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Full Length Research Paper

Leaf anatomy of *Crambe abyssinica* Hochst. during *in vitro* shoot induction

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This study aimed to characterize and evaluate possible modifications in the leaf anatomy of crambe during the process of shoot induction based on micropropagation protocol. The anatomic characteristics of the leaves, and also the morphological characteristics of crambe plantlets, were evaluated during the shoot induction phase of the micropropagation. The shoots were induced by the cytokinins, 6-benzylaminopurine (BAP), kinetin (KIN) and thidiazuron (TDZ), at distinct concentrations on Murashige and Skoog (MS) medium during 120 days of culture. Comparing the leaf anatomy, it was observed that, at day 30, only the adaxial epidermis and the palisade parenchyma presented significant differences in relation to the tested concentrations, independently of the type of cytokinin employed. At 120 days, the anatomic analysis of the mesophyll demonstrated no marked difference among the cytokinins at 5 µM. Therefore, the various sources and concentrations of cytokinins applied in this work did not promote marked changes in the sense of altering the organization and/or thickness compared to the control.

Key words: Oilseeds, micropropagation, mesophyll, histology, plant morphology.

INTRODUCTION

Crambe abyssinica (Brassicaceae) is a species of this genus, which is most economically important, with 34 representatives (Wang et al., 2004). Its seeds contain 45 to 50% of crude protein and up to 35% of oil, which

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consists of up to 55 to 60% of erucic acid (Palmer and Keller, 2011). Such characteristics make it highly valuable for animal feed, industrial and pharmaceutical applications, biodiesel production, as well as fabrication of coatings, detergents, gliding products, cosmetics, lubricants and nylon (Vargas-Lopez et al., 1999; Capelle and Tittonel, 1999; Palmer and Keller, 2011).

The genetic breeding of species like *C. abyssinica*, with the aim of increasing their oil production or altering any compound of interest, has been often employed (Wang and Peng, 1998; Murphy, 1999; Wang et al., 2003; Wang et al., 2004; Li et al., 2010). However, in order for the genetic breeding to be successful, biotechnological steps beforehand must be efficiently concluded. For crambe, the lack of protocols suitable for micropropagation (*in vitro* culture) and the need for large amount of plant material have hampered the development of transgenic plants (Gao et al., 1998; Li et al., 2010).

In previously published protocols, the *in vitro* regeneration of crambe was achieved by using hypocotyls as explants to enable studies related to genetic breeding of the species, such as transgenesis and somatic hybridization (Wang et al., 2004, 2003; Wang and Peng, 1998). However, studies related to the induction of shoots and regeneration of whole plants from the apical segment have not been described yet. The *in vitro* environment offers low light density, high humidity, presence of sugar and growth regulators in the substrate, and low gas exchange, with the lack of CO_2 and excess of ethylene (Kadlecek et al., 2001). These factors induce disruptions in the growth, development and morphology, involving anatomic and physiological alterations in the plant (Kozai, 1991).

The anatomic abnormalities may result from excess of growth regulators present in the culture medium (Hronkova et al., 2003). Only few studies exist regarding these characteristics during the process of shoot induction in the successive phases of micropropagation (Appezzato-Da-Glória et al., 1999; Apóstolo et al., 2005; Hazarika, 2006). There are no anatomic studies of *C. abyssinica* available in the specific literature yet. Hence, the present study aimed to characterize and evaluate possible modifications in the leaf anatomy of *C. abyssinica* and morphology of the plantlets during the process of induction of shoots obtained through micropropagation protocol.

MATERIALS AND METHODS

The experiment was carried out at the Laboratory of Plant Tissue Culture at the Center of Agronomic Sciences, and at the Laboratory of Plant Anatomy at the Department of Biological Sciences, both at the Federal University of Espírito Santo, ES – Brazil. Seeds of *C. abyssinica* Hochst cv. 'FMS brilliant', harvest of 2012, were used, supplied by the Fundação MS, located in Maracaju, Mato Grosso do Sul – Brazil.

The seeds of crambe were initially washed under running water with neutral detergent. Subsequently, they were transferred into aseptic conditions under laminar flow hood. Next, the seeds were disinfested by immersion in antibiotic solution containing penicillin (10 mg L^{-1}) and rifampicin (10 mg L^{-1}) for 30 min, followed by 70% alcohol for 1 min, commercially available 50% sodium hypochlorite (active chlorine: 2 to 2.5%) for 30 min, then washed three times in autoclaved distilled water. The used culture medium was half-strength MS medium (Murashige and Skoog, 1962), supplemented with 15 g L^{-1} sucrose and 7 g L^{-1} agar. pH of the media was adjusted to 5.8 prior to autoclaving under 1.1 atm. and 121°C, for 20 min. The seeds were inoculated into 25 × 150 mm test tubes containing 10 ml of medium, and incubated in growth room with photoperiod of 16/8 h (light/dark), under fluorescent lamps providing flow of 25.2 µmol m^{-2} s^{-1} photosynthetic photons flux's, and temperature of 25 ± 1°C.

After 30 days, the apical segments of the obtained suitable plantlets were removed and used as explants in the process of induction and subculture of shoots. The shoots were induced by the cytokinins 6-benzylaminopurine (BAP), kinetin (KIN) and thidiazuron (TDZ) during 120 days of culture. The experiment followed a factorial scheme 3 × 3, having three levels in the factor cytokinin (BAP, KIN and TDZ), and three levels in the factor concentration (0, 5 and 10 µM), in a completely randomized design with five repetitions, each consisting of ten test tubes of 25 × 150 mm with one explant each and 10 ml of culture medium.

The adopted culture medium was MS, supplemented with 30 g L^{-1} sucrose, 0.5 mg L^{-1} silver nitrate ($AgNO_3$) (LI et al., 2010) and 7.0 g L^{-1} agar, and pH fixed to 5.8. The tubes were autoclaved and stored under the growth room conditions already described. The number of shoots per explant (NSE) and percentage of survival (%SUR) were evaluated after 30, 60, 90 and 120 days. The removal of leaves per treatment for the anatomic analyses was done at 30 and 120 days.

Anatomic analysis

The anatomic analyses were performed in five more developed leaves for each treatment, in the midrib region and internervural area. The samples were fixed in formalin-acetic acid-alcohol (FAA_{50}; Johansen, 1940) for 48 h, stored in ethyl alcohol 70%, later subjected to dehydration in increasing ethanol series (70, 90, 95 and 100%), and embedded in hydroxyethyl methacrylate (Leica®, Germany). The blocks were cross sectioned on a rotating microtome, with thickness varying between 8 and 10 µm. The sections were stained with 0.05% toluidine blue in acetate buffer, pH 4.3 (O'brien et al., 1964), and mounted in synthetic resin Entellan®. Measurements of the thickness of the blade, mesophyll, adaxial and abaxial epidermis, palisade parenchyma, spongy parenchyma and midrib region were performed. Also, the vascular tissue area of the midrib was measured. The observations and measurements were carried out under microscope Nikon E200 with coupled digital camera connected to a computer containing the software Tsview® (China). The photo documentation was done in a photomicroscope Nikon 50i (Nikon, Japan). The design for anatomic evaluations was completely randomized, with five repetitions per treatment, each repetition corresponding to one leaf and comprising the mean value of three slides with eight sections each.

Statistical analysis

All obtained data was subjected to analysis of variance, after verification of normality and homogeneity. After confirming the significance of the treatments, the Tukey test of averages was applied, adopting 1 and 5% probability, with the software Assistat (Silva and Azevedo, 2009). The regression analyses in function of the cytokinins and the evaluation times were performed with the software SigmaPlot 10.0 (2006). However, none of the evaluated

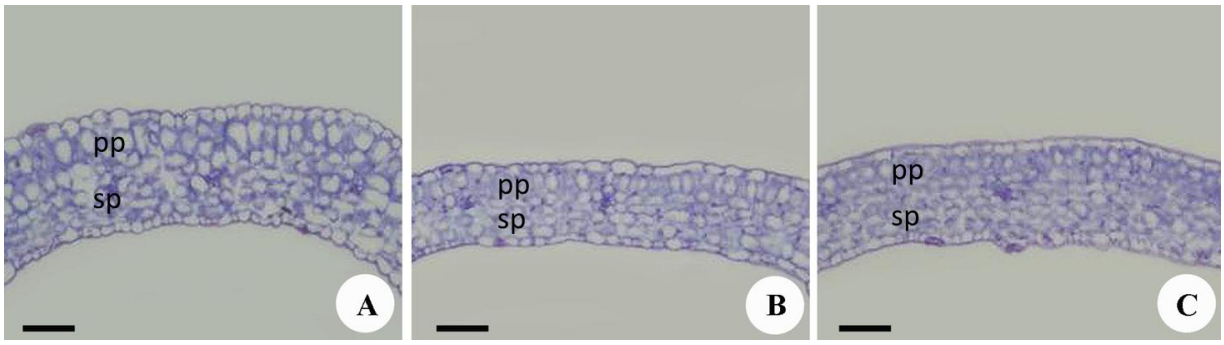

Figure 1. Cross section of the leaves, showing the mesophyll (A to C) of *C. abyssinica* leaves *in vitro* at 30 days, subjected to treatments with different concentrations of cytokinin. A: 0 µM; B: 5 µM; C: 10 µM. pp - palisade parenchyma; sp - spongy parenchyma. Bar 100 µm.

variables adjusted to the linear model based on the significance of the regression coefficients and determination coefficient (R2), assuming 5% probability by the F-test and also considering the biological phenomenon under study. For this reason, the Tukey test of averages was also adopted in the quantitative factor to support the discussion of this work.

RESULTS AND DISCUSSION

General anatomic description

The leaf of *C. abyssinica* is amphistomatic, with uniseriate epidermis constituted by cells of varying shapes and sizes, covered by a thin cuticle. Glandular and tector trichomes occur sparsely on both surfaces of the epidermis (data not shown). The mesophyll is dorsiventral, formed by one or two cell layers of palisade parenchyma, and spongy parenchyma with three to four cell layers. Vascular bundles of collateral type and of small size occur across the mesophyll. The midrib, in cross section, appears concave-convex, with epidermis formed by round cells. The vascular system is predominantly formed by a single collateral vascular bundle (Figures 1 and 2).

The leaf anatomy of *C. abyssinica* is similar to that described for other representatives of Brassicaceae, such as *Brassica napus* L. (colza), *Brassica gravinae* Ten., *Brassica rapa* L. (turnip), *Raphanus sativus* L. (radish), *Diplotaxis tenuifolia* (Ueno, 2011) and *Arabidopsis thaliana* (L.) Heynh (Boeger and Poulson, 2006).

Survival and morphological evaluations

The survival percentage of plantlets originated from the four subcultures did not have any significant difference among the treatments, varying from 96.66% (at 90 days) to 100% (at 30, 60 and 120 days). These results disagree with those obtained by Pennell (1987), who relates that the higher the number of subcultures in the micropropagation, the lower the rate of survival. With this result, it can be highlighted that the utilization of apical segments obtained from plantlets germinated *in vitro*, in active growth, is preferable for the establishment of a micropropagation process, since the explants present very high rates of survival with the increasing number of subcultures. The oxidation, contamination and eventual death of the explant are problems for the *in vitro* establishment of some species owing to their peculiar characteristics (Sato et al., 2001).

By analyzing the summary of the analysis of variance, the induction of shoots in *Crambe* was more significantly influenced by the tested concentrations than by the types of cytokinins applied. The analysis of variance for the number of shoots per explant (NSE) after 30, 60, 90 and 120 days of cultivation *in vitro* are shown in Table 1. This analysis did not demonstrate significance in the interactions between the factors cytokinins and concentration in any of the evaluation times. In the factor cytokinin, significance was only found at 90 days of cultivation (Table 1). For the factor concentration, significance was found at 60, 90 and 120 days (Table 1).

For the morphologic variables evaluated in crambe, it is noted that there was difference among the cytokinins BAP, KIN and TDZ, influencing the development of new shoots and their growth. The differences in the factor concentration showed the necessity of cytokinin in the culture medium at the tested concentrations to stimulate the shoot in apical segments of crambe (Table 1).

For NSE at 90 days (Table 2) in the factor cytokinin, BAP (10.46) and TDZ (10.03) did not differ statistically. Thus, the application of BAP or TDZ at the tested concentrations, promoted significant increase in the induction of shoots in comparison with KIN. The production of nodal segments is an important variable in the *in vitro* propagation of *Crambe*, as it reflects the production of new plants at every subculture. According to Flores et al. (2009), BAP is the most efficacious cytokinin for the multiplication of aerial parts of most species. Furtado et al. (2007) described that TDZ and

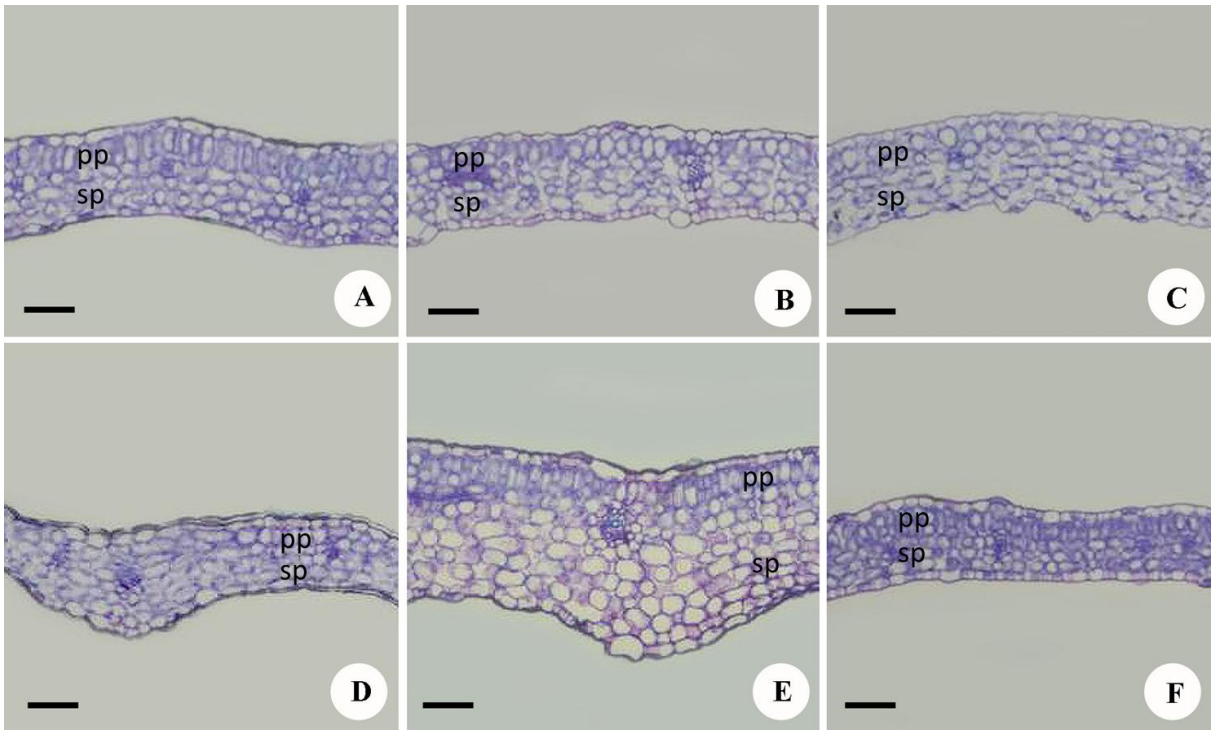

Figure 2. Cross section of the leaves, showing the mesophyll of *C. abyssinica* leaves *in vitro* at 60 days, subjected to treatments with different cytokinins and concentrations. A: 5 µM BAP; B: 5 µM KIN; C: 5 µM TDZ; D: 10 µM BAP; E: 10 µM KIN; F: 10 µM TDZ. pp - palisade parenchyma; sp - spongy parenchyma. Bar 100 µm.

Table 1. Summary of the analysis of variance for the number of shoots per explant (NSE) in *C. abyssinica* plants after 30, 60, 90 and 120 days of cultivation with different cytokinins at distinct concentrations.

VD	DF	Test F			
		30 d	60 d	90 d	120 d
Cytokinin (A)	2	0.17^{ns}	1.20^{ns}	3.98*	0.10ns
Concentration (B)	2	2.23^{ns}	28.26**	19.49**	38.97**
Int. A × B	4	0.58^{ns}	0.87^{ns}	1.25^{ns}	0.38^{ns}
Residue	36	-	-	-	-
General mean	-	10.08	7.56	9.56	8.52
CV (%)	-	29.47	31.51	24.40	26.14

**, * significant at 1% and 5% probability, respectively; ns: non-significant; VD: variance factors; DF: degrees of freedom; CV(%): coefficient of variation in percentage.

BAP induced greater formation of shoots on the cotyledonary bud explant in all treatments of peanut (*Arachis hypogaea* L.– Fabaceae), showing higher mean values than for KIN. Marino and Bertazza (1990) obtained greater proliferation of shoots when using BAP than KIN or zeatin for micropropagation of kiwi (*Actinidia deliciosa* A. Chev. - Actinidiaceae). Grattapaglia and Machado (1998) indicated that the higher efficiency of BAP in comparison with KIN might rely on the capacity of the plant tissues of metabolizing synthetic growth regulators more rapidly than plant hormones. According to the authors, this may vary depending on the used plant species. Krikorian (1991) comments that, at present, BAP has been more used than the cytokinins KIN and TDZ due to being a more active compound which can be easily found and at reasonable price.

