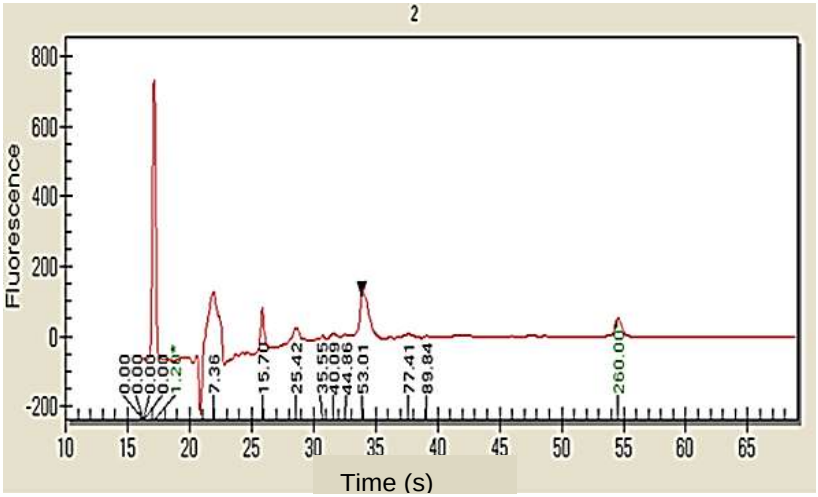

Figure 6. Total proteins bands and molecular weight of *Scorzonera papposa.*

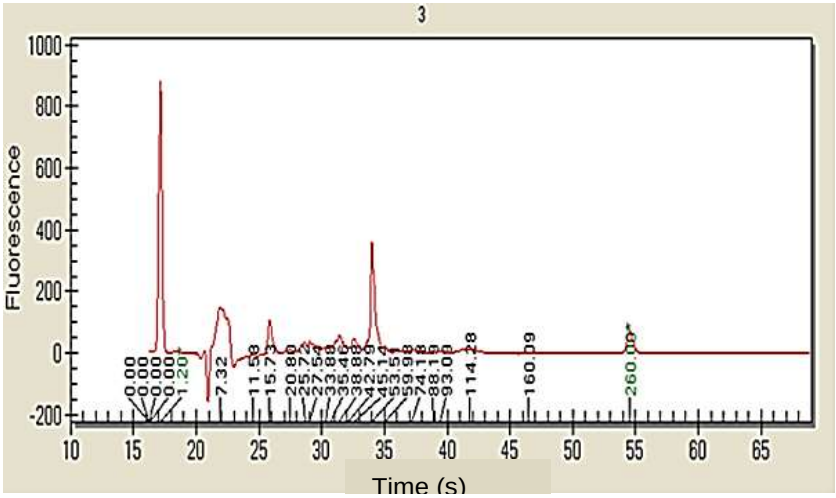

Figure 7. Total proteins bands and molecular weight of *Echinops hussoni*.