In the tested concentrations of cytokinins, the highest values of NSE (10.43) were found at 5 µM (Table 3),

Table 2. Mean for the number of shoots per explant (NSE) in *C. abyssinica* plantlets on medium with different cytokinins after 90 days of cultivation *in vitro*.

Cytokinins	Mean
BAP	10.46[a]
KIN	8.20[b]
TDZ	10.03[ab]

There is no difference among means followed by the same letter by Tukey test at 5%.

Table 3. Mean of the number of shoots per explant (NSE) in *C. abyssinica* plantlets at different concentrations of cytokinin after 60, 90 and 120 days of cultivation *in vitro*.

Concentration (µM)	Days		
	60	90	120
0	4.00[c]	6.50[b]	4.40[b]
5	10.43[a]	11.26[a]	10.96[a]
10	8.26[b]	10.93[a]	10.20[a]

There is no difference among means followed by the same letter by Tukey test at 5%.

being statistically superior to 10 µM at 60 days. After 90 days, NSE value at the concentration of 5 µM (11.26) did not differ from 10 µM (10.93), which did not change at 120 days, with NSE values of 10.96 for 5 µM and 10.20 for 10 µM. In all time points, the lowest values were observed in the control, in the absence of the growth regulator, demonstrating the importance of an exogenous cytokinin to stimulate the shoot induction in the process of micropropagation in crambe. This way, according to Grattapaglia and Machado (1998), the choice of cytokinin type and its concentration are factors that influence the success of multiplication *in vitro*, indispensibly, during the process of overcoming the apical dominance and induction of proliferation of axillary buds. This way, the concentration of 5 µM for the tested cytokinins was sufficient to induce efficient shooting in apical explants of crambe.

Li et al. (2010), working with hypocotyls of *C. abyssinica* cv. Galactica, described a protocol for direct induction of shoots in MS medium supplemented with 10 µM TDZ and 2.7 µM α-naphthaleneacetic acid (NAA), promoting a regeneration frequency of up to 60%. In a new work, Li et al. (2011) obtained 95% of regeneration of shoots from hypocotyl segments, using the medium Lepiovre (Quoirin and Lepoivre, 1977) again supplemented with 10 µM TDZ and 2.7 µM NAA. Chhikara et al. (2012) demonstrated that the regeneration of shoots from calli obtained from hypocotyls of crambe presented greater efficiency in the medium, with 8.8 µM BAP and 0.10 µM NAA, with a frequency of 70%. Although a different explant was used in the present work than in the aforementioned works with crambe, it was noticed that the concentrations of the used cytokinins were close in both works, and efficiently promoted the expected morphogenetic response.

Anatomic evaluations

Regarding the leaf anatomy characteristics evaluated after 30 days (Table 4) in the plantlets cultivated in MS medium with different cytokinins at distinct concentrations, the analysis of variance has revealed no significant interaction between the employed factors. The various sources of cytokinins × concentrations used in this work did not exhibit significant effects on the thickness of the blade, mesophyll, abaxial epidermis, spongy parenchyma, midrib and area of the vascular bundle of crambe leaves at 30 days of cultivation. Only the adaxial epidermis and the palisade parenchyma presented significant differences in relation to the tested concentrations independently of the employed cytokinin type.

Table 5 shows the summary of the analysis of variance for the anatomic characteristics of crambe leaves, cultivated *in vitro* with different cytokinins at distinct concentrations, after 120 days. There was significant interaction between the tested factors for the thickness of the mesophyll and palisade parenchyma. Analyzing the factor concentration, only the thickness of the adaxial epidermis demonstrated statistical difference. Just as observed in the analysis carried out at 30 days, variations among the treatments were only observed for the adaxial epidermis and the palisade parenchyma. It is suggested that, at 30 days, owing to the short time of contact of the cytokinin with the explant, this factor may thus far not have influenced in such way as to alter effectively the thickness or structure of the evaluated characteristics. However, in analysis at 120 days, it was noted that few alterations remained. This result corroborates the fact that the *in vitro* multiplication of *Crambe* shoots under effect of the tested cytokinins does not alter the morphoanatomy of the leaves until day 120 of cultivation.

The thickness of the adaxial epidermis after 30 days and 120 days, and of the palisade parenchyma after 30 days of crambe leaves cultivated *in vitro* with cytokinins at different concentrations is represented in Table 6. The thickness of the adaxial epidermis (30 days) and of the palisade parenchyma presented similar behavior, in that their highest values were recorded for the concentration of 0 µM (21.69 and 47.74 µm, respectively); this differed statistically from the values obtained for 5 µM (16.16 and 29.68 µm, respectively), which were lowest. The

Table 4. Summary of the analysis of variation for the thickness (µm) of blade, mesophyll, adaxial and abaxial epidermis, palisade and spongy parenchyma, midrib and area (µm^2) of the vascular bundle of *C. abyssinica*, obtained by cultivation *in vitro* with different cytokinins and concentrations after 30 days.

VD	DF	Test F							
		Blade	Mesophyll	Adaxial epidermis	Abaxial epidermis	Palisade parenchyma	Spongy parenchyma	Midrib	Vascular bundle
Cytokinin (A)	2	1.10^{ns}	1.06^{ns}	1.44^{ns}	1.65^{ns}	0.70^{ns}	1.19^{ns}	1.14^{ns}	1.67^{ns}
Concentration (B)	2	2.80^{ns}	2.70^{ns}	3.77*	0.82^{ns}	4.74*	1.38^{ns}	0.65^{ns}	0.73^{ns}
Int. A × B	4	0.53^{ns}	0.64^{ns}	0.38^{ns}	0.46^{ns}	0.28^{ns}	0.74^{ns}	0.36^{ns}	0.47^{ns}
Residue	36	-	-	-	-	-	-	-	-
General mean	-	139.49	106.21	18.50	15.30	36.82	71.88	381.28	14442.44
CV (%)	-	42.10	44.83	30.85	40.92	46.36	46.87	38.46	90.68

**, * significant at 1% and 5% probability, respectively; ns: non-significant; VD: variance factors; DF: degrees of freedom; CV(%): coefficient of variation in percentage.

Table 5. Summary of the analysis of variation for the thickness (µm) of blade, mesophyll, adaxial and abaxial epidermis, palisade and spongy parenchyma, midrib and area (µm^2) of the vascular bundle of *C. abyssinica leaves,* obtained by cultivation *in vitro* with different cytokinins and concentrations at 120 days.

VD	DF	Test F							
		Blade	Mesophyll	Adaxial epidermis	Abaxial epidermis	Palisade parenchyma	Spongy parenchyma	Midrib	Vascular bundle
Cytokinin (A)	2	1.37^{ns}	1.68^{ns}	0.33^{ns}	0.42^{ns}	3.48*	0.77^{ns}	0.80^{ns}	1.16^{ns}
Concentration (B)	2	0.48^{ns}	0.19^{ns}	3.87*	3.24^{ns}	1.60^{ns}	0.05^{ns}	0.68^{ns}	1.03^{ns}
Int. A × B	4	2.55^{ns}	3.33*	0.20^{ns}	0.18^{ns}	3.24*	2.13^{ns}	0.58^{ns}	1.09^{ns}
Residue	36	-	-	-	-	-	-	-	-
General mean	-	156.31	123.69	17.98	16.05	45.22	81.24	343.14	9473.68
CV (%)	-	24.71	26.87	20.13	20.40	31.26	30.57	30.90	57.00

**, * significant at 1% and 5% probability, respectively; ns: non-significant; VD: variance factors; DF: degrees of freedom; CV(%): coefficient of variation in percentage.

Table 6. Mean thickness (µm) of the adaxial epidermis (DE) after 30 days and 120 days, and of the palisade parenchyma (PP) after 30 days of *C. abyssinica* leaves obtained by cultivation *in vitro* with cytokinins at different concentrations.

Concentration (µM)	DE (30 d.)	DE (120 d.)	PP (30 d.)
0	21.69^{a}	20.10^{a}	47.74^{a}
5	16.16^{b}	17.10^{ab}	29.68^{b}
10	17.65^{ab}	16.75^{b}	33.04^{ab}

There is no difference among means followed by the same letter by Tukey test at 5%.

Table 7. Mean thickness (µm) of the mesophyll of *C. abyssinica* leaves, obtained through *in vitro* cultivation with different cytokinins at distinct concentrations at 120 days.

Cytokinins	Concentration (µM)		
	0	5	10
BAP	127.76^{aA}	98.63^{aB}	109.23^{aAB}
KIN	127.76^{aA}	117.04^{aAB}	157.17^{aA}
TDZ	127.76^{abA}	153.32^{aA}	94.53^{bB}

There is no difference among means followed by the same letter, small in the rows and capitalized in the columns, by Tukey test at 5%.

Table 8. Mean thickness (µm) of the palisade parenchyma of *C. abyssinica* leaves, obtained through *in vitro* cultivation with different cytokinins at distinct concentrations at 120 days.

Cytokinins	Concentration (µM)		
	0	5	10
BAP	50.45^{aA}	36.57^{aA}	31.92^{aB}
KIN	50.45^{aA}	44.51^{aA}	63.49^{aA}
TDZ	50.45^{aA}	49.38^{aA}	29.72^{aB}

There is no difference among means followed by the same letter, small in the rows and capitalized in the columns, by Tukey test at 5%.

treatments with 0 µM and 5 µM yielded the highest values of the palisade parenchyma after 120 days (20.10 and 17.10 µm, respectively), though not differing between themselves, and the latter not differing from the thickness at 10 µM (16.75 µm).

The use of cytokinins in the culture medium may induce the production of cells with decreased size during cell division (Pereira et al., 2000), explaining the reduction in the thickness of the adaxial epidermis and of the palisade parenchyma in the culture media that contained exogenous cytokinin, in comparison with the control at 30 days. The increase in thickness of the leaf epidermis seen in the control is one of the protection mechanisms through reducing the penetration of radiation into the sensitive tissues of the leaf mesophyll (Sullivan et al., 1996).

For the mean thickness of the mesophyll after 120 days of cultivation (Table 7), only TDZ demonstrated difference among its tested concentrations, with the highest value (153.32 µm) being observed at 5 µM, though not differing from the control (127.76 µm) or KIN (117.04 µm). In turn, at the concentration of 10 µM, the highest value (157.17 µm) was observed for KIN, not differing from BAP (109.23 µm). According to Araujo et al. (2009), the greater the thickness of the mesophyll, the higher the photosynthetic efficiency. Since one of the main problems of acclimatization is related to the low photosynthetic rates, the excess of cytokinins may hamper this step. In accordance with Oliveira et al. (2008), in their work with pond-apple (*Annona glabra* – Annonaceae) using different cytokinins in the concentration of 5 µM, the thickness of the mesophyll was affected by the presence of BAP and KIN, and not by TDZ and ZEA (zeatin). This result diverges from that obtained in the present work at 120 days, in which only the presence of TDZ differed from the control.

In the thickness of the palisade parenchyma after 120 days (Table 8) no significant difference was observed among the tested cytokinin concentrations. However, among the cytokinins, difference occurred at 10 µM KIN (63.49 µM), which differed significantly from the others. Overall, the highest thickness values were found at the concentration of 0 µM, repeating the results observed at 30 days. Prolonged use of cytokinins may exert phytotoxic effect, which is mainly characterized by the lack of elongation and reduction in the size of the leaves (Grataplaglia and Machado, 1998). The palisade parenchyma is responsible for the photosynthesis, with its thickening being essential for allowing higher fixation of CO_2, contributing to the autotrophy of the plant after transfer into *ex vitro* environment (Silva et al., 2008; Castro et al., 2009).

Cross sections of the mesophyll of crambe leaves collected at the beginning of the *in vitro* multiplication (30 days), under effect of the cytokinins × concentrations, are shown in Figure 1. Although very discrete, it is still possible to notice the mesophyll limit, which consists of palisade parenchyma, with one or two layers of juxtaposed cells showing little elongation, and of spongy parenchyma, with three to four layers of weakly organized cells.

In Figure 1, it is remarkable that the appearance of the mesophyll *in vitro* treated with 0 µM cytokinin presented greater length compared to other concentrations. It is also possible to observe that, in the treatment with 10 µM cytokinin, a higher concentration of cells exists (variable not evaluated) (Figure 1C). The cytokinins are associated with the stimulation of cell division (Howell et al., 2003), which explains the smaller length and higher number of cells observed in the treatments with cytokinins.

The anatomic observations of mesophyll at 120 days demonstrated that there is no marked difference among the effects of the cytokinins at 5 µM (Figure 2A to C). However, at 10 µM KIN is superior to other cytokinins (10 µM BAP and TDZ) (Figure 2D to F), and the palisade parenchyma presents more elongated cells, characterizing more markedly the mesophyll limit of the mesophyll and demonstrating greater differentiation. Oliveira et al. (2008) also verified that leaves of plants subjected to treatment with BAP and KIN presented greater differentiation of the mesophyll compared to the treatments with TDZ and ZEA, with reduced proportion of intercellular spaces; this suggests higher efficaciousness

of these two sources of cytokinin on the development of the assimilatory system of the plants during cultivation *in vitro*. The data from the aforementioned author corroborates that described for the present work at 120 days, in which the treatments with BAP and KIN at 5 and 10 µM demonstrated greater differentiation between the parenchyma.

The various sources of cytokinins at different concentrations used did not promote changes in the sense of modifying the organization and/or thickness of the leaves compared to the control. Consequently, the treatments employed in this experiment did not alter the leaves morphologically.

Conflict of interests

The authors have not declared any conflict of interest.

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Characterization of biochemical behavior of sorghum (*Sorghum bicolor* [Moench.]) under saline stress conditions using multivariate analysis

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The aim of this research was to characterize the biochemical behavior of sorghum plants under saline stress using multivariate statistical analysis methods for efficient management of *Sorghum bicolor* [Moench.]). The experimental design was completely randomized design composed of three saline concentrations (0, 1.5 and 2.0 M) in 10 replications. In the multivariate analysis (hierarchical method), there were distinct and sub-groups in the sorghum plant treatments. Group 1 consisted of the root parts and under this group there were two subgroups: 1.5 to 2.0 concentration (Group 1) and 2 concentration (Group 2). The increase of NaCl concentration in the roots and leaves has inverse correlation with decrease of nitrate reductase, amino acids, protein and starch. The amounts of amino acids, carbohydrates, sucrose and proline in the roots and carbohydrates, sucrose and proline in the leaves of sorghum plants are reliable biological indicators of saline stress conditions in the soil. The nitrate compound differed ($p \leq 0.05$) in the sorghum plant roots; it had an average value of 0.04 µmol kg^{-1} of nitrate in the control treatment dry matter. The nitrate average was between 0.04 and 0.06 µmol kg^{-1}, but without statistical difference for all concentrations.

Key words: Multivariate statistics, salt concentration, proline, carbohydrate.

INTRODUCTION

Knowing the effects of salts on plants and soil, as well as the phenomena involved, is essential when the aim is to adopt appropriate water management practices and cultivation aimed at higher production. In addition, it causes an improvement in quality of the final product and benefits the families that make a living marketing this

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crop, especially in regions with salinity problems. The increased concentration of salts in soil decreases soil productivity state (Iqbal et al., 2014).The occurrence of excess salts in the soil can directly impair nitrogen absorption by plants, especially when they occur in the form of nitrate (NO^{3-}) or ammonium (NH^{4+}), limiting the growth of forage (Debouba et al., 2006; Lea and Azevedo, 2006; Grattana and Grieveb, 1998). Soils with excess sodium (Na^{+}) in the root environment affect plant growth because it alters membrane integrity (Silva et al., 2010) and causes changes in nutrient absorption, thus leading to great photosynthetic changes (Reddy et al., 2015).

Studies related to the effect of salinity on the biochemical behavior of sorghum (*Sorghum bicolor* [Moench.]) are still incipient, especially in relation to the nitrogen metabolism in the plant physiology. In Brazil, sorghum is used in human and animal nutrition, and as raw material for production of anhydrous alcohol, alcoholic drinks, colas and sugar extraction (Ribas, 2003; Cruz et al., 2015). Approximately 10 to 15% of the area planted with sorghum in Brazil is used as silage and has stood out due to its high production (Avelino et al., 2011). The good sorghum productivity results are due to its moderate tolerance to abiotic stresses, among which are saline stresses (Aquino et al., 2007). It is a crop that has bromatological characteristics similar to corn. This leads to its suitable fermentation and accumulation in silage, high concentration of soluble carbohydrates and crude protein in some varieties, and agronomic characteristics that present high biomass productivity (Von Pinho et al., 2006).

The assumption is that under high saline soils, some biochemical compounds present in the sorghum plants are significantly altered, and thus affect crop production. The present study aimed to characterize the biochemical behavior of sorghum plants under saline stress using multivariate statistical analysis methods, to efficiently manage *S. bicolor* [Moench.]).

MATERIALS AND METHODS

Experimental set up

The experiment was conducted in a greenhouse at the Federal Rural University of Amazonia (UFRA), Capitão Poço Decentralized Unit located between geographic coordinates of 01°44'04''S and 47°03'28''W, with an average altitude of 96 m asl. A completely randomized design (CRD) was established in the experiment in 2013, consisting of three saline concentrations of NaCl (0, 1.5 and 2.0 M), with 10 repetitions using forage sorghum plants (*Sorghum bicolor* [Moench.]). Plant Max variety totaling 30 experimental units was made up of two plants per pot^{-1}. The pots were placed in a spacing of 0.60 m between rows and 0.40 m between plants in a random distribution. The sorghum plants were grown in Leonard pots containing modified silica substrate: vermiculite (1:2) and irrigated with Hoagland and Arnon (1950) nutrient solution.