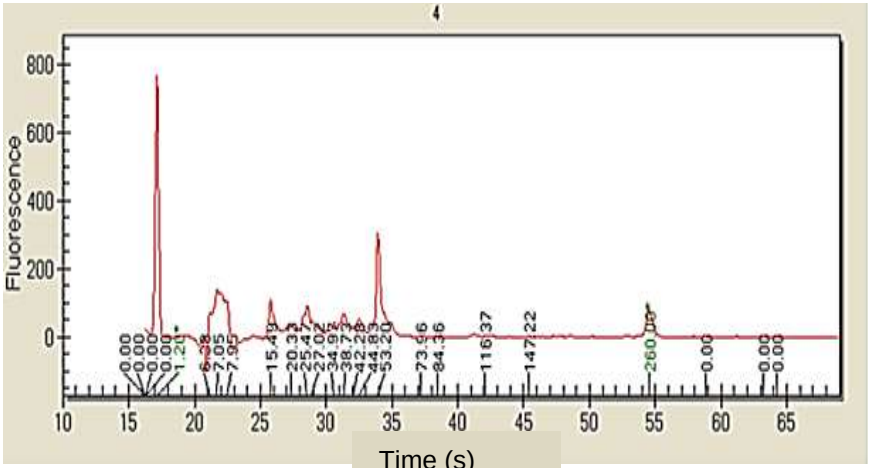

Figure 8. Total proteins bands and molecular weight of *Launaea capitat.*

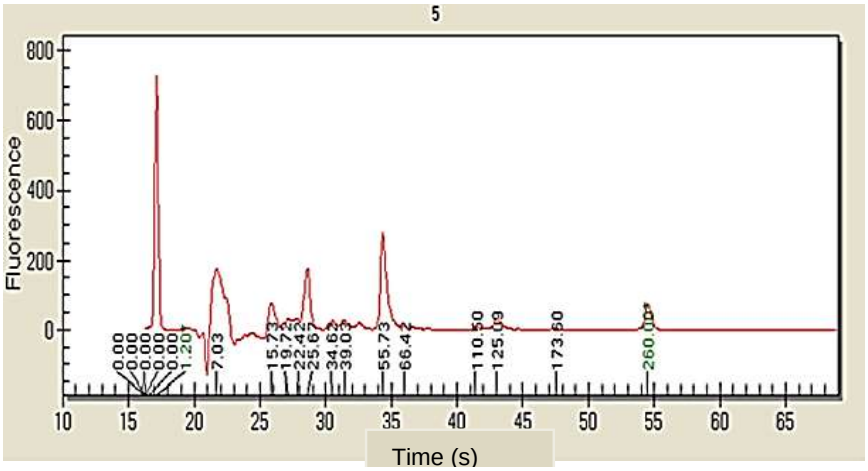

Figure 9. Total proteins bands and molecular weight *of Senecio desfontainei*.

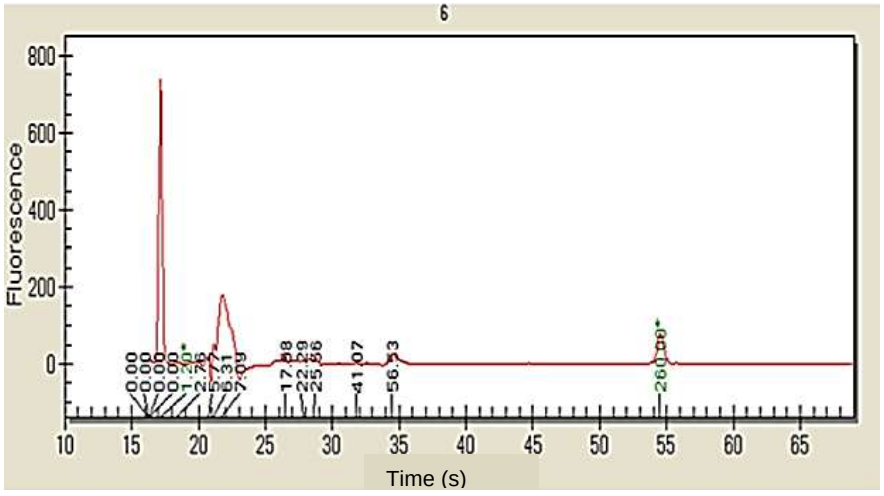

Figure 10. Total proteins bands and molecular weight of *Osteospermum vaillantii*.