Increase in the doses of sodium chloride (NaCl) was done from 18th day after seedling germination (11 days) until plant collection. The preliminary test with this species (sorghum) showed that it could not tolerate sodium chloride (NaCl) after 11 days.

Collections of destructive plants in the vegetative stage were carried out (33 days), at 9:00 am, where the plants were separated into roots and leaves. Samples of each part were taken to a forced-air oven at 70°C (±5°C), and were subsequently crushed in a mill to perform the analysis.

Variables analyzed

The nitrate reductase activity was recorded using the method described by Hageman and Hucklesb (1971). To obtain the total soluble proteins, the method described by Bradford (1976) was used. For soluble amino acids, the method described by Peoples et al. (1989) was used and the free ammonia was obtained by the method described by Weatherburn (1967). The concentration of proline was obtained via the method described by Bates et al. (1973); nitrate, by Cataldo et al. (1975); total soluble carbohydrates, by Dubois et al. (1956); glycine betaine, by Grieve and Grattan (1983); and sucrose, by Van Handel (1968).

Statistical analysis

Initially, the variability in the data was evaluated using descriptive statistics. This involved calculating the average, standard deviation of the mean and coefficients of variation. The associations between biochemical compounds and saline concentrations applied in different parts of the plant (leaf and root) were analyzed by multivariate techniques. Thus, the data were subjected to multivariate exploratory cluster analyses by hierarchical method and principal components. The hierarchical cluster analysis method is an exploratory multivariate technique which gathers the sample units in groups, so that there is uniformity and heterogeneity within the groups. The structure of the groups contained in the data is seen on a graph called dendrogram, constructed from the similarity matrix between samples (Sneath and Sokal, 1973). The similarity matrix was constructed with Euclidean distance and the connection of the groups was performed using the Ward method.

The principal component analysis is a multivariate exploratory technique that condenses the information contained in a set of original variables into a set of smaller dimensions consisting of new latent variables, preserving a relevant amount of original information. The new variables are the eigenvectors (principal components) generated by linear combinations of the original variables constructed from the eigenvalues of the covariance matrix (Hair et al., 2005). After the standardization of data (zero mean and unit variance), the analyses were conducted in the program STATISTICA 7.0 (StatSoft. Inc., Tulsa, OK, USA). Simultaneously with the statistical analyses, the basic assumptions of analysis of variance, normality of errors and homogeneity of variances were tested for all variables (data not shown).

RESULTS AND DISCUSSION

Biochemical compounds in the leaf and root of sorghum plants

The nitrate compound (NIT) only differed ($p \leq 0.05$) in the roots of the sorghum grain plant, with an average value of 0.04 μmol kg^{-1} in the dry matter (DM) of the control (without application of saline concentrations) and with a 50% increase in relation to the saline concentrations of

1.5 to 2.0 M. In leaves, the NIT quantity ranges from 0.04 to 0.06 μmol kg^{-1} nitrate in the DM, but with no distinction among concentrations (Table 1). Sorghum plants treated with ion concentrations (NaCl) caused the highest nitrate accumulation in the stem tissue, which is an important storage organ for accumulation of these ions and their subsequent use in the leaves (Lobo et al., 2011). The decrease in NIT concentration in plants grown under salinity (NaCl) can be related to competition from the chloride ions (Cl^-) for the nitrate conveyors (NO^{3-}) (Ogawa et al., 2000). Aragon et al. (2011) observed that in saline conditions via the addition of NaCl at a concentration of 100 mM (-0.45 MPa) solution, the nitrate content in leaves was significantly low only on the 8th and 10th day of the experiment, whereas in the roots saline stress caused significant difference throughout the experiment.

Regarding the availability of ammonium (AMO), the highest values were observed in the roots, especially at the applied saline concentration of 2.0 M (24.09 μmol ammonium kg^{-1}) and with increases of 26.85% in relation to the control treatment (17.62 μmol ammonium kg). In the leaf there was no distinction among concentrations, the average ranging from 5.96 to 7.80 μmol ammonium kg^{-1} (Table 1). Although the accumulation of AMO was not observed in the leaves in the study results, Wolf et al. (2011), in a similar study, found the opposite: under saline concentration conditions there was ammonium storage both in sorghum leaves as well as in the forage sorghum stalk.

The nitrate reductase activity (NRA) had higher average values in the leaves and roots in the control treatment, with 0.11 and 0.04 μmol NO_{2-} g^{-1} h^{-1}, respectively; demonstrating that the NRA decreases as the saline concentration increases (Table 1). The same behavior can be observed for the availability of amino acid (AMINOA), protein (PRT) and starch (AMID), in both the root and leaves of sorghum plants (Table 1). Decreases in the NRA under saline stress conditions were also obtained by Aragon et al. (2011) and Lobato et al. (2009), with reductions in soluble protein concentrations compared to the root and leaf control treatments. Furthermore, an increase in AMINOA content in the leaves and roots was verified, but with no distinction between treatments (Aragão et al., 2011).

The concentration of proline (PROL), carbohydrates (CAR), sucrose (SAC) and glycine betaine (GLIC) had higher average values under high saline concentrations (Table 1). The SAC showed increases of 16.69 and 23.02%, in leaves and roots, respectively, with 2.0 M concentration compared to the control treatment (Table 1). Higher increases were reached in the PROL available in leaves with 47.57 and 59.39% in the control treatment at concentrations of 1.5 and 2.0 M respectively. Increases in the PROL content in the aerial part of plants subjected to saline stress were also found by Wolf et al. (2011). These increases are proportional to the salt content in the culture medium, the concentration of PROL in the sorghum leaves and also CAR, PROT and AMINOA concentrations (Oliveira et al., 2006). Under hydric or saline stress conditions there was initially loss of cell turgor, resulting in PROL accumulation to help reduce the osmotic potential in plant tissue (Munns and Tester, 2008). Furthermore, the addition of such an osmoregulator in plant tissue is associated with the plant osmotic stress behavior (Parida and Das, 2005) and to different sorghum genotypes (IPA SF-25, IPA 02-03-01, IPA 42-70-02, CFS-4, CSF-5, CSF-6, CSF-7, CSF-8, CSF-9 and CSF-10) (Oliveira et al., 2006).

Biochemical compounds in the leaf and root of sorghum plants analyzed by multivariate analysis

An alternative to better represent the relationship of inter and intradependence of qualitative and quantitative variables is the multivariate analysis because it allows simultaneous analysis of multiple measures that facilitate the characterization and principally the visualization of certain behaviors that would hardly be identified using only basic statistics (Hair et al., 2005). In the multivariate analysis (hierarchical method), there were distinct and sub-groups in the sorghum plant treatments. Group 1 consisted of the root part and within this group there was the formation of two subgroups: Subgroup 1 (saline concentrations 1.5 to 2.0) and subgroup 2 (0 saline concentration). The leaf part was concentrated in group 2, however without formation of subgroups, suggesting a different pattern by the nature of these formations (Figure 1). The distinction between leaf and root is related to the difference in the accumulation of ions (Na^+ and Cl^-) in the plant. In the leaf blades the amount of water absorbed during stress application is higher (Larcher, 2000), and also the distinction of the exclusion and retention capacity of ions from these tissues (Boursier and Lauchli, 1989).

The sorghum plant has the ability to retain Na^+ in the stem, resulting from stress, and remains constant for 20 days under saline stress conditions. While, there is minimal increase in toxic ion content in the leaf blades. The sorghum plant has the capacity to export the Na^+ present in the stalk to the leaves (Trindade et al., 2006). This behavior can be characterized as a mechanism for tolerating salinity stress conditions. When in the same group, it is considered that these groups have similarities. In addition, between one group and another group (G1 and G2, for example), there is dissimilarity, being that both measurements take into consideration various characteristics simultaneously. Within this context, Figure 1 represents the natural grouping structure of variables analyzed in this study. It is noted that although Group 1 presents a similarity that distinguishes it from Group 2, this subdivides by the saline concentrations evaluated, and it can be inferred that when 1.5 and 2.0 M concentrations were applied at the root of the sorghum plant they present similar behavior, but are dissimilar in

Table 1. Descriptive statistics of biochemical compounds in the shoot and root of sorghum plants grown in saline concentrations.

SC (M)	Leaf			Root		
	Average	SD	CV	Average	SD	CV
Nitrate (µmol kg^{-1})						
0	0.06[a]*	0.01	15.38	0.04[a]	0.001	2.50
1.5	0.06[a]	0.02	34.48	0.02[b]	0.001	5.00
2.0	0.04[a]	0.02	47.62	0.02[b]	0.001	5.00
Ammonium (µmol kg^{-1})						
0	7.80[a]	0.97	12.44	17.62[b]	3.14	17.82
1.5	5.96[a]	2.22	37.25	21.72[ab]	2.31	10.64
2.0	5.99[a]	2.44	40.73	24.09[a]	2.11	8.76
Nitrate reductase activity (µmol g^{-1})						
0	0.11[a]	0.03	27.27	0.04[a]	0.001	2.50
1.5	0.05[b]	0.02	40.00	0.02[b]	0.001	5.00
2.0	0.04[b]	0.02	50.00	0.01[b]	0.001	10.00
Amino acid (µmol g^{-1})						
0	63.59[a]	26.85	42.22	40.27[a]	7.15	17.76
1.5	43.51[a]	18.73	43.05	35.65[a]	7.22	20.25
2.0	36.74[a]	13.98	38.05	29.76[a]	4.96	16.67
Protein (mg g^{-1})						
0	3.89[a]	1.06	27.25	1.57[a]	0.41	26.11
1.5	2.89[ab]	0.61	21.11	1.11[ab]	0.45	40.54
2.0	2.42[b]	0.52	21.49	0.73[b]	0.21	28.77
Carbohydrate (mmol g^{-1})						
0	1.03[b]	0.20	19.42	0.84[b]	0.27	32.14
1.5	1.57[a]	0.18	11.46	1.21[ab]	0.26	21.49
2.0	1.84[a]	0.19	10.33	1.45[a]	0.34	23.45
Starch (mmol g^{-1})						
0	0.34[a]	0.02	5.88	0.06[a]	0.001	1.67
1.5	0.25[b]	0.02	8.00	0.04[b]	0.001	2.50
2.0	0.21[c]	0.01	4.76	0.04[b]	0.001	2.50
Sucrose (mmol g^{-1})						
0	59.73[b]	1.61	2.70	54.95[c]	2.62	4.77
1.5	68.75[a]	1.61	2.34	63.96[b]	2.14	3.35
2.0	71.70[a]	2.55	3.56	71.39[a]	2.88	4.03
Glycine betaine (µmol g^{-1})						
0	9.77[a]	0.70	7.16	5.56[a]	0.50	8.99
1.5	12.10[a]	4.57	37.77	11.44[a]	5.22	45.63
2.0	12.15[a]	6.11	50.29	12.05[a]	7.03	58.34
Proline (mmol kg^{-1})						
0	4.32[b]	0.24	5.56	0.77[c]	0.05	6.49
1.5	8.24[a]	1.01	12.26	2.62[b]	0.30	11.45
2. 0	10.64[a]	2.46	23.12	3.61[a]	0.27	7.48

N = 05; SC = saline concentration in molar concentration (M); SD = standard deviation of the mean; CV = coefficient of variation. * Means followed by the same letter in the column do not differ by Tukey's test at 0.5% probability.

relation to non-application (Concentration 0). On the other hand, in sorghum leaves the concentrations (0, 1.5 and 2.0 M) are similar, and therefore have the same behavioral pattern.

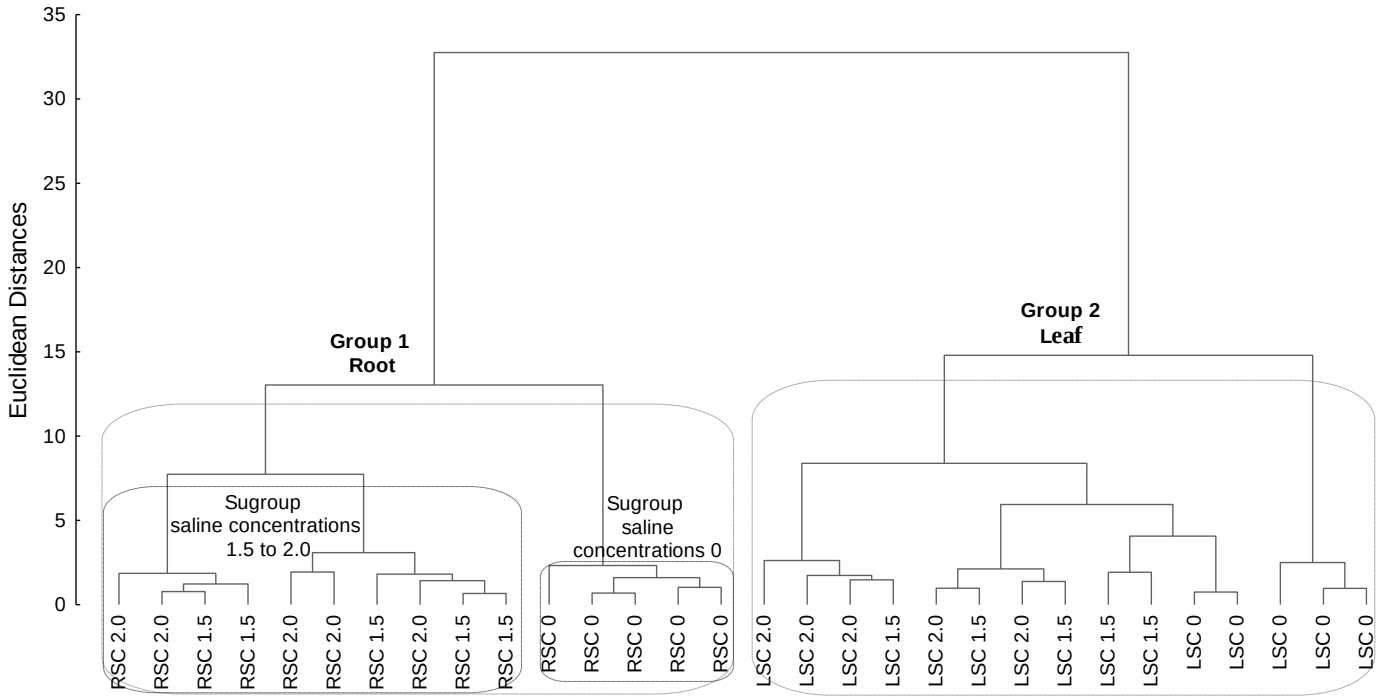

Figure 1. Dendrogram showing the hierarchy of groups in the aerial part of the leaf (L) and root (R) of sorghum plants subjected to saline concentrations (SC 0, SC 1.5 and SC 2.0 M) resulting from the cluster analysis by the hierarchical method.

Biochemical behavior in the leaf

The results obtained with the cluster analysis (Figure 1) confirm the results obtained in the principal component analysis (PCA), since in this analysis it was also possible to distinguish the leaf (Figure 2) and root areas (Figure 3). Through the PCA, it was possible to identify which biochemical compounds are characteristic, that is, are more expressive or not in a specific saline concentration when assessing both the sorghum root as well as the plant leaf. The two-dimensional plane generated with the first two principal components (PC) corresponds to 86.26% of the information contained in the original data: 61.79% in Principal Component 1 (PC1) and 24.47% in principal component 2 (PC2) when analyzing the sorghum plant leaf (Figure 2). These results are consistent with the criteria established by Sneath and Sokal (1973), wherein the number of PCs used in the interpretation must be such that they explain at least 70% of the total variance.

In the first principal component and in order of importance, the compounds that showed higher correlation coefficients were CAR (-0.98), AMID (0.97), SAC (-0.97), NRA (0.91), PROL (-0.84), PROT (0.83), NIT (0.77) and AMINOA (0.72). In the second principal component, we have GLIC (0.91) and AMO (0.78). The correlations are presented in Table 2 and represented by the arrows of each attribute and their projections are given in Figure 2. Compounds having charges (correlation values) with the same sign are directly correlated (positively) among themselves, while those with different signals have an inverse relationship; for example, carbohydrate (CAR), sucrose (SAC) and proline (PROL) have a direct relationship; on the other hand, they are negatively correlated to starch (AMID), nitrate reductase activity (NRA), protein (PRT), nitrate (NIT) and amino acid (AMINOA).

In PC1, AMINOA, PROT, NRA and AMID compounds located to the right of the PC1 are characteristics of sorghum leaves without the use of saline concentrations, that is, these compounds are found in greater amounts in the leaves under such conditions (Figure 1); a similar behavior is shown in Table 1; but, by the Tukey test, the AMINOA and PROT compounds do not differ in relation to the concentrations, although they present higher values in the treatment without saline concentration (Table 1). This fact reinforces the discussion on the relationships that are observed in the multivariate analysis and sometimes do not appear in basic statistics. In contrast, the compounds PROL, CAR and SAC located to the left of PC1 indicate that saline concentrations 1.5 and 2.0 favor greater quantities of these compounds in the sorghum leaves, especially at the 2.0 concentration (Table 1).

Piza et al. (2003) observed reduction in total soluble protein content in pineapple plants after 15 days of evaluation at all salinity levels (0, 0.57, 1.15 and 2.30 g L^{-1} de NaCl) studied. According to the authors, the

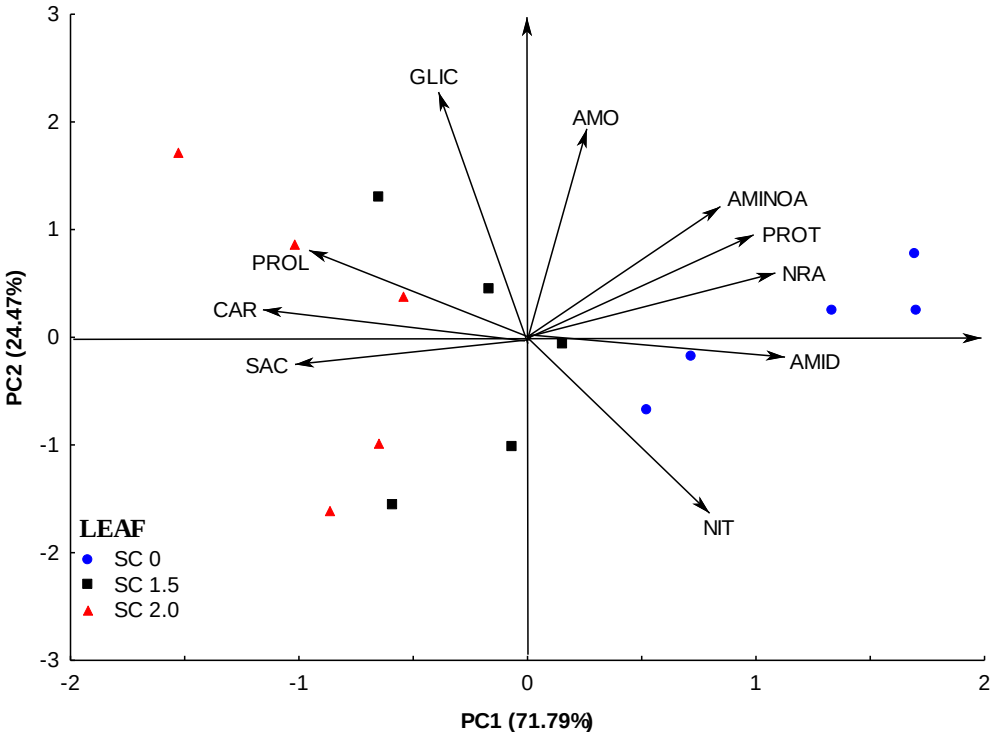

Figure 2. Biplot graphs containing the biochemical compounds in the leaf and the respective saline concentrations (SC) for the evaluated variables. NIT, nitrate; AMO, ammonium; NRA, nitrate reductase activity; AMINOA, amino acid; PROT, protein; CAR, carbohydrate; AMID, starch; SAC, sucrose; GLIC, glycine betaine; PROL, proline. In the Figure: (L) is the leaf. SC 0: is the 0 M saline concentration; SC 1.5: 1.5 M saline concentration; and SC 2.0: 2.0 M saline concentration.

Table 2. The correlation coefficient between the principal component scores for the variables in the shoot (leaf) of sorghum plants subjected to saline concentrations (0, 1.5 and 2.0 M).

Variable	PC1 (61.79%)*	PC2 (24.47%)*
Nitrate	**0.77**	-0.65
Ammonium	0.22	**0.78**
Nitrate reductase activity	**0.91**	0.26
Amino acid	**0.72**	0.48
Protein	**0.83**	0.40
Carbohydrate	**-0.98**	0.09
Starch	**0.97**	-0.04
Sucrose	**-0.97**	-0.06
Glycene betaine	-0.31	**0.91**
Proline	**-0.84**	0.34

*Percentage of the variability of the original data set retained by their respective principal components. Correlations in bold (> 0.70 in absolute value) were considered in the interpretation of the principal component.

reduction in protein synthesis can delay or even accelerate protein degradation, leading to an increase in the concentration of free amino acids. Jampeetong and Brix (2009) found that the proline content increased in *Salvinia natans* plants under saline conditions. The GLIC and AMO compounds retained in PC2 present a direct relationship with each other, but are not related to saline concentrations in the sorghum leaves.

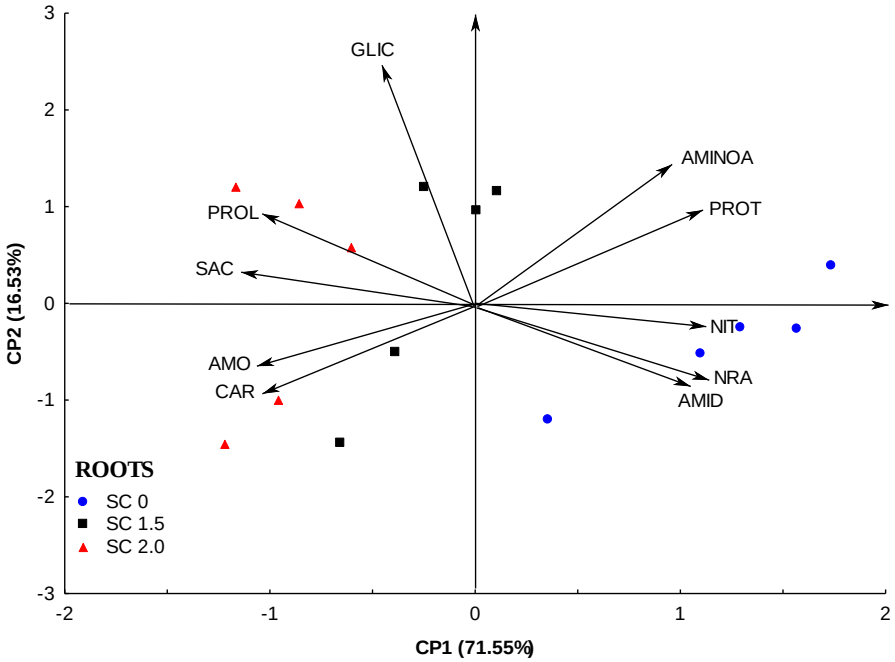

Figure 3. Biplot graph containing the biochemical compounds in the roots and their respective saline concentrations (SC) for the evaluated variables. NIT, nitrate; AMO, ammonium; NRA, nitrate reductase activity; AMINOA, amino acid; PROT, protein; CAR, carbohydrate; AMID, starch; SAC, sucrose; GLIC, glycine betaine; PROL, proline. In the figure: R represents the root. SC 0: the 0M saline concentration; SC 1.5: the 1.5 M saline concentration; and SC 2.0: the 2.0 M saline concentration.

Biochemical behavior in the root

The two-dimensional plane generated in the biochemical behavior analysis of the sorghum plant roots due to saline concentrations accounts for 88.08% of the variability of the original data: 71.55% retained in PC1 and 16.53% in PC2 (Figure 3). Most of the biochemical compounds were retained in PC1, with the exception of glycine betaine (GLIC) in PC2 (Table 3). In PC1, the compounds AMO, CAR, SAC and PROL (same negative sign) are directly correlated and are found in greater amounts in the roots of sorghum plants that received saline concentrations of 1.5 and 2.0 (Figure 3 and Table 3). In the root, the compounds, AMO, CAR, SAC and PROL, are responsible for characterizing the sorghum plants that received saline concentrations (1.5 and 2.0 M), and the control treatment (Figure 1). While in sorghum leaves these compounds are CAR, SAC and PROL (Figure 2).

The effect of proline accumulation in plants on both root and leaf subjected to stress has been studied for over 45 years (Kavi Kishor et al., 2005), and is currently considered as an indicator of acquired tolerance of the plants (Bellinger et al., 1991). The relative availability of K^+/Na^+ ions (Maathuis and Amtmann, 1999) and peroxidase phenols are also considered indicators of stress in plants (Lee et al., 2001). NIT, NRA, AMINOA, PROT and AMID (same positive sign) compounds were located to the right in PC1 (Figure 3), as occurred in sorghum leaves (Figure 2), and have higher availability in the roots of plants of the control treatment. Salinity conditions promote plant stress contributing to reduction in the NIT (Aragão et al., 2011; Lobato et al., 2009) and AMINOA activity (Elshintinawy and Elshoubagy, 2001). With reduced availability of AMINOA, the PROT amount decreases. The variance of glycine betaine (GLIC) was retained and isolated from other variables in PC2 (Figure 3). The amount of GLIC was higher in sorghum roots subjected under 1.5 M saline concentration. According to Khan et al. (2000), the GLIC levels increase in the leaves and roots of plants under saline stress, confirming the observations in the present study.

The changes in the biochemical behavior of the sorghum plant leaves and roots occur because the stimulus to saline stress causes excess Na^+ and Cl^- in the protoplasm. This causes physiological disturbances that affect photophosphorylation, respiratory chain, nitrogen assimilation and protein metabolism (Trindade et al., 2006). In addition, excess Na^+ inhibits many enzymes requiring potassium (Greenway and Munns, 1980), since there is a competition between Na^+ and K^+ for the active site. Consequently, the alterations observed in the sorghum plants under saline stress cause low productivity, decrease in the stand of the plants and, in

Table 3. The correlation coefficient among the principal component scores for the variables in the root part (root) of sorghum plants subjected to saline concentrations (0, 1.5 and 2.0 M).

Variables	PC1 (71.55%)*	PC2 (16.53%)*
Nitrate	**0.86**	-0.02
Ammonium	**-0.87**	-0.28
Nitrate reductase activity	**0.88**	-0.28
Amino acid	**0.75**	0.51
Protein	**0.88**	0.34
Carbohydrate	**-0.85**	-0.39
Starch	**0.94**	-0.26
Sucrose	**-0.96**	0.09
Glycene betaine	-0.40	**0.89**
Proline	**-0.91**	0.33

*Percentage of the variability of the original set of data retained by their respective principal components. Correlations in bold (> 0.70 in absolute value) were considered in the interpretation of the principal component.

severe cases, death of seedling (Silva and Pruski, 1997). A similar situation occurs in other crops, such as rice - *Oryza sativa spp. japonnica* (Carvalho et al., 2011), bean - *Vigna unguiculata (L.) Walp*, corn - *Zea mays* (Willadino et al., 1999; Azevedo Neto and Tabosa, 2000) and soy bean - *Glycine max L*. (Carvalho et al., 2012).

Conclusion

Under salinity condition, there occurs a distinction in the biochemical behavior of sorghum plants between the position of the leaf and root, with the formation of two dissimilar groups in the cluster analysis. The salinity increase causes an adverse effect on the biochemical behavior in the sorghum roots and leaves, a reduction of nitrate reductase activity, amino acids, proteins and starches, while there is an increase in the amount of proline, carbohydrates, sucrose and glycine. In the multivariate analysis of principal components, the direct correlation of carbohydrates, sucrose and proline in the leaves and roots of sorghum plants is verified, while there is an inverse correlation with the availability of amino acids, nitrates, protein and nitrate reductase activity.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Short-term phyto-toxicity consequences of a non-selective herbicide glyphosate (Roundup™) on the growth of onions (*Allium cepa Linn*.)

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This study examined the phyto-toxic effects of a commonly named non-selective herbicide glyphosate (Roundup™) on onions (*Allium cepa Linn*.). The study was necessitated due to the indiscriminate use and release of Roundup™ for weed control in the Niger Delta soils of Nigeria. The Organisation for Economic Co-operation and Development (OECD) standard protocol # 208 was adopted. The short-term phyto-toxic consequence on onion (*A. cepa L.*) was determined after a 4-day exposure to varying concentrations of the test chemical at 0.625, 1.25, 2.5, 5 and 10 mg/L, respectively. The percentage growth rate decreased as percentage growth rate inhibition efficiency increased, which implies that the effect of the herbicide was concentration dependent. The mean percentage growth rate inhibition efficiency relative to the control was 28, 47, 61, 73 and 96%, respectively. The effective concentration (EC_{50}) for % Inhibition efficiency was 1.550 mg/L with a 95% confidence interval of 1.269 to 1.848 mg/L. The biological alterations on the onions in the test solutions varied in order of increasing concentrations. Some observations made include decolouration of the test solutions and stunted growth especially at the highest concentration of 10 mg/L. Other effects include: bulb deformation, tissue and root damage. There was significant difference between the exposed species and the control at $P < 0.05$. The use of the herbicide Roundup™ with such hazardous effects can harm plants especially onion which is a very viable food product of man. This could lead to likely distortion on the ecosystem balance of onions (*A. cepa*) and similar plants and subsequent effect on human, the major end user.

Key words: Herbicide, glyphosate (roundup), onion (*Allium cepa Linn*.), phytotoxicity.

INTRODUCTION

Pesticides are substances or mixture of substances intended for preventing, destroying, repelling, mitigating or controlling any pest. They could interfere with the production, processing, storage, transport or marketing of

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food, agricultural commodities, wood products and animal feedstuffs (FAO, 2002; USEPA, 2007). Subclasses of pesticides and their target pest include: herbicides (weeds), insecticides (insects), pediculicides (lice), biocides (micro-organisms), algicides (algae), avicides (birds), molluscicides (snails), nematicides (nematodes) and rodenticides (rodents) (Gilden et al., 2010). Herbicides commonly known as weed-killers are pesticides, substances or cultured biological organisms used to kill or suppress the growth of unwanted plants and vegetation (Cork and Krueger, 1992). Herbicides may be selective and non-selective. Selective herbicides affect only certain types of plants (that is, they kill specific targets while leaving the desired crop relatively unharmed). Non-selective herbicides kill all plants with which they come into contact (that is, inhibits a very broad range of plant types). In Nigeria, herbicides have been used effectively to control weeds in agricultural systems (Adenikinju and Folarin, 1976). Herbicides could accumulate to toxic levels in soils and become harmful to microorganisms, plants, wildlife and man (Amakiri, 1982; Wilson and Tisdell, 2001). Herbicides may directly contact surface water *via* aerial drift, accidental spills, or surface runoff polluting water bodies thereby causing havoc to aquatic habitats amongst others. Over 95% of herbicides reach a destination other than their target species, including non-target species, air, water, bottom sediments and food. The amount of herbicide that migrates from the intended application area is influenced by the particular chemical's properties such as: propensity for binding to soil, vapour pressure, water solubility, and resistance to being broken down over time. Glyphosate (Roundup™), which was assessed in this study is one of the most widely used non-selective herbicide with applications in agriculture, forestry, industrial weed control, garden and aquatic environments (Baerson, 2002; Tomlin, 2006; Cavusoglu et al., 2011). In the soil environment, glyphosate is resistant to chemical degradation, stable to sunlight, relatively non-leachable, and has a low tendency to runoff. It is relatively immobile in most soil environments as a result of its strong adsorption to soil particles. It is also known to cause damage to soil organisms (Dalby et al., 1995).

Onion (*Allium cepa*) belongs to the bulb crops, a group belonging to the family of *Alliaceae*. It is one of the most important edible vegetable crops used for seasoning not only in Nigeria but all over the world. Weeds are one of the main plant protection problems in onion fields. They compete with onions for light, nutrients, water and space. Many researchers have reported that onion plants are poor competitors (Ghosheh, 2004; Carlson and Kirby, 2005; Qasem, 2006; Smith et al., 2011). This poor competitive ability with its initial slow growth and lack of adequate foliage (shallow roots and thin canopy) makes onions weak against weeds. In addition, their cylindrical upright leaves do not shade the soil to block weed growth (Wicks et al., 1973). Mainly, chemical control is applied against weeds in onion producing areas, but possible phyto-toxicity on onion is also a main problem. Root growth inhibition and adverse effects upon chromosomes provide indications of toxicity and genotoxicity (Rank and Nielsen, 1993; Bonciu, 2012; Olorunfemi and Ehwre, 2010).

The purpose of this study was to determine the deleterious effects of a commonly used non-selective herbicide; glyphosate (Roundup™) on the growth characteristics of onion (*A. cepa*). The use of herbicides in the environment has been of a great benefit in weed control however, their effects on onions and other plants as well as their fate in the environment is of important consideration due to the fact that humans are the major end users of plant produce. Onion (*A. cepa*) was chosen because they are relatively abundant in Nigeria and worldwide, available all the year round, sensitive to pollutants and are daily consumed by man and other organisms (Beeby, 2001; Rank and Nielsen, 1993).

MATERIALS AND METHODS

Test chemical

Roundup™ (Glyphosate, N-phosphonomethyl glycine), a non-selective herbicide was obtained locally from the vendors in Warri, Delta State, Nigeria. The test chemical is liquid, soluble in water and contains 360 g/L glyphosate. The chemical is currently used by farmers for weed control in the Niger Delta area of Nigeria.

Test species

The test species was common onion (*A. cepa* L.) of the purple variety. The onions used had a mean size of 6.30 ± 0.08 cm in diameter with a mean weight of 78.33 ± 0.87 g.

A. cepa assay

The *A. cepa* assay was assessed using the Organisation for Economic Co-operation and Development, (OECD) protocol 208 (OECD, 2003).

Procurement and preparation of onions

Onion bulbs (*A. cepa*) of the purple variety with average size were purchased locally in Effurun, Warri, Delta State and the same batch of bulbs were used throughout the experiment. The onions were sun-dried for a week and the dried roots present at the base of the onion bulbs were carefully shaved off, with a sharp razor blade to expose the fresh meristematic tissues. Bulbs attacked by fungi were discarded at the beginning of the experiment. The bulbs were then placed in freshly prepared distilled water to protect the primordial cells from drying up (Rank and Nielsen, 1993).