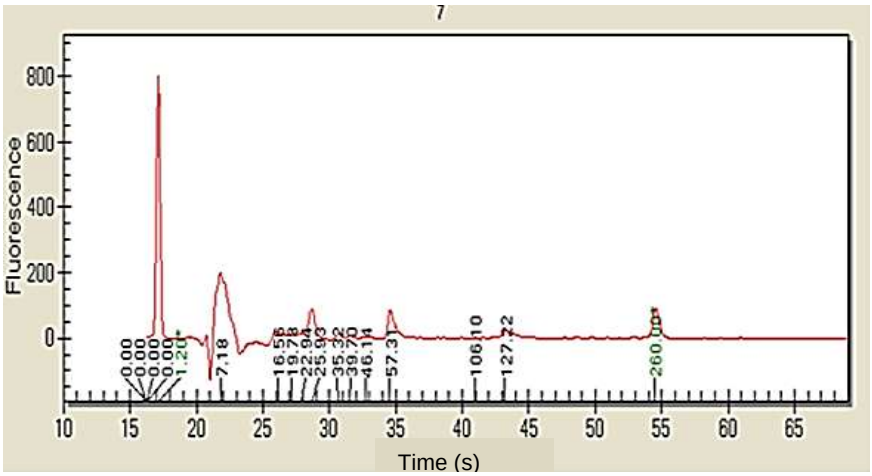

Figure 11. Total proteins bands and molecular weight of *Sonchus oleranceus.*

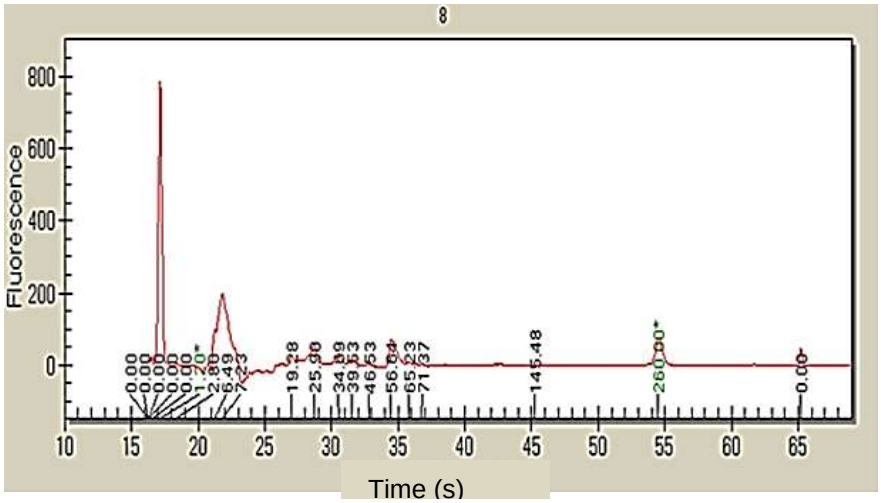

Figure 12. Total proteins bands and molecular weight of *Artemisia inculta.*

attributed that the appearance of a common band in individual plants in population is due to the fact that the gene expression of the protein (enzyme) does not vary in these plants, and there is polymorphism on the basis of differences in protein intensity among genotypes (Munazza et al., 2009). The presence and absence of proteins bands due to genes expression are in response to environmental factors, this difference in genes activation will lead to evolutionary genetic diversity that is reflected in plant phenotype. The similarity coefficient between studied genera, minimum 0.5 and maximum 0.95 indicates that genetic diversity exists within the studied genera of the family (Alege, 2015; Funk et al., 2005). Mossa et al. (1987) and Natarajan (2014) found differences in protein profiles of some *Brassica* species and in soybeans, respectively. The hierarchical cluster of studied plants showed two major clusters, the first major cluster grouped into two sub-cluster, each genus under the same sub-cluster indicates close genetic affinity and common ancestry (Alege, 2015), while the second major cluster contained only one genus. The presence of two clusters suggests more than one evolutionary line (Alege et al., 2014). Several researches used hierarchical cluster to detect genetic diversity among plants (Irfan et al., 2007; Alege et al., 2014; Bruneau et al., 2001; Amouri et al., 2014).

Conclusion

The result of protein patterns of eight genera of Astreaceae revealed genetic diversity that was supported by the hierarchical cluster; the concentrations and intensity with the presence and absence of some proteins

Table 4. Presence (+), absence (_) of proteins bands and the molecular weight of eight genera of Asteraceae.

Mig. Tim (s)	Ladder		*Anthemis melampodina*		*Scorzonera papposa*		*Echinops hussoni*		*Launaea capitata*		*Senecio desfontainei*		*Osteospermum vaillantii*		Sonchus oleranceus		*Artemisia inculta*	
	Presence /Absence	Mol. Wt	Presence /Absence	Mol. Wt.	Presence /Absence	Mol. Wt.	Presence /Absence	Mol. Wt.	Presence /Absence	Mol. Wt.	Presence /Absence	Mol. Wt.	Presence /Absence	Mol. Wt.	Presence /Absence	Mol. Wt.	Presence /Absence	Mol. Wt.
21.4-21.94	+	6.66	_		+	7.36	+	7.32	+	6.38	+	7.03	+	7.09	+	7.1	+	7.23
22.1-22.41	+	7.56	+	7.51	_		_		+	7.95	_		_		_		_	
24-24.51	+	10	+	10.9	_		+	11.58	_		_		_		_		_	
27.25-27.99	+	20	+	22.06	_		+	20.8	+	20.33	+	22.42	+	22.82	+	22.99	+	19.28
28.5-28.69	+	25	+	25.51	+	25.42	+	25.72	+	25.47	+	25.67	+	25.56	+	25.93	+	25.9
31.39-31.8	+	37	+	38.82	+	40.09	+	38.88	+	38.73	+	39.03	+	41.07	+	39.7	+	39.53
33.45-33.95	+	50	+	52.98	+	53.01	_		+	53.2	_		_		_		_	
37.35-37.88	+	75	+	79.52	+	77.41	+	74.18	+	73.96	_		_		_		_	
40.3-40.96	+	100	_		_		_		_		_		_		+	106.1	_	
45.4-45.4	+	150	_		_		_		+	147.22	_		_		_		+	145.48

bands indicate that the difference between studied plants was due to genotype and the response to environmental factors, and also the appearance of two common bands can be used as a marker for these genera of Asteraceae family.

CONFLICT OF INTEREST

The author has no conflict of interest.

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Full Length Research Paper

Evaluation of cytotoxic and mutagenic effects of two artificial sweeteners by using eukaryotic test systems

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Considering the vast use of sweeteners by the Brazilian population and the constant need for toxicological studies of food additives, this study aimed to evaluate the cytotoxic and mutagenic potentials of the sodium saccharin and/or sodium cyclamate sweeteners in plant (*Allium cepa)* and animal (*Mus musculus*) test systems based on concentrations permitted by the Brazilian laws. In *A. cepa*, both the sweeteners individually and their combinations concentration and exposure time (ET: 48, 72 and 168 h) dependently exerted cytotoxic and mutagenic effects. Similarly, an increased in micronuclei formation was also observed by the sweeteners in peripheral blood cells in mice. More toxic effects were observed with the combination doses at 168 ET. There may be a synergistic effect and DNA damage with an increasing concentration and ET. In conclusion, the concentrations considered safe by the Brazilian laws had significant cytotoxic and mutagenic activity on the eukaryotic cells.

Key words: *Allium cepa*, *Mus musculus*, sweeteners, toxicity.

INTRODUCTION

Food additives are the substances intentionally added to food without the nourishing purpose. In Brazil, the governing rules of the use of food additives are controlled by the National Health Surveillance Agency (ANVISA)

based on the international benchmarks, such as the Codex Alimentarius, the European Union and, complementarily, the US Food and Drug Administration (ANVISA, 2009). Sweeteners are the food additives that are added with technological or organoleptic intention to give the sweet taste without adding calories at any stage of food processing (Jain and Grover, 2015).

Among the sweeteners accepted for the use by ANVISA, sodium saccharin and sodium cyclamate stand out. Saccharin was introduced in 1878, which is 300 times sweeter than the sucrose, and not metabolized in the human body. It is synthesized from the toluene sulfonic acid, derived from petroleum (Pearson, 2001). In aqueous solution, it may be used in a conjunction with other sweeteners (Fitch and Keim, 2012) such as salts of cyclamic acid or cyclamates. These acids are prepared using chlorosulfonic acid and were discovered by Sveda and Audrieth (Jain and Grover, 2015).