Root growth inhibition evaluation

For root growth inhibition evaluation, the 4-days semi-static renewal assay started with a range finding test to determine the range of concentrations to be used for the definitive test. Freshly prepared

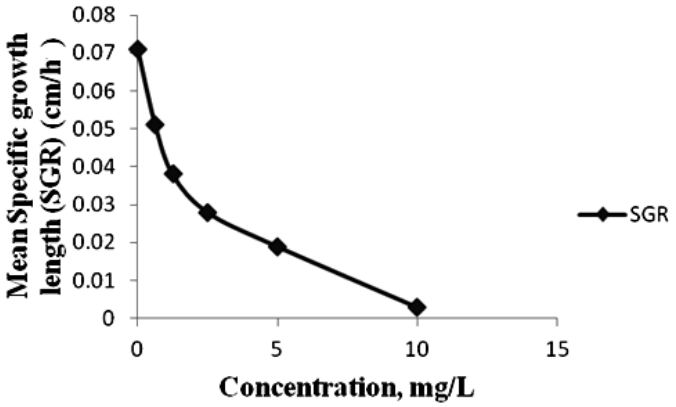

Figure 1. Means of specific growth length of *onions (Allium cepa)* exposed to glyphosate (Roundup™) in comparison with the control.

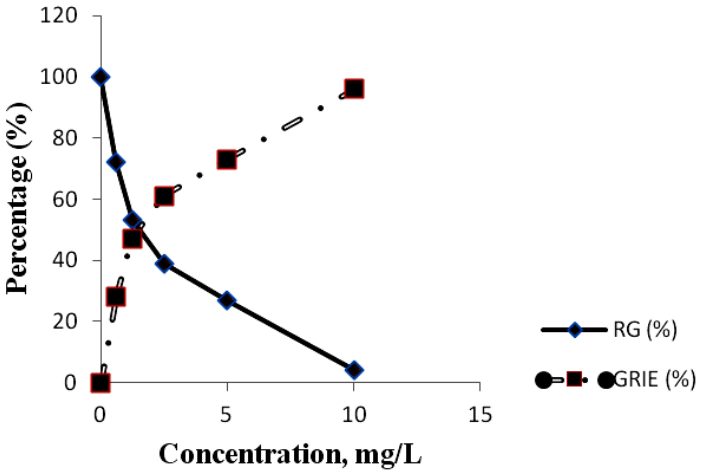

Figure 2. Means of percentage root growth relative of control (RG) in comparison with mean growth root inhibition efficiency (GRIE).

stock extracts of the test chemical was serially diluted into five concentrations of 10, 5, 2.5, 1.25 and 0.625 mg/L, respectively. Triplicate for each concentration and the control (tap water) were used for the 96 h bioassay. The negative control was set up with tap water of good quality. The tap water was ascertained to be with pH of 6.7±0.3 and had a relatively soft hardness concentration of 16 ± 0.01 mg $CaCO_3$/L and free from any chlorine compounds and toxic ions (Fiskesjo, 1985). The base of each of the bulbs was suspended on the extracts inside 200 mL beakers in the dark for 96 h. Test extracts were changed daily to maintain the same concentration. At the end of the exposure period, the roots of onion bulbs at each concentration were removed with forceps and their lengths measured (in cm) with a metre rule. From the weighted averages for each test concentration and the control, the percentage root growth inhibition in relation to the negative control and the EC_{50} (the effective concentration where the percentage inhibition efficiency amounts to 50% of the controls) for each extract was determined (Fiskesjo, 1985). The effect of each sample on the morphology of growing roots was also examined.

Statistical analysis

The effective concentration (EC_{50}) for root inhibition efficiency was used to determine the vulnerability of onions to the test chemical at 4 d (OECD, 2003). In addition, the analysis of variance (ANOVA) in Statistical Package for Social Science (SPSS) statistical software in Version 16.0 was also used to test the mean statistical difference between the controls and treated groups at significance levels of $P < 0.05$.

RESULTS

The results of root growth inhibition evaluation of glyphosate (Roundup™) on onion (*A. cepa*) for the 4-days phytotoxicity assay are presented in Figures 1 to 3. There was significant growth in the control experiment for the test duration, which indicated that the test conditions were appropriate and thus growth inhibition recorded in the test solutions could be attributed to the effect of the test chemical. Although, the assay was carried out in the dark, at each test renewal, observations were made and recorded. At experimental renewal hour of 24, there was no growth in the highest concentration of 10 mg/L while the other concentrations recorded gradual growth. Figure 1 shows the mean root lengths of *A. cepa* cultivated in varying concentrations of the test chemicals and control. The mean root lengths of *A. cepa* obtained for the test chemical ranged from 0.29 ± 0.03 to 4.90 ± 0.12 cm while the control recorded 6.82 ± 0.07 cm. At test termination of the 4-days experiment, the mean percentage growth rate relative to the control recorded for the five concentrations 0.625, 1.25, 2.5, 5 and 10 mg/L were 72, 53, 39, 27 and 4%, respectively (Figure 1). There was a decrease in mean growth rate from the lowest concentration to highest concentration in the test chemical, an indication that growth rate was concentration dependent. The percentage growth rate between the control experiment and the test chemical was significantly different at levels of $P< 0.05$. However, the effect of the glyphosate was most exhibited at the highest concentration which recorded the highest percentage inhibition efficiency where growth was most inhibited. The percentage growth rate decreases as percentage growth rate inhibition efficiency increases, which implies that the effect of the herbicide was concentration dependent. The mean percentage growth rate inhibition efficiency relative to the control recorded 28, 47, 61, 73 and 96%, respectively (Figure 2).

In the various concentrations used in this assessment, the onion manifested varying degree of stress to the test chemical. Phytotoxicity of the test chemical was shown by decolouration of the test solutions and stunted growth in the highest concentrations. Daily observations showed that there was root damage in the test chemical vessels especially at higher concentrations. The roots were also characterized by malformations and these were mostly broken roots, twists, roots bent upwards and stunted growth. Other effects include: bulb deformation, tissue damage, etc. The effective concentration (EC_{50}) of the

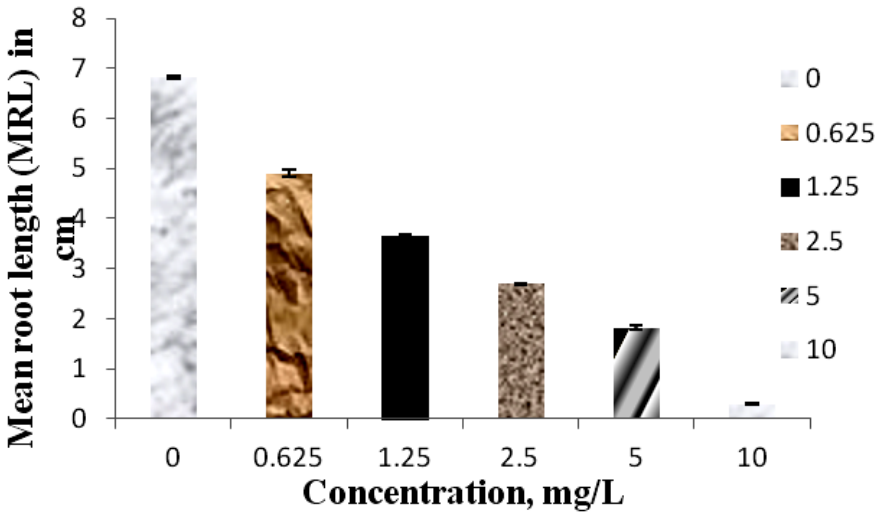

Figure 3. Means of root length of onions (*Allium cepa*) exposed to glyphosate (Roundup™) in comparison with the control.

chemical was evaluated using estimated 96 h EC_{50} values in varying concentrations (Figure 3). From the result, 0.625 mg/L test solution has the minimum growth inhibition efficiency of about 28% while 10 mg/L has the maximum 96% in relation to the control. Safety factors are arbitrarily built in around the EC_{50} values in order to arrive at environmentally tolerable concentrations. The concentration of a chemical in the environment should not exceed 10% of the EC_{50}. The estimated EC_{50} value after 96 h for glyphosate (Roundup™) was 1.550 ± 0.04 mg/L with a safe limit of 0.155 mg/L and 95% confidence interval of 1.269 to 1.848 mg/L.

DISCUSSION

As farmers continue to realize the usefulness of herbicides, larger quantities would be applied to the soil. However, the fate of these compounds in the environment is becoming increasingly important, since they could accumulate to toxic level and become harmful to plants as well as the environment (Wilson and Tisdell, 2001; Ayansina et al., 2003). However, most of the commonly used herbicides have not been characterized for their phytotoxicity consequence. The material safety data sheet (MSDS) of most chemicals / pesticides contain little or no details of their toxic levels to aquatic, terrestrial and plant species. Since a large number of herbicides are in use, there is significant concern regarding health effects to plants and man. From the data obtained for this assessment, there is the likelihood of this occurring. As the concentration of the pesticide is increasing, the percentage growth rate decreases while the percentage growth rate inhibition efficiency increases. This implies that uncontrollable use and release of the test chemical into the environment would lead to depletion of these viable species onion (*A. cepa*) serving as food for man. In addition to the health effects caused by herbicides, commercial herbicide mixtures often contain other chemicals, including non-active ingredients, which could have negative impacts on human health (Bonciu, 2012).

Herbicides present the only group of chemicals that are purposely applied to the environment with aim to suppress weed and animal pests and to protect agricultural and industrial products. However, the majority of herbicide is not specifically targeting the pest only and during their application they also affect non-target plants and animals (non-selective herbicides). Repeated application could lead to loss of biodiversity. Many herbicides are also not easily degradable and thus persist in soil, leach to groundwater, surface water and contaminate wide environment. Depending on their chemical properties they can bioaccumulate in food chains, enter organisms and consequently influence human health. The overall intensive non-selective herbicide application results in several negative effects in the environment that cannot be ignored (Carlson and Kirby, 2005; Olorunfemi and Ehwre, 2010). It has been proposed that glyphosate interferes with absorption and utilization of Mn, thus increasing a plants susceptibility to disease. However, the majority of research has not found reductions in Mn concentrations within plants following glyphosate applications (Bott et al., 2008; Nelson, 2009; Rosolem et al., 2009). From the discussion above, it is apparent that the use of herbicides in the environment has negative impacts on onion cells and likely soil nutrients which might affect the growth and yield of onions as well as other similar plant species (Kellogg et al., 2000; Cavusoglu et al., 2011).

However, due to the root growth and sensitive response to the tested chemical, *A. cepa* test could be useful for a water and soil quality assessment; that is, as a bio-indicator informing one of the environmental conditions in the event of application, spill and unintentional release.

Conclusion

The indiscriminate use of herbicides for pest control would be difficult to eliminate in Nigeria since the regulators: National Agency for Food and Drug Administration and Control (NAFDAC) and the Federal Ministry of Agriculture and Rural Development (FMARD) have not enforced the characterization of all the organic and inorganic herbicides used in Nigeria prior to sale and use by farmers. However, only registration of the chemicals is ensured, this does not give details of the ecological damage the chemicals can cause to aquatic, terrestrial and plant species. Thus, the fate of these pesticides in the environment is an important consideration due to the fact that humans are the end users of plant produce. The assessment is with a view to safe-guiding the environment, its components and humans.

Conflict of Interests

The authors have not declared any conflict of interests.

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Abbreviations: NAFDAC, National Agency for Food and Drug Administration and Control; **OECD,** Organisation for Economic Co-operation and Development; **EC_{50},** effective concentration; **FMARD,** Federal Ministry of Agriculture and Rural Development.

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Morphological and molecular genetic diversity of Syrian indigenous goat populations

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Domestic goats in Syria may provide an interesting source of genetic variability due to its proximity to the centers of domestication. This study aimed to assess the morphological variation, genetic diversity and population substructure of the Syrian goat populations. Commonly, three goat genotypes are distinguished in Syria, namely Jabali or mountain goat, Baladi or local goat and Shami or Damascus (a well-known dairy goat). A pre-tested semi-structured questionnaire was used in recording both qualitative (coat color, eye color, horn length, horn orientation, nose profile) and quantitative (height at wither, chest girth, cannon length, body length, ear length and ear width) morphological data. Data from a total of 5,730 individual goats of the three goat populations reared in ten representative provinces of Syria were collected and analyzed using GenStat version 14 statistical packages. Results of the morphological analysis confirmed that there were clear morphological variations among the three goat populations. The three goat populations are mainly distinguished by their straight (Baladi, 71.1% and Jabali, 82.8%) and curved (Shami, 89.5%) nose profiles. Substantial phenotypic variability was found among and within the breeds suggesting that these goat breeds have not yet undergone an organized breeding program. The genetic variability and population substructures from 398 individual animals of the three breeds were genotyped using 12 DNA microsatellite markers from Food and Agricultural Organization (FAO) panel. All microsatellites typed were found to be polymorphic and a total of 41 distinct alleles were detected on Baladi, Jabali and Shami goat populations. The Syrian goat populations had observed and expected heterozygosity values that ranged from 0.50 to 0.62 and 0.74 to 0.85, respectively, and an average of 13.97 alleles per locus across breeds. For all loci, an average inbreeding values (F_{IS}) of low to moderate level was obtained across the three goat breeds, which ranged from 0.29 (Shami goats) to 0.34 (Baladi goats) indicating the absence of mating between close relatives within these populations. The observed positive F_{IS} coefficients among the studied goat breeds also suggested heterozygote deficiencies. The analyses of the molecular data using STRUCURE program indicated there were two primary populations, which did support the results based on morphological data of the same goat populations that clustered these goat populations into two main groups and confirmed the admixture nature of the Baladi and Jabali goat populations, while the Shami goat breed was well differentiated and grouped into a separate cluster that suggests its evolutionary and genetic uniqueness. The analysis of molecular variance (AMOVA) results detected genetic variations within individuals in a population (96%). The high genetic variability within individuals in a population provides a good base for designing genetic improvement programs under the existing goat management systems.

Key words: Characterization, cluster, genetic differentiation, population structure, Syria.

INTRODUCTION

Syria covers an area of 18.6 million ha and nearly half of Syria's land mass is classified as rangeland. The agriculture sector accounts for about 26% of Syria's Gross Domestic Product. Livestock provides over 34% of the total value of agricultural production, and contributes 17.7% of the value of agricultural exports. The livestock industry also provides employment to about 20% of the national workforce (Hajar, 2006) and is the main source of income and livelihood for Bedouin herders. Goats (*Capra hircus*) are common and the most important livestock species in Syria. They often possess great adaptability to harsh local environmental conditions and represent important genetic resources. These native breeds did not undergo extensive artificial selection by humans and are generally well-adapted to semi-arid or even arid conditions and such adaptive characteristics contribute to a growing interest on indigenous species for conservation and breeding programs.

The majority of Syria's goat population of 2.29 million heads consists of indigenous goats which are distributed all over the country (FAOSTAT, 2011) and raised for multipurpose uses. The economic importance of small ruminants has been increasing in Syria in recent years due to the erratic rainfall and related risk of crop failures. Besides, indigenous goat populations are known in general for heat tolerance, disease resistance, mothering and walking abilities, and the ability to efficiently metabolize low quality feeds (Trail and Gregory, 1984). Thus, indigenous goats are very valuable genetic resources adapted and suited for low-inputs-outputs agricultural production systems of the developing countries like Syria. The Mediterranean countries, namely Lebanon, Syria and Cyprus are considered as the main goat milk and cheese producer next to India (Dubeuf et al., 2004) and the Shami (Damascus) goat is well appreciated for its high milk yield and twinning ability. Recognizing the value of the Shami goats, the Syrian Ministry of Agriculture and Agrarian reform established seven Shami goats' improvement and conservation research and development centers in representative provinces of the country to maintain and improve this unique goat breed. The genetic relationships and differentiation between Shami and other native goat populations available in Syria have not yet been characterized and established in a way to contribute to the declared aim of the Food and Agriculture Organization (FAO) of the United Nations to preserve the genetic diversity of domestic animals including goats.

Phenotypic and molecular characterizations have been widely used to quantify morphological and genetic diversity in small ruminants (Gizaw et al., 2007; FAO, 2012). Morphological polymorphisms are the first to be used to determine the relationship between breeds (Weigend and Romanov, 2002) and considered as an essential component of breed characterization that can be used to physically identify, describe, and recognize a breed, and also to classify livestock breeds into broad categories (FAO, 2012). The magnitude of phenotypic variability differs under different environmental conditions, in which morphometric characters are continuous characters describing aspects of body shape. In addition, microsatellite markers have also been successfully used to study the biodiversity and genetic relationship and differentiation between and within domesticated livestock populations or breeds (Ruane, 1999; Baumung et al., 2006; Toro et al., 2006; Gizaw et al., 2007; Bizhan and Majnoun, 2009; Visser and van Marle-Koster, 2009; Hassen et al., 2012). Their abundance, high level of repeat number of polymorphism, suitability for amplification by polymerase chain reaction (PCR), co-dominant inheritance and random distribution in the organism's genome have facilitated their extensive use for molecular characterization of domestic animals. Therefore, the present study was aimed to analyse the Syrian goat genetic diversity and differentiation using recommended morphological traits (FAO, 2012) and microsatellite markers (FAO, 2004) to inform the design of rational goat breeds improvement and utilization strategies.