In recent decades, the consumption of these types of sweeteners has been increased. It may be due to their synergistic sweetening properties and improved taste of the product. Dietetic products (especially diet sodas and diet sweeteners) have been the main sources of intake of non-caloric sweeteners (Alzin, 2003). However, there is no consensus on the safety of the use of cyclamate and saccharin. Studies on the toxicologic and carcinogenic potential of these substances are still quite controversial. There are studies that show that, these sweeteners cause testicular atrophy with an increase in the incidence of bladder tumors in mice. Although, there is no evidence in human risks, but animal studies have encouraged the prohibition of these sweeteners in some countries, such as Canada, USA, England, France and Japan (Uçar and Yilmaz, 2015; Mishra et al., 2015).

Despite all this controversy, the use of sodium cyclamate and sodium saccharin is authorized in more than 50 countries. Brazil is one of them (ASAE, 2006; CODEX, 2007). The last toxicological evaluation of sweeteners was performed by JECFA in 1982, following a setting of a safety limit (JECFA, 2000). However, in Brazil, ANVISA has not conducted any study related to safety concentrations other than allowing a considerable limits use in foods; thus, the essence of conduction of the toxicological studies on these types of sweeteners.

Bioassays with plants and animals have been considered highly sensitive and simple in monitoring cytotoxic and mutagenic effects of food additives (Iganci et al., 2006). Among them, *Allium cepa* is an excellent test system to indicate possible toxicological effects of a wide variety of substances. It is popularly used due to the fact that the cells in the meristematic region of roots have kinetic properties of proliferation and have a small number of chromosomes (2n = 16), leading to raise reliability and agreement with the other toxicological studies using higher eukaryotic test systems (Tabrez et al., 2011).

On the other hand, among the genotoxicity assessment tests recommended by the international agencies and government health institutions, the micronucleus test in rodents' peripheral blood is widely accepted (Ribeiro et al., 2003), which allows identification of increased mutation frequency exposed to a particular genotoxic agent for short or long terms (Yadav et al., 2014).

By considering the above mentioned facts, this study aimed to evaluate the cytotoxic and mutagenic effects of the above mentioned sweeteners using *A. cepa* and *Mus musculus* test systems.

MATERIALS AND METHODS

Selection and preparation of test concentrations

The concentrations of the tested sweeteners were calculated in accordance with the limits set by ANVISA through the law (RDC) No. 18 on March 24, 2008. The maximum allowed values for each sweetener were 100 g or 100 ml of ready-to-eat food. Based on the legislation, we have defined three concentrations of each food additive in this study as shown in Table 1. The exposing effect in both system tests was based on the maximum daily consumption level considered safe by ANVISA.

A. cepa assay

A. cepa assay in this study was done according to the method described by Islam et al. (2016). Briefly, small, uniform, same origin, not germinated and healthy onions was used. The onions were placed in vials containing distilled water at room temperature for rooting. Then the onions (5/each concentration) with multiple roots at least 0.5 cm of each, were selected for the treatment solutions. The exposure time (ET) was set at 48, 72 and 168 h. Copper sulfate (0.0012 g/L) was used as a standard in this test. After the elapsed ET, the root tips (meristem) were removed and fixed in Carnoy (3: 1 ethanol: acetic acid) solution. For photomicroscopic analysis, the cells were harvested and stained with 2% acetic orcein. Slides were evaluated using an optical microscope (100X), where 1000 cells were evaluated per repetition (5,000 cells per treatment). The mitotic index (MI) and chromosomal aberrations (CA) were evaluated to determine the cytotoxic and mutagenic effects, respectively.

MN assay in mice

In this purpose, male *Swiss* mice (30-35 g body weight, 2 months old) were obtained from the animal facility of the Federal University of Piauí (UFPI, Piauí / Brazil) and acclimatized under standard environmental conditions (12 h light/dark cycle and temperature of 23 ± 2°C) with free access to food and drinking water *ad libitum*. The experimental protocol was approved by the Ethics Committee for Animal Research Experimentation (CEEA) of the Federal University of Piauí, Brazil (No. 156/2016).

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Table 1. Tests and concentrations tested for sodium saccharin and/or sodium cyclamate and the test systems.