MATERIALS AND METHODS

Description of the study area

The Syrian Arab Republic is one of the Mediterranean countries and is located in the Middle East between 32° 19' and 37° 30'N and 35° 45' and 42° E with a total area of 185,400 km^2. Ten out of the fourteen Syrian provinces were selected following purposive sampling techniques by considering agro-ecology, socioeconomic importance of the indigenous goat populations, goat production systems, types of indigenous goats, the main challenges and opportunities of goat keeping. The study areas were also chosen based on previous informal and/or formal studies carried out on Syrian Jabali goats (Wurzinger et al., 2008) and other goat breeds elsewhere in North Africa and West Asia (Iniguez, 2005a, b). The ten targeted provinces were Aleppo, Al-Raqua, Al-Hasakeh, Idleb, Hama, Homs, Damascus Rural, Al-Seweida, Deir Al Zour and Tartous (Figure S1). The districts in each province were randomly selected and fifty-seven districts from the total sixty-four districts were chosen for collecting morphological data and blood samples from the targeted goat populations.

Goat populations and morphometric measurements

The agricultural development experts who were involved in the

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actual field survey were trained on characterization of the indigenous goat populations at morphological levels. The targeted goat populations were Baladi, Jabali and Shami, which are raised and distributed almost all over the country. The populations were varied on their physical appearance and in population sizes. The Baladi goats were the most dominant followed by the Jabali goats, while the Shami goats were the least in number per flock in each targeted study areas. These goats' breeds were normally kept together by smallholder farmers, which were managed under extensive management practices and studied goat populations were sampled from farmers' flocks. To capture the largest possible representation of the existing genetic diversity, we sampled relatively unrelated animals from several flocks in each population for morphological and molecular characterizations of the aforementioned goat breeds. In these flocks, veterinary health care and proper small ruminant feeding are rarely practiced, mating was uncontrolled and performance recording scheme was also not existing.

Field surveys were carried out in ten representative and accessible provinces across Syria covering all agro-ecological zones for generating socioeconomic data, goat herd structure (results of the goat production system analysis and goat management systems are not presented here), and recording the physical appearances and also for measuring selected phenotypic traits. In addition, in each of the study provinces, goat owners, key informants and extension agents were interviewed using a pre-tested semi-structured questionnaire and 3 to 5 mature unrelated animals per herd were randomly selected (that would help to avoid sampling of closely related individuals) and used to measure morphological traits. In addition, a list of physical descriptors was used to record morphological variations on goats coat color, eye color, horn length, horn orientation and nose profile. Moreover, quantitative data on linear body measurements (cm) such as height at wither, chest girth, cannon length, body length, ear length and ear width were measured using a measuring tape following the descriptor list of the Food and Agriculture Organization of the United Nations FAO (1986). Besides, focus group discussions were held with livestock keepers and knowledgeable key informants for generating general information regarding the history of the various goat types and special distinguished features of the targeted goats. These descriptors were used for identifying and refining the goat populations available in Syria. Depending upon the number of each goat population within a flock (data not shown in this paper) data on a total of 5,730 individual goats of both sexes (male, n=1,071 and female, n= 4,659): 409, 2,071 and 3,250 goats of Shami, Jabali and Baladi, respectively were recorded from ten provinces following the FAO goat descriptor manual (FAO, 1986).

Blood sampling and DNA extraction

The blood samples were collected from eight representative provinces, namely Aleppo, Al-Raqua, Al-Hasakeh, Idleb, Hama, Homs, Damascus Rural and Al-Seweida which followed the distribution and the density of the Baladi, Jabali and Shami goat populations across the country (Figure S1). A total of 398 blood samples from the three targeted goat populations (Baladi, n=236, Jabali, n= 90 and Shami, n= 12) were collected from different villages and herds (one blood sample per herd). Samples were also collected from 60 Shami goats (30 samples from each station) captured in Hmemeh Shami research station in Aleppo and Karahta Shami goat research station in Damascus provinces. Samples were taken from mature genetically unrelated herds that represented the three morphologically identified goat populations. The samples were collected by puncturing the jugular vein of each animal using the 10 ml vacutainer tubes having K3-EDTA as anticoagulants, field collection, all samples were extracted following the DNA extraction procedure described for peripheral blood lymphocytes (PBL) stored with Urea (Tapio et al., 2007). Subsequently, DNA concentrations were estimated by a Nano-DNA spectrophotometer (Pharmacia LKB-Ultraspec III) in which the quality of DNA was assessed using the ratio of A260/A280.

PCR amplification and genotyping

The DNA was amplified by PCR in a thermal cycler (GeneAmp PCR System 2700 96-well cyclers) using 12 microsatellite markers selected from FAO/ISAG panel list (FAO, 2004) described in Table 3. The forward primers of each pair were labeled with either NED (Yellow), FAM (Blue), and VIC (Green) dyes, which were supplied by Applied Biosystems Company, Warrington, UK. The primers were screened from the list of microsatellite markers recommended by FAO/ISAG in which only 12 fluorescently labelled microsatellite markers were chosen based on the degree of polymorphism obtained using genomic DNA extracted from Syrian goats. These markers were also used by different researchers for livestock genetic variability studies such as on Ethiopian goats (Hassen et al., 2012), Hamdani sheep (Al-Barzinji et al., 2011) and the characterization of other goat populations elsewhere (Visser and van Marle-Koster, 2009; Martinez et al., 2006). The 20 µl amplification reactions contained genomic DNA, 10× standard polymerase chain reaction (PCR) buffer, dNTPs, Amplicon *Taq* DNA polymerase, forward and reverse primer mix and double-distilled sterilized water, which was formulated following a standard protocol (Sambrook et al., 1989) and were performed in a single PCR amplification for each locus. The reactions were processed on a thermal cycler following a step down amplification method. The PCR products were initially visualized in agarose gels stained with Ethidium bromide to identify possible imperfections and to decrease the rate of failure in capillary electrophoresis. Only the amplified PCR products were multiplexed based on their reported allele size range and the type of fluorescent labels used for each locus which were finally analyzed by capillary electrophoresis using an automated ABI Prism® 3100 DNA genetic analyzer. The data was captured using GeneScan version 3.1 software with 350 ROX labeled internal size standards and the allele sizes (bp) were determined using GeneMapper version 3.7 software (Applied Biosystems, Foster City, CA, USA). Finally, an allelic table was created from this dataset and exported to Microsoft Excel for further statistical analysis using appropriate molecular genetic analyses software.

Statistical data analyses

The General Linear Model (GLM) procedures of GenStat (version 14) statistical package were employed to analyze the quantitative data collected from Syrian goat breeds in which breed, sex, age and their interactions were fitted as fixed effects. In other words, the goat breeds, sex and age were fitted as independent variables, whereas body weight and other linear body measurements were fitted as dependent variables. The qualitative traits were analyzed in GenStat as descriptive statistics (as percentage). The interactions between breed, age and sex were found to be statistically non-significant; hence, the statistical analysis results for breed by age and sex interactions are not presented in this paper. As farmers did not have birth record of their animals, age of each sampled goat was estimated using dentition as described by Wilson and Durkin (1984); hence, four age groups were identified (Table 2). For each goat population, morphometric data from different villages and flocks across the different districts in the targeted

provinces were collected from unrelated goats of both sexes and breeds. The quantitative traits considered were height at withers, chest girth, cannon length, ear width, body length, ear length and ear width while the qualitative traits recorded were coat color, eye color, horn length, horn orientation and nose profile. Hierarchical cluster analysis was conducted and a dendrogram constructed based on Euclidean distances between populations derived from morphological variables using the unweighted pair-group method to describe morphological clustering pattern of the targeted goat populations. In the hierarchical cluster analysis, the major quantitative (height at wither, chest girth, cannon length, body length, ear length and ear width) and qualitative (coat color, eye color, horn length, horn orientation and nose profile) morphometrical variables were included, in which the data for individual traits were pooled for both sexes in each breed.

The Genetic Analysis in Excel (GenAlEx) version 6.5 (Peakall and Smouse, 2012) program and PowerMarker version 3.25 (Liu and Muse, 2005) statistical package were used to generate population genetic diversity parameters (total and average number of alleles, allele frequency, observed and expected heterozygosity). The Genetic diversities within and among goat populations were measured as the mean number of alleles (MNA) per locus and breeds as well as effective number of alleles (Ne). The effective number of alleles (Ne) per locus and breed were computed using PopGene version 1.32 software (www.ualberta.ca/~fyeh/popgene). The observed mean number of alleles (MNA), observed (H_o) and expected heterozygosity (gene diversity) (H_e) values were also computed. Using the variance-based method of Weir and Cockerham (1984) fixation indices (F_{IS}, F_{IT}, F_{ST}), estimated genetic differentiation (Gst), Shannon diversity index (I) and gene flow (Nm) values for each locus and overall loci were calculated based on GenAlEx software. P-value for inbreeding coefficients (F_{IS}) within samples was calculated on FSTAT version 2.9.3.2 (Goudet, 2001). The MNA detected in each population and the H_e values are in general good indicators of genetic polymorphism. MNA was counted as the average number of alleles observed in a population, while the H_e value is estimated as the proportion of heterozygote expected in a population. The GenAlEx program was used for testing deviation or departure from Hardy-Weinberg Equilibrium (HWE) at each locus for each studied goat populations.

Pair-wise Reynolds' linearized standard genetic distances between populations were calculated from allele frequencies following Nei et al. (1983) procedure in GenAlEx package. Distances obtained were used to construct a dendrogram following unweighted pair group method with arithmetic mean (UPGMA). Bootstrap (n=1,000) re-sampling was carried out to check the robustness of the phylogenetic tree and the resulting tree was visualized in TREEVIEW version 1.6.6 software. The analysis of molecular variance (AMOVA) was also performed by the GeneAlEx using the codominant allelic distance matrix with 999 permutations to partition the total covariance components into inter-group, inter-populations within group and within population components.

The Bayesian cluster analysis using STRUCTURE version 2.3.4 software (Pritchard et al., 2000) was run to infer the genetic relationships between the Syrian goats and also to assess level of admixture. To estimate the number of subpopulations (K), ten independent runs of K = 1 to 15 were carried out with a 100,000 burn-in period at 100,000 Markov Chain Monte Carlo iterations by considering correlated allele frequencies and an admixture model. The averaged likelihood at each K was used to calculate ΔK (Evanno et al., 2005) to further investigate results from STRUCTURE using STRUCTURE HARVESTER version 0.6.94 website based program (Earl and vonHoldt, 2012), which was used as an *ad hoc* indicator of population number or cluster number. Results from replicate runs at the optimal K were combined in CLUMPP (Jakobsson and Rosenberg, 2007) and then CLUMMP results were used to plot bar graphs based on DISTRUCT (Rosenberg, 2004) program.

RESULTS AND DISCUSSION

It is not possible to provide research results on the characterization of Syrian indigenous goat production and management systems. Rather, this part will be attempted to cover as a separate manuscript. This paper provides a range of research results on phenotypic and molecular characterization of Syrian goat populations (Baladi, Jabali and Shami) which identified substantial phenotypic and genotypic variability among and within the targeted breeds. As mentioned earlier in the methodology part, agro-ecological zones, distributions and goat densities were the main criteria used for collecting morphological data and blood samples from the purposely targeted goats breed of the Baladi, Jabali and Shami goat populations across the country (Figure S1).

Qualitative variations

The physical body characteristics for Syrian goat breeds and their coat colors as well as their appearances are presented in Table 1. The majority of the Syrian indigenous goat populations had varying percentages of different coat color types and compositions, which could be used in most cases to distinguish the three goat populations (Figure 1). Commonly, three goat genotypes are distinguished in Syria, namely Jabali, Baladi and Shami. The majorities of the Baladi, Jabali and Shami goat populations of both sexes have predominant brown, black and brown coat colors, respectively. The most common and frequent body coat colors for these goats were black (32.23%) and brown (31.93%). Most Syria male (29.3%) and female (32.83%) goats had black coat color followed by brown. In addition, goats with crème and admixture of black, grey and brown coat colors were also observed. Such colors might have resulted from continuous selection practiced by goat owners for developing breeds with preferred colors. The most common eye color observed on Syrian goat breeds of Baladi, Jabali and Shami were crème (35%), brown (23.9%) and crème (76.9%), respectively along with the presence of various percentages of other eye colors.

Most Syrian Baladi (62.1%) and Shami (81.2%) goat breeds were polled (hornless) (Table 1), while the remaining goats have horns of short to long size orienting upward, backward and sideward. Moreover, the majority of the Syrian Jabali goats had backward horn-orientation (41.3%) with the presence of various percentages of other horn-orientation types. Most Baladi (71.1%) and Jabali (82.8%) goats' populations have had straight nose profile, which is in line with the findings reported for Syrian Jabali goats (Wurzinger et al., 2008). Most of the Shami goats had curved nose structure (89.5%) and similar nose profile of a Roman nose structure was also reported by Hancock and Louca (1975) for Shami goat breed. Most of the Syrian goat breeds have long and drooping ears and the owners of the goats practice ear

Table 1. Observed mean frequency (%) values for selected qualitative traits on Syrian goat populations.

Qualitative traits		Breed			Sex by breed					
					Male			Female		
		Baladi (3250)*	Jabali (2071)	Shami (409)	Baladi (512)	Jabali (487)	Shami (87)	Baladi (2738)	Jabali (1584)	Shami (322)
Coat color	Black	25.4(826)	52.9(1096)	18.4(76)	17.2(88)	50(244)	20.7(18)	26.9(737)	53.7(851)	17.9(58)
	Brown	34.1(1109)	17.2(356)	44.5(182)	39.1(200)	18.1(88)	41.5(36)	33.2(909)	17(269)	45.2(146)
	Crème/Grey	26.2(852)	18.8(389)	27.5(113)	28.7(147)	20.3(99)	26.8(23)	25.7(704)	18.4(291)	27.7(89)
	Mixed	0.4 (13)	0.7(15)	0.7(3)	0.6(3)	0.2(1)	1.2(1)	0.4(11)	0.8(13)	0.6(2)
Eye color	Crème	35.0(1138)	23.9(495)	76.7(314)	43.8(224)	31.1(151)	76.8(67)	33.4(914)	21.7(344)	76.7(247)
	Brown	28.2(917)	46.9(972)	7.8(32)	21(108)	39.4(192)	7.3(6)	29.5(808)	49.2(779)	8(26)
Horn length	No horn (polled)	62.1(2018)	34.7(719)	81.2(333)	77.3(396)	41(200)	75.5(66)	59.1(1618)	32.8(520)	82.1(264)
	Cut	4.3(140)	1.6(34)	7.0(29)	2(10)	0.6(3)	11.3(10)	4.7(129)	1.9(30)	6(19)
Horn orientation	Backward	27.9 (907)	41.3(855)	10.9(45)	11.5 (59)	16.3(79)	5.3(5)	30.8(843)	49.0(776)	12.2(39)
Nose profile	Straight	71.1(2311)	82.8(1715)	10.5(43)	56.9(291)	76.6(373)	15.9(14)	73.7(2018)	83.7(1326)	9.2(30)
	Curved	28.9(939)	17.2(356)	89.5(366)	43.1(221)	23.4(114)	84.2(73)	26.3(720)	16.3(258)	90.8(292)

*- numbers in bracket refers to sample sizes taken for each qualitative trait per breed.

cutting to avoid the damage of goat ears during browsing and/or grazing. A set of distinguishable and unique characters to each goat breed is presented in pictures (Figure 1).

Quantitative variations

Least squares means (± SE) of the body measurements (cm) for Syrian goat breeds are depicted in Table 2. Age, breed and sex consistently showed a highly significant ($p<0.001$) effect on height at wither, chest girth, cannon and body length. In addition, a highly significant difference was obtained ($p<0.001$) among goat breeds on ear length and difference on ear width were obtained on age and breed. The linear body measurements like height at wither, chest girth, cannon, and body length and ear width of the Syrian Shami goats were the highest followed by Baladi goats, while the Jabali goats were the smallest for all the aforementioned measured traits. Mavrogenis et al. (2006) reported comparable results regarding the body length of the Damascus (Shami) goats, which were imported into Cyprus more than 70 years ago with the aim to upgrade the local Cypriot goat population.

Majority of the Shami, Baladi and Jabali goat breeds can be distinguished by their dominant qualitative and quantitative traits. For instance, most of the Shami goats have brown coat color, no horn and curved nose profile, while the Jabali goats have black coat, and brown eye color, no horn and have straight nose structure (Table 1 and Figure 2).

Microsatellite marker polymorphism and allelic variations

The present work investigated for the first time the genetic variability and the population structure of the Syrian indigenous goat populations using microsatellite markers from FAO panel. The

Figure 1. Physical appearance of indigenous Syrian goat populations

Table 2. Least squares means (± SE) for effects of breed, sex and age of Syrian goat breeds on quantitative traits.

Effect and level	Height at withers	Chest girth	Canon length	Body length	Ear length	Ear width
N	5730	5730	5730	5730	5730	5730
Overall	74.67±0.31	85.42±0.42	10.81±0.08	70.82±0.54	20.46±0.45	9.22±0.15
CV (%)	7.6	8.88	14.86	13.84	27.87	21.2
Age group	***	***	***	***	NS	***
G1	75.52 ±0.23	85.16±0.31	11.26±0.06	70.7±0.39	20.15±0.26	9.65±0.08
G2	77.58 ±0.2	87.89±0.29	11.41±0.05	73.69±0.37	20.1±0.23	9.49±0.08
G3	78.25 ±0.21	89.08±0.28	11.46±0.05	74.4±0.37	20.52±0.23	9.43±0.08
G4	77.55±0.02	89.05±0.21	11.22±0.04	73.76±0.27	20.15±0.2	9.27±0.07
Breed	***	***	***	***	***	***
Baladi	76.99±0.29	87.41±0.4	11.40±0.08	73.54±0.52	21.04±0.44	9.48±0.15
Jabali	74.68±0.31	85.16±0.41	10.89±0.09	69.81±0.53	19.25±0.46	8.79±0.16
Shami	80.01±0.21	90.82±0.37	11.73±0.07	76.03±0.32	20.38±0.4	10.12±0.11
Sex	***	***	***	***	NS	NS
Male (M)	80.04±0.19	90.22±0.26	12.04±0.05	75.75±0.34	20.03±0.23	9.49±007
Female (F)	74.42±0.17	85.38±0.22	10.67±0.03	70.51±0.25	20.41±0.21	9.44±0.06

***, p<0.001; NS, Non-significant; *- G1, G2, G3 and G4 are referred to the age of the goats from 1 to 2, >2 to 3, > 3 to 4 and > 4 years old, respectively.

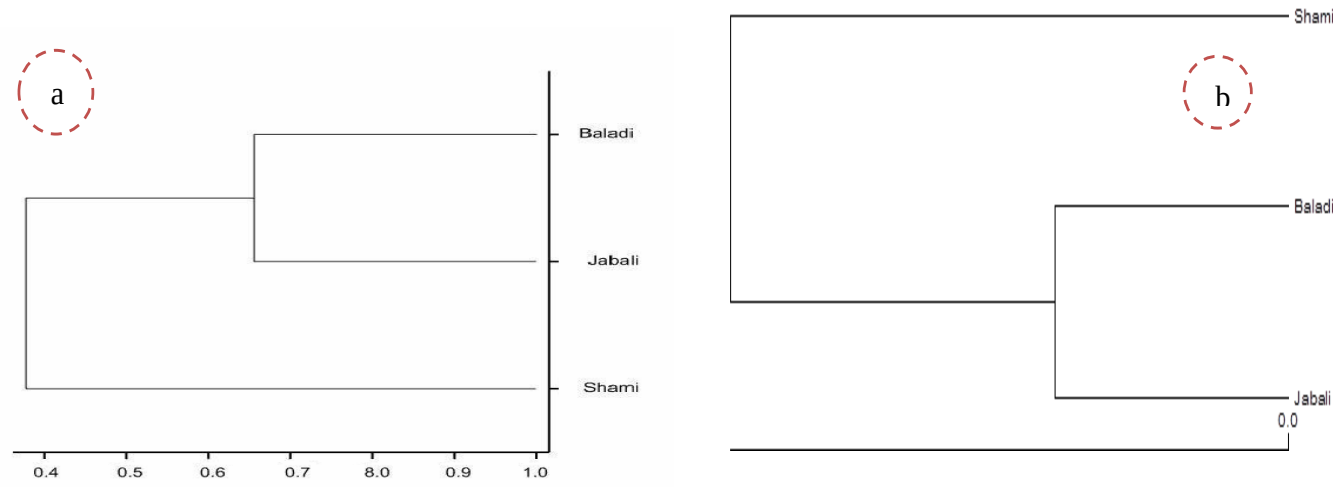

Figure 2. (a) Dendrogram showing morphological and **(b).** molecular variabilities among Syrian goat populations.

Table 3. Basic characteristics of each microsatellite loci used, annealing temperature (AT), observed allele size (AS) range, major allele frequency (AF), total number of alleles (TNA), mean number of alleles (MNA), effective number of alleles (Ne), polymorphic information content (PIC), Observed heterozygosity (Ho), Expected Heterozygosity (He), Wright's F-statistics (F_{IT}, F_{IS}, F_{ST}), observed genetic differentiation (G_{ST}), Shannon diversity index (I) and gene flow (Nm) values by locus across studied Syrian goat populations

Locus	AT (°C)	AS (bp)	AF (%)	TNA	MNA	Ne	PIC	Ho	He	FIS	FIT	FST	GST	I	Nm
ILSTS044	65-55	138-172	0.38	20	11.33	4.38	0.68	0.31	0.72	0.47	0.50	0.06	0.05	1.66	4.33
ILSTS087	68-58	114-167	0.25	27	15.33	6.19	0.83	0.59	0.85	0.34	0.38	0.06	0.05	2.31	4.24
SRCRSP8	65-55	112-255	0.47	22	12.00	4.36	0.72	0.25	0.74	0.70	0.73	0.09	0.08	1.9	2.66
ILST019	65-55	118-176	0.37	22	13.67	6.70	0.80	0.29	0.82	0.57	0.58	0.04	0.03	2.16	6.99
OARFCB48	68-58	96-180	0.25	23	14.00	6.17	0.83	0.62	0.85	0.22	0.26	0.04	0.04	2.27	5.76
ILSTS005	68-58	96-196	0.56	20	9.67	4.05	0.58	0.19	0.62	0.59	0.63	0.09	0.09	1.41	2.47
BM6444	68-58	118-182	0.12	34	23.67	13.08	0.94	0.61	0.94	0.34	0.37	0.04	0.03	3.08	6.05
ETH10	68-58	121-212	0.41	13	7.33	3.50	0.63	0.60	0.69	0.11	0.19	0.09	0.08	1.43	2.55
MAF209	68-58	89-122	0.51	12	6.33	3.15	0.64	0.55	0.68	0.11	0.21	0.11	0.11	1.52	1.96
MAF70	68-58	95-182	0.19	41	23.33	11.74	0.89	0.75	0.90	0.11	0.13	0.03	0.02	2.67	9.68
P19(DYA)	65-55	116-203	0.18	24	16.33	8.50	0.90	0.81	0.91	0.02	0.06	0.04	0.03	2.57	6.86
SRCRSP9	68-58	92-144	0.14	23	14.67	8.68	0.90	0.68	0.91	0.17	0.21	0.04	0.03	2.56	6.04
Mean/Breed			0.32	23.42	13.97	6.71	0.78	0.52	0.80	0.32	0.35	0.06	0.05	2.13	4.97

characteristics and the nature of the twelve selected microsatellite markers used in this study are summarized in Table 3. Results of molecular genetic analysis showed that all twelve loci were polymorphic; none of the markers were monomorphic and were adhered to the parameters for studying genetic diversity. A total of 281 alleles were detected at the 12 microsatellite loci on three studied goat breeds. The observed mean number of alleles per locus ranged from 6.33 (MAF 209) to 23.67 (BM6444), with an average of 13.97 alleles per locus across breeds. The observed number of alleles at a locus (Table 3) and the genetic distance values (Table 5) indicated genetic variability at that locus, which suggested the appropriateness of the loci to be used to analyze genetic diversity on goats. It was also suggested that for studies of genetic diversity and genetic distance within and among populations, microsatellite markers should have no fewer than four alleles which could help to reduce standard errors of distance estimate (Barker et al., 2001).

The effective number of alleles (Ne) ranged from 3.15 (MAF209) to 13.08 (BM6444), with an overall mean of 6.71 alleles per locus. All the loci used to analysis the Syrian goat diversity were highly informative which make them useful in genetic diversity studies. This is due to the fact that if a locus has PIC value > 0.5 that locus is considered as highly polymorphic loci; while a locus with PIC values ranging from 0.25 to 0.5 is clustered as a medium polymorphic marker (Vanhala et al., 1998). In this investigation, all loci were highly polymorphic, with PIC value range from 0.58 (ILSTS005) to 0.94 (BM6444),

Table 4. Breed level allelic and genetic diversity: mean number of alleles (MNA), Polymorphic Information Content (PIC), effective number of alleles (Ne), Shannon's diversity index (I), observed (Ho), and expected (He) heterozygosity and inbreeding coefficient (F_{IS}) values for Syrian goat populations.

Breed	Allelic diversity			Genetic diversity			
	MNA	Ne	PIC	Ho	He	I	FIS
Baladi	15.33±1.92	6.32±1.22	0.75	0.52±0.06	0.78±0.04	1.92±0.17	0.34±0.06*
Jabali	12.50±1.80	5.93±1.27	0.71	0.50±0.07	0.74±0.04	1.84±0.17	0.33±0.09*
Shami	14.08± 1.70	7.87±1.09	0.83	0.62±0.07	0.85±0.01	2.26±0.11	0.29±0.07*
Mean	13.97±1.87	6.71±1.19	0.78	0.52±0.07	0.80±0.03	2.01±0.15	0.32±0.07

*$p < 0.05$.

with a mean of 0.78 per locus. The average expected heterozygosity value ranged between 0.62 (ILSTS005) and 0.94 (BM6444) with an overall mean of 0.80, whereas the observed heterozygosity value ranged from 0.19 (ILSTS005) to 0.81 (P19) with an average value of 0.52 (Table 3).

Genetic diversity and differentiation

The genetic variability of each subpopulation was initially studied in terms of observed and effective number of alleles, as shown in Table 3 and the three goat breeds were having varied mean number of alleles. Genetic diversity analyses also suggested substantial genetic variability within the studied goats breeds (96%) (Table S2) in which its value supported by the morphological characterization study carried out on the same goat populations (Tables 1 and 2). The genetic diversity of each goat breed also showed considerable differences when measured in MNA per breed across all loci that ranged between 12.50 for Jabali goat and 15.33 for Baladi goat breeds. The Syrian Jabali goats had less than the average number of allele, while the Shami and Baladi goat breeds had higher mean number of alleles (Table 4). It was suggested that the observed MNA over a range of loci across different population is considered to be a good indicator of genetic variation within a given animal population (MacHugh et al., 1997).

Observed mean heterozygosity value was lower than the expected mean heterozygosity for all studied goat populations. The average observed and expected heterozygozity values ranged from 0.50 to 0.62 and 0.74 to 0.85, respectively. Moreover, the mean observed and expected heterozygosity values across all loci on studied goat populations were 0.52 and 0.80, respectively (Table 4), which were comparable with studies carried out on other domestic goat breeds (Kumar et al., 2005; Aggarwal et al., 2007; Dixit et al., 2008; Hassen et al., 2012). Due to its geographical location, Syria is considered as one of the countries believed to be home for small ruminant populations including the present domesticated goat breeds. The presence of long term natural selection for adaptation and the existence of interbreeding as result of the free movement of animals in the area are thought to contribute at large to the broad genetic diversity of the Syrian goats. The genetic diversity observed in Syrian goat breeds was comparable with the results reported on South-East Asian goats (Barker et al., 2001), Chegu goats (Behl et al., 2003), Indian goats (Kumar et al., 2009) and Ethiopian goats (Hassen et al., 2012). In addition, the PIC values across all loci ranged from 0.71 (for Jabali goats) to 0.83 (for Shami goats), with an average PIC value of 0.78 (Table 4), which suggested that all loci used in this study showed relatively high polymorphism for analyzing genetic diversity in goats.

Populations genetic differentiation were evaluated base on fixation indices (F_{IS}, F_{IT} and FST) according to Weir and Cockerham (1984), genetic differentiation (G_{ST}), Shannon diversity index (I) and gene flow (Nm) values using 12 microsatellite markers across three goat breeds (Table 3). The mean estimates of F-statistics obtained over loci were $F_{IS} = 0.32$, $F_{IT} = 0.35$ and $F_{ST} = 0.06$. The within breed deficit or excess in heterozygosity value was assessed by the inbreeding coefficients (F_{IS}), ranged between 0.02 (P19) to 0.70 (SRCRSP8), with an average of 0.32 across all loci, while the degree of differentiation within breed was estimated by the F_{IT} value, which was 0.35 and the extent of differentiation among subpopulations was measured by F_{ST} that was 0.06. All loci having positive F_{IS} values indicate an excess of homozygotes across the studied goat breeds. The highest value of F_{ST} (0.11) was observed for MAF209, while the lowest value of 0.03 was obtained at MAF70. The mean F_{ST} value (0.06) indicates that most of the total genetic variation corresponds to differences among individuals within goat populations (Table S2). Both high F_{IS} and F_{ST} values imply considerable degree of inbreeding and genetic differentiation among goat populations, respectively. Values of G_{ST} ranging from 0.02 (MAF70) to 0.11 (MAF209), with a mean of 0.05 (Table 3), reveal that genetic variation among the studied goat breeds was relatively low (5%).

At goat breed level, the inbreeding coefficients (F_{IS} values) showed heterozygote deficiencies occurring in all

Table 5. Pair wise estimate of genetic identity (above diagonal) and genetic distance (below diagonal) values for Syrian goat populations.

Breed	Baladi	Jabali	Shami
Baladi	***	0.961	0.458
Jabali	0.043	***	0.466
Shami	0.780	0.763	***

the three goat breeds. The mean F_{IS} value ranged from 0.29 for Shami to 0.34 for Baladi goat breeds that represented an average increase in the number of homozygous loci of 29% and 34% in Shami and Baladi goat populations (Table 4), respectively. It was suggested that less inbreeding values of less than 0.5 or closer to zero (Wang, 1996) might have happened due to the absence of mating between close relatives and/or within individuals. Similarly, such comparable inbreeding values were also reported for Asian (Barker et al., 2001), Indian goat populations (Kumar et al., 2005; Aggarwal et al., 2007; Dixit et al., 2008; Dixit et al., 2010) and local Albanian goat breeds (Hoda et al., 2011).

The HWE test results revealed that most of the markers used to study Syrian goats were not in HWE, except one locus (that is, ETH10) did show adherence to HWE for Jabali goats (Table S1). Several researchers (Laval et al., 2000; Barker et al., 2001) reported similar results of deviation from HWE for goat diversity studies using microsatellite markers. A population is considered to be within HWE when it is able to maintain its relative allele frequencies. Departure from HWE occurred when there is no excess heterozygotes due to the presence of null alleles (Pemberton et al., 1995), small sample size, migration, selection intensity, uncontrolled goat breeding practices, presence of less heterozygosity (Wahlund effect) and decreased heterozgosity due to inbreeding (Kumar et al., 2006).

Genetic relationships and phylogenetic analyses based on morphological and molecular data

The mean morphological values of both qualitative and quantitative characters were used as classifying variable, and the three goat populations were grouped into two main clusters as indicated in the dendrogram (Figure 2a). Cluster one contains Baladi and Jabali goat populations, while cluster two contains the Shami goats indicating that the Shami goats is relatively different from the two goat populations and was consistent with our earlier assumptions. Further subdivisions between Baladi and Jabali goats showed that these subpopulations were developed from different but closely related goat populations which were also consistent with our earlier hypothesis. Substantial phenotypic variability was found among and within these breeds suggesting that these goat breeds are not under an-organized breeding program.

The Syrian goat populations genetic dissimilarity was computed using molecular data and the smallest genetic distance value was detected between Baladi and Jabali goat populations (0.043), while the largest genetic distance value was recorded between the Baladi and Shami (0.780) and also between Jabali and Shami (0.763) goat populations (Table 5). These genetic distance values were used to construct a phylogenetic tree using UPGMA method and the results revealed the clustering of the three goat populations into two major separate groups. The first cluster contains the Shami goat breed, while the second group includes the Baladi and Jabali goat subpopulations (Figure 2b), which was also supported by classification based on the morphological dataset from the same goat populations.