Treatments	Test systems	
	Allium cepa	***Swiss* mice**
Negative control	Distilled water	
Sodium saccharin	10 mg/ 100 g/ml 15 mg/ 100 g/ml 30 mg/ 100 g/ml	
Sodium cyclamate	20 mg/ 100 g/ml 40 mg/ 100 g/ml 80 mg/ 100 g/ml	
Sodium saccharin + Sodium cyclamate	10 mg/100 g/ml sodium saccharin + 20 mg/100 g/ml sodium cyclamate (dosages below the limits permitted by ANVISA for daily consumption)	
	15 mg/100 g/ml of sodium saccharin + 40 mg/100 g/ml sodium cyclamate (maximum dosage allowed by ANVISA for daily consumption)	
	30 mg/100 g/ml of sodium saccharin + 80 mg/100 g/ml Sodium cyclamate (dosages above those limits permitted by ANVISA for daily consumption)	
Positive control	Copper sulfate 0.0012 g/L	Cyclophosphamide (5 mg/ 100 g/ml) equivalent to 36.4% of lethal dose (137 mg/kg)

A total of 55 mice was grouped accordingly, five for each concentration. The negative control (distilled water, 1 ml/ 100 g) and test samples were administered *via* oral gavage (0.5 ml), while the positive control (cyclophosphamide, 5 mg/ 100 g/ml) was given by an intraperitoneal (i.p.) injection. All the treatments were given once a day for 7 consecutive days. The peripheral blood was collected by the puncturing of the tail vein at the times of 48, 72 and 168 h after the treatment initiation. Collected blood was divided into two slides for each animal and followed by drying for 12 h at 22°C. The slides were then fixed in methanol P.A. and subsequently stained for 10 min in a solution of 3% Giemsa. A total of 2000 cells (1000 cells per slide) were observed for each concentration of the test and controls. Slides were evaluated using an optical microscope with 100X magnification (Taimo et al., 2016).

Statistical analysis

The results of the two tests were performed by the RM-MANOVA test, followed by Tukey post-test, using the program STASTISTIC 7.0, considering $p < 0.05$.

RESULTS

According to Table 2, it is clear that the PC and sodium saccharin and/or sodium cyclamate concentration and ET dependently decreased MI but increased in CA formation in *A. cepa* test. The NC did not show such type of regularity in the MI and CA values. The lowest MI (4.1 ± 1.1 at 168 ET) was observed at 40 mg/100 g/ml of sodium cyclamate, while highest CA (28.2 ± 2.5) with the combined treatment of sodium saccharin (10 mg/100 g/ml) and sodium cyclamate (20 mg/100 g/ml) group. However, more cytotoxic and mutagenic effects were attributed with the combined treatments with these sweetners. The standard copper sulphate at 168 ET exhibited the highest CA by 50.0 ± 1.0.

Table 3 depicts a MN profile with the treatments at ET 48, 72 and 168 h in the peripheral blood cells of mice. Here is also a concentration and time-dependent augmentation in MN values of the test samples and cyclophosphamide (CP). The highest MN (14.4 ± 3.6) was observed in the co-treatment group of sodium saccharin and sodium cyclamate (30 + 80 mg/100 g/ml) group at 168 ET. Sodium cyclamate at 168 ET was found to produce MN by 13.1 ± 3.1 at 80 mg/100 g/ml. The NC attributed a reduced number of MN at 72 h other than 48 and 168 h.

DISCUSSION

The sodium cyclamate and sodium saccharin with a ration of 2:1 are popularly used to mask the bitter taste of some products (Zanini et al., 2011). In 2001, ANVISA decided to reduce the maximum amount of these two sweeteners in drinks and foods. With this decision the RDC n. 3/2001 was repealed and the maximum cyclamate usage ranged from 97 to 130 mg to 40 to 56 mg per 100 ml or 100 g, while saccharin by 22 to 30 mg to 10 to 15 mg. This decision was made according to the raised concerns about the possible toxicological and neoplastic effects on the human body.