Population genetic structure analyses

The AMOVA indicated that 4 % of the total variation was present among goat populations, and the majority of genetic variation was found within the goat population (Table S2), hence, similar partitioning of variance has also been reported (Vahidi et al., 2014). The population genetic structure of the Syrian goat populations was analyzed using STRUCUTRE program (Pritchard et al., 2000) with the number of expected clusters (K) ranging from 1 to 15 and bar plots were generated (Figure 3). Such analysis was carried out to detect the potential presence of substructures within breeds. Figure 3 shows the admixture plot of all individuals revealing the admixture patterns of the Syrian goat breeds ranging from K = 2 to 5. At K = 2, the admixture plot of all goat breeds reveals two distinct population patterns, which show strong support for a true subpopulation structure. Particularly, the Shami admixture plot represents a different pattern from those of Baladi and Jabali goat breeds show some degree of genetic dilution with these goat breeds. The true population substructure could be hindered by the sample sizes considered. At K = 3, the Shami goat exhibited similar admixture pattern as observed at K=2 showed more admixture pattern with Jabali goats (Table 5). Whereas, Baladi and Jabali goat breeds revealed different admixture pattern indicate substantial gene flow between the Syrian Baladi and Jabali goat breeds. At K = 4, Shami goats were clearly separated from the other goat breeds as before, while the Baladi and Jabali goat breeds admixture pattern was inseparable and additional unclear subpopulation were observed. The number of blood samples taken for both Jabali and Shami goats might hinder to point out the true population structures of these goat breeds. These were represented as admixture individuals without showing any notable subdivision among the different groups when K = 4. At K = 5 the admixture patterns revealed relatively similar patterns to that of K = 4, however, further

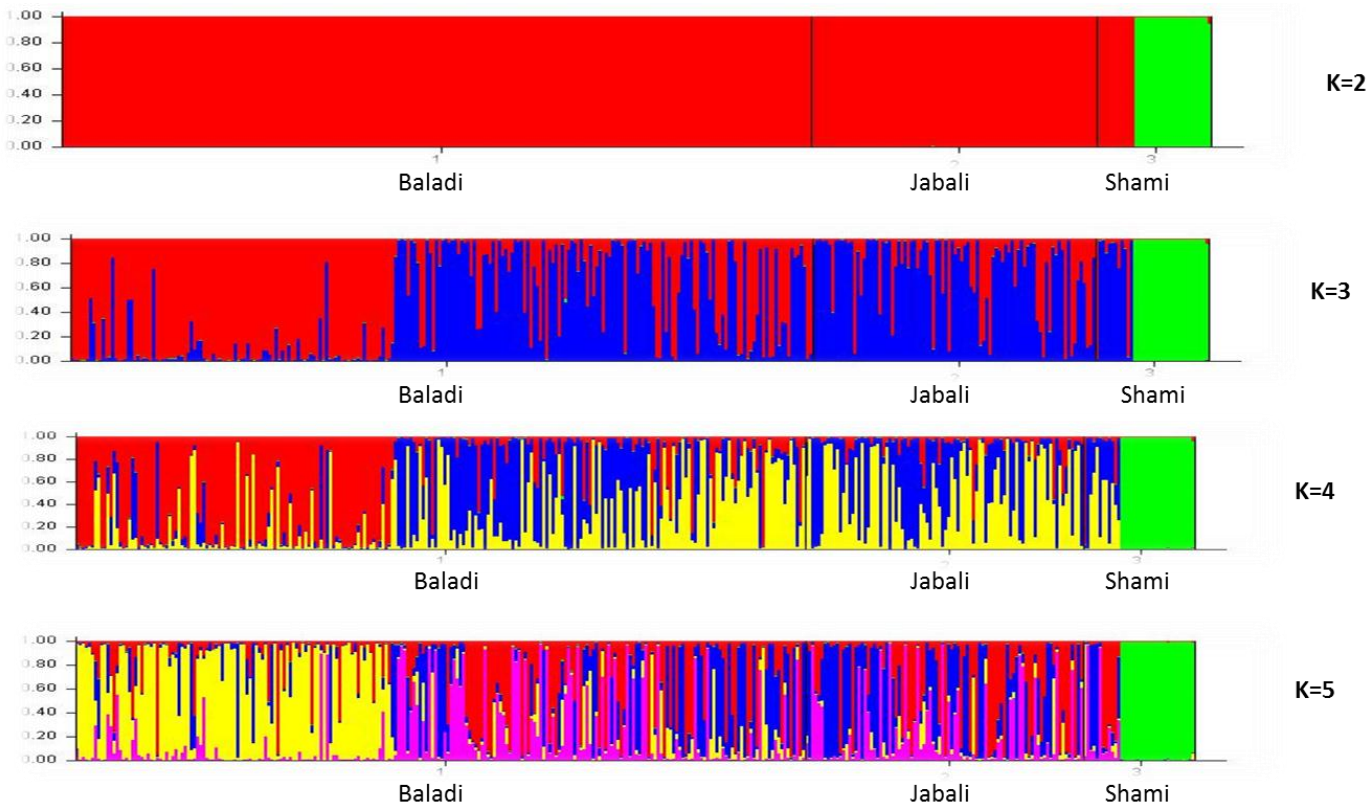

Figure 3. Depicts the clustering of all samples at k= 2, k=3, k= 4 and k=5, and each individual is represented by a vertical bar and partitioned into different colored segments with the length of each segment representing the proportion of the individual's genome, and populations are separated by backlines and each population is labeled.

inseparable subpopulation were also created. Hence, at K = 3, all three goat breeds appeared to have distinguishable admixture patterns, especially the Shami goat population was clearly separated from the rest of the Syrian goat populations at K= 2 to K= 5 with some levels of genetic dilution present with other Syrian goats. The Shami goat population had the least cases of genome admixtures, which is consistent with the phylogenetic tree constructed using the morphological data generated from the same goat populations (Figure 2a). This might be due to the fact that the Shami goats are raised mainly for milk and used as a milk-improver breed for crossing with other goat breeds in Syria and beyond (for example, in Cyprus). This might be due to the fact that the Shami goat owners might not erode and dilute the genetic identity of this goat breed by crossing with other goats breeds kept in a flock, which actually needs further investigation. The admixture plot at K = 3 could best describe the Syrian goat structure in which the three indigenous goat populations were represented by three predominating colors and distinct admixture patterns. These results suggest that Syrian indigenous goat breeds may descend from the same ancestor(s).

Further, to elucidate the relationships between the Syrian goat populations and provide finer quantification of the different ancestral contributions, STRUCTURE HARVESTER analysis was conducted by evaluating replicate likelihoods and resultant ΔK statistics for different values of K (K= 1 to 15). The Ln (K) values increased from K= 1 to K=2, and somewhat less quickly from K= 2 to K= 3, before reaching a plateau at successive values (Figure S2a). The largest value of ΔK occurred for K= 2 (ΔK = 488.67) and, secondarily, for K= 3 (ΔK =85.26) (Figure S2b).

Conclusion

This paper describes the first attempt to compare the morphological and molecular genetic variability of the Syrian indigenous goat populations. The Syrian goats were well differentiated into two major groups by their morphological appearances, genetic distance and population structure values, suggesting the genetic diversity of these groups. Phenotypically, the three goat breeds can be distinguished by their coat color, nature of their horn orientation and nose profile. It is remarkable that the Baladi (local) and Jabali (mountain) goats have higher genetic similarity both at morphological and molecular levels even though they have sub-grouped into

separate subpopulations. However, only the Shami goat population was structurally different and clustered into a separate group as a result of its high genetic distance compared to the other two goat breeds of Syria. The higher genetic variability in Shami goats may mean the presence of private or unique alleles suggesting the presence of certain functional genes which may result to better adaptability and performances. The results also reveal the presence of higher genetic variability within the goat population (96%) and such high variation within breed provides an excellent base for designing genetic improvement program.

Conflict of Interests

The authors have not declared any conflict of interests.

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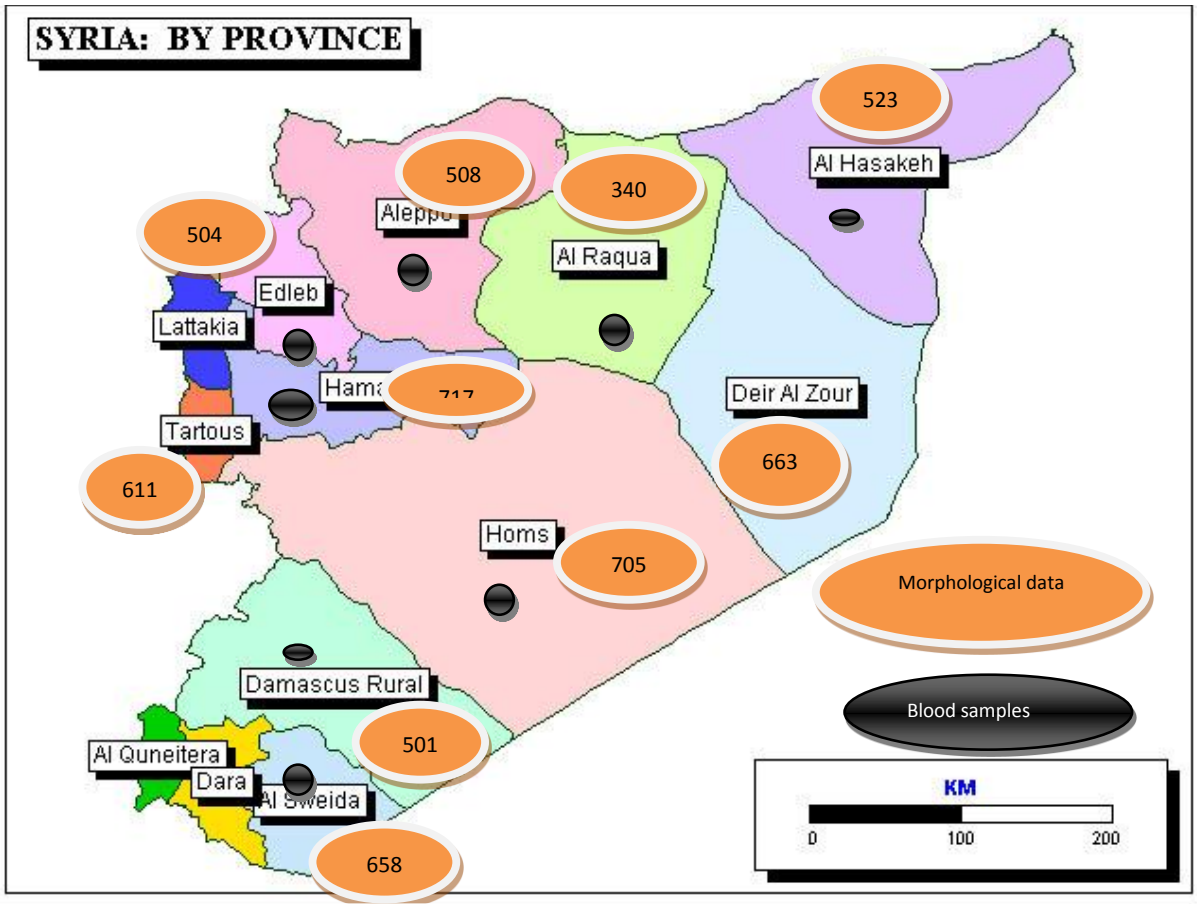

Figure S1. Map of Syria showing sampled provinces.

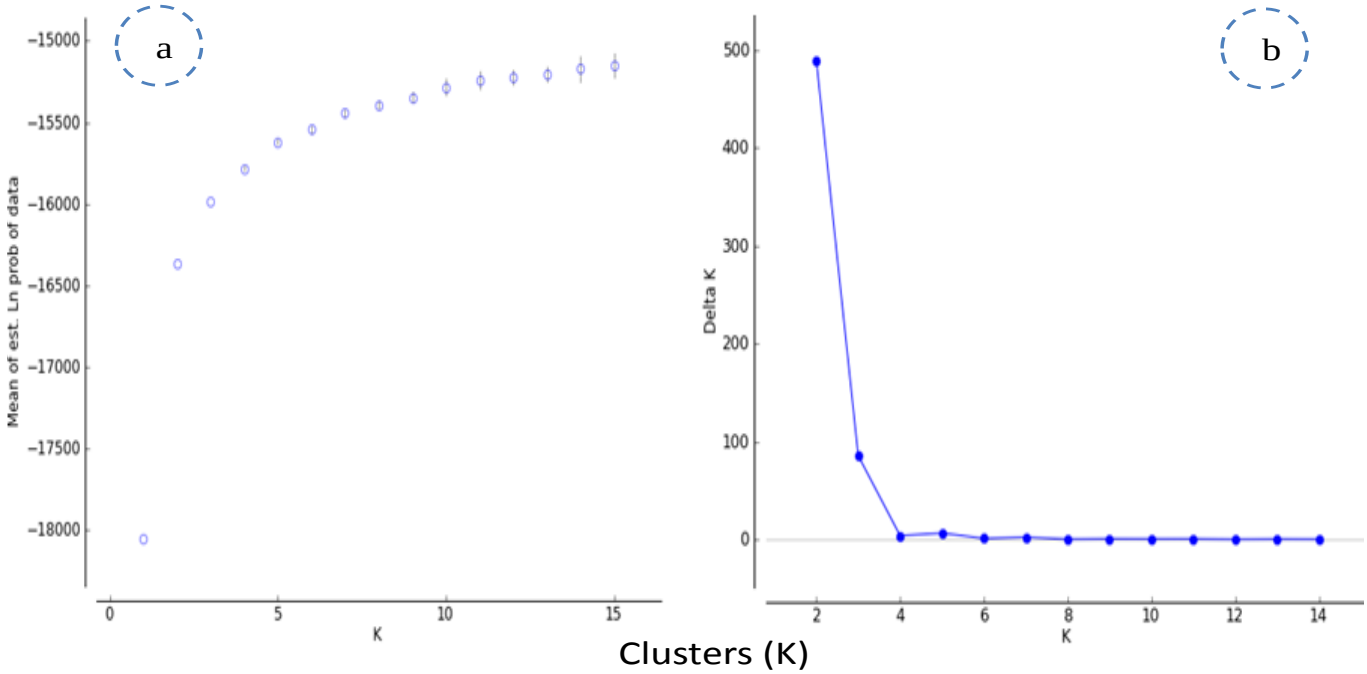

Figure S2 (a, b). Mean Log-likelihood (left-a) and ΔK (right-b) based on 10 replicated STRUCTURE runs for Syrian goat breeds.

Table S1. Analyzed mean number of alleles (MNA), effective number of alleles (Ne), Polymorphic Information Content (PIC), expected (He) and observed heterozygosity (Ho) values, Shannon diversity index (I), inbreeding coefficient (F_{IS}) and Hardy Weinberg equilibrium (HWE) p-values.

Locus	Baladi							Jabali							Shami						
	MNA	Ne	PIC	He	Ho	I	HWE	MNA	Ne	PIC	He	Ho	I	HWE	MNA	Ne	PIC	He	Ho	I	HWE
ILSTS044	10	3.16	0.65	0.69	0.30	1.41	0.000***	12	3.52	0.62	0.67	0.26	1.54	0.000***	12	6.46	0.82	0.84	0.56	2.14	0.000***
ILSTS087	18	5.46	0.8	0.82	0.61	2.02	0.000***	15	5.72	0.79	0.81	0.60	2.11	0.000***	13	7.38	0.84	0.86	0.39	2.26	0.000***
SRCRSP8	14	3.41	0.72	0.74	0.30	1.66	0.000***	10	2.88	0.53	0.56	0.20	1.53	0.000***	12	6.80	0.81	0.83	0.09	2.26	0.000***
ILST019	17	4.85	0.79	0.80	0.26	1.97	0.000***	10	4.70	0.74	0.76	0.19	1.87	0.000***	14	10.56	0.89	0.89	0.69	2.52	0.000***
OARFCB48	14	6.20	0.8	0.82	0.59	2.11	0.000***	13	5.00	0.81	0.83	0.63	1.90	0.003**	15	7.32	0.87	0.88	0.86	2.18	0.000***
ILSTS005	9	2.15	0.5	0.54	0.14	1.09	0.000***	7	2.52	0.5	0.58	0.19	1.09	0.000***	13	7.49	0.85	0.86	0.50	2.22	0.000***
BM6444	31	15.99	0.94	0.94	0.61	2.99	0.000***	26	18.08	0.92	0.93	0.69	3.05	0.000***	14	5.17	0.77	0.78	0.47	2.27	0.000***
ETH10	7	2.79	0.58	0.65	0.58	1.17	0.030*	7	2.93	0.57	0.64	0.72	1.22	0.948ns	8	4.77	0.76	0.79	0.46	1.80	0.000***
MAF209	8	2.85	0.63	0.67	0.58	1.34	0.000***	4	2.27	0.42	0.47	0.39	0.97	0.000***	7	4.32	0.73	0.77	0.76	1.62	0.000***
MAF70	21	7.92	0.86	0.87	0.73	2.36	0.000***	21	8.64	0.88	0.89	0.75	2.46	0.000***	28	18.65	0.94	0.94	0.86	3.16	0.000***
P19(DYA)	18	10.57	0.9	0.91	0.81	2.48	0.000***	15	6.93	0.85	0.86	0.80	2.20	0.001***	16	8.00	0.87	0.88	0.89	2.31	0.000***
SRCRSP9	17	10.51	0.89	0.90	0.67	2.47	0.000***	10	8.00	0.86	0.88	0.61	2.14	0.000***	17	7.54	0.87	0.88	0.89	2.34	0.000***
Mean	15.33	6.32	0.75	0.78	0.52	1.92		12.5	5.93	0.71	0.74	0.50	1.84		14.08	7.87	0.83	0.85	0.62	2.26	

ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001 did not adhere to HWE.

Table S2. Analysis of molecular variance (AMOVA) for Syrian goat populations using genotyped data from twelve microsatellite markers.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Explained variation (%)
Among populations	2	90.452	0.212	4
Among individuals within populations	359	2347.857	1.763	35
Within individuals	362	1091.000	3.014	61
Total	723	3529.309	4.989	

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Lipase-producing fungi for potential wastewater treatment and bioenergy production

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The use of fungal biomass as a lipase biocatalyst represents an attractive approach for the treatments of oil wastewater as well as for the production of biodiesel from oil and residual grease, due to its greater stability, possibility of reuse, and lower cost. In this work, 20 filamentous fungi were isolated from the grease trap scum of a restaurant at the Federal University of Espírito Santo, Brazil. The fungi included those belonging to the genera *Aspergillus*, *Beauveria*, *Botrytis*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Geotrichum*, *Penicillium*, *Rhizomucor*, and *Verticillium*. Fungal lipase activity and biomass production were quantified. Lipase activity ranged from 0.13 U mg^{-1} protein of *Rhizomucor* sp. ECGF18 to 18.06 U mg^{-1} protein of *Penicillium* sp. ECGF02, and the biomass production ranged from 7.61 mg mL^{-1} for *Cladosporium* sp. ECGF19 to 12.68 mg mL^{-1} for *Rhizomucor* sp. ECGF18. In the sequence, *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18, were previously select and, further evaluated in solid-state fermentation. Results confirmed the high extracellular lipase-activity of *Penicillium* sp. ECG02 and the high intracellular lipase activity of *Rhizomucor* sp. ECG18. *Rhizomucor* sp. ECG18 showed potential for use in future research, in the form of whole-cell lipases, wastewater treatment, and as a biocatalyst in the production of biodiesel from oil residues.

Key words: Lipase-producing fungi, wastewater treatment, bioenergy.

INTRODUCTION

Lipases (such as triacylglycerol acyl hydrolase, E.C. 3.1.1.3) work