Table 2. Cytotoxicity and mutagenicity of sodium saccharin and sodium cyclamate in *Allium cepa.*

Treatments		ET 48 h		ET 72 h		ET 168 h	
		MI	CA	MI	CA	MI	CA
NC		15.1 ± 0.4	5.5 ± 1.5	13.0 ± 1.4	9.0 ± 2.5	15.87 ± 0.9	7.0 ± 0.5
PC (0.0012 g/L)		12.5 ± 1.6	26.0 ± 4.5[a]	7.2 ± 1.1[a]	33.5 ± 4.5[a]	6.9 ± 0.8[a]	50.0 ± 1.0[a]
Sodium saccharin (in 100 g/ml)	10 mg	9.2 ± 2.8[a]	6.7 ± 2.7	7.7 ± 1.5[a]	8.2 ± 3.2	5.62 ± 1.8[a]	11.7 ± 4.7
	15 mg	10.2 ± 0.9[a]	9.25 ± 2.8	7.2 ± 0.8[a]	10.0 ± 4.0	6.6 ± 2.5[a]	12.5 ± 2.5
	30 mg	10.8 ± 2.4	10.5 ± 4.5	6.8 ± 1.6[a]	12.5 ± 2.5	6.3 ± 0.8[a]	13.2 ± 2.2[a]
Sodium cyclamate (in 100 g/ml)	20 mg	11.5 ± 3.3	9.0 ± 3.9	9.3 ± 1.9	12.0 ± 1.8	5.9 ± 1.4[a]	13.7 ± 15
	40 mg	11.0 ± 4.8	9.2 ± 3.1	8.6 ± 1.5[a]	12.5 ± 8.1	4.1 ± 1.1[a]	16.7 ± 7.8[a]
	80 mg	7.7 ± 1.1[a]	7.2 ± 4.6	6.1 ± 2.7[a]	11.7 ± 3.1	5.4 ± 1.8[a]	14.2 ± 7.5[a]
Sodium saccharin + Sodium cyclamate (in 100 g/ml)	10 + 20 mg	13.5 ± 3.8	5.2 ± 1.5	5.57 ± 1.1[a]	19 ± 1.4[a,b]	5.07 ± 1.4[a]	28.2 ± 2.5[a,b]
	15 + 40 mg	8.9 ± 1.7[a]	18.5 ± 1.2[a]	5.4 ± 0.8[a]	19.7 ± 1.7[a,b]	4.3 ± 1.7[a]	21.2 ± 1.6[a,b]
	30 + 80 mg	15.4 ± 2.6	16.07 ± 2.1[a]	6.0 ± 0.6[a]	16.2 ± 0.9[a,b]	5.1 ± 0.4[a]	19.2 ± 2.6[a]

MANOVA with Tukey *post-test*. ET, exposure time; NC, negative control (distilled water); PC, positive control (Copper sulfate 0.0012 g/L); MI, mitotic index; CA, chromosomal aberrations; [a]p <0.05 compared to the NC (same TE analyzed); [b] p <0.05 compared to the individual sweeteners in the same concentration and ET.

Table 3. Micronuclei formation by the sodium saccharin and sodium cyclamate in mice.

Treatments		MN		
		ET 48 h	ET 72 h	ET 168 h
NC (1 ml/ 100 g)		3.7 ± 1.6	3.2 ± 1.8	2.8 ± 1.6
PC (5 mg/ 100 g/ml)		10.3 ± 2.6[a]	10.6 ± 1.8[a]	11.9 ± 3.3[a]
Sodium saccharin (in 100 g/ml)	10 mg	1.4 ± 1.6	4.1 ± 1.7	8.5 ± 2.5[a]
	15 mg	3.3 ± 1.2	3.7 ± 1.6	8.9 ± 2.9[a]
	30 mg	3.2± 1.1	3.3 ± 2.1	7.3 ± 1.4
Sodium cyclamate (in 100 g/ml)	20 mg	2.8 ± 1.7	4.8 ± 2.1	5.9 ± 1.6
	40 mg	3.9 ± 2.2	9.0 ± 1.4[a]	9.1 ± 1.7[a]
	80 mg	3.2 ± 1.6	5.4 ± 1.1	13.1 ± 3.1[a]
Sodium saccharin + sodium cyclamate (in 100 g/ml)	10 + 20 mg	5.7 ± 3.1	5.8 ± 1.8	6.0 ± 2.1
	15 + 40 mg	8.6 ± 2.6	8.6 ± 2.1[a]	9.2 ± 2.4[a]
	30 + 80 mg	13.1 ± 3.2[a,b]	14.0 ± 3.2[a]	14.4 ± 3.6[a,b]

RM, MANOVA with Tukey *post-test*. ET, exposure time; MN, micronuclei; NC, negative control (distilled water); PC, positive control (cyclophosphamide, 5 mg/100 g/ml); AC, chromosomal aberrations; [a]p <0.05 compared to the NC (same ET analyzed); [b]p <0.05 compared to the individual sweetener in the same concentration and ET.

In the earlier studies, it has been shown that these two sweeteners have genotoxic effects on a number of test systems (Bandyopadhyay et al., 2008). Before that, Sasaki et al. (2002) evaluated five sweeteners, including these two using comet assay in cells of various organs of rats exposed for 3 and 24 h of treatment revealed that the sodium cyclamate induced an increase in DNA damage in the glandular cells of the stomach, colon, kidney, and bladder. The authors also reported saccharin-induced alterations in the glandular cells of the stomach and colon even at the lowest concentration tested (1000 mg/kg). In this study, in *A. cepa* test, a concentration and ET-dependent alterations of MI and CA values in the sodium cyclamate and/or sodium saccharin groups was also found. More alterations were observed in the combination group of the sweeteners at 168 h. However, the activity was lower than the PC group.

Furthermore, an increased risk of bladder cancer (30% of experimental animals treated with a dose of 7.5% of sodium saccharin in their diet) was consistently proven to the second generation of rodents with these sweeteners (Weihrauch and Diehl, 2004). In the US, since 1981, products containing saccharin are needed to be labeled with a warning that saccharin may cause cancer in

laboratory animals. Moreover, cyclohexylamine, a metabolite of cyclamate is evident to exert toxic effects on the animals, possibly due to its cytotoxic and mutagenic effects (Bastaki, 2015). In a study, cyclohexylamine caused testicular atrophy and reduced spermatogenesis in rats and dogs (Weihrauch and Diehl, 2004). Additionally, Martins et al. (2010) found a late fetal development and pancreas hypertrophy in rat fetuses exposed to sodium cyclamate.

A study conducted by Van Eyk (2015) found that aspartame, sodium cyclamate, acesulfame K and sodium saccharin are cytotoxic, mutagenic and genotoxic to Caco-2 cell lines (colonic cells), HT-29 (colon cells) and HEK-293 (renal cell). These results corroborate with those of Sasaki et al. (2002) that used the comet assay and reported that sodium saccharin and sodium cyclamate exerted genotoxic and mutagenic effects on rodent colon cells. Demir et al. (2014) also suggested that, both acesulfame K and sodium saccharin may induce DNA damage in rodents. A case-control study by Andreatta et al. (2008), demonstrated that the incidence of tumors of the urinary tract and the consumption of sweeteners in Cordoba in Argentina. Our study conducted on *Swiss* mice, suggesting that both sodium saccharin and/or sodium cyclamate has mutagenic effects in peripheral blood lymphocytes. Both of them with or without combining treatments augmented a concentration and ET-dependent MN production in the test systems. In some cases, we have seen that the middle concentration/dose exerted more cytotoxic or mutagenic effects in comparison to the lowest and highest treated concentration/dose. It may be due to the adaptation responses in the test systems.

Conclusion

Sodium saccharin and sodium cyclamate sweeteners showed cytotoxic and mutagenic effects both in vegetal (*A. cepa*) and animal (*Swiss* mice) cells at the concentrations allowed by the Brazilian law. More toxic effects were observed with their combined treatments. There may be a synergistic effect. Further studies are urgently needed, using more intensive bioassays, such as human cell lines, to say exact mechanisms of cytotoxic, genotoxic and mutagenic effects of these popularly used artificial sweeteners.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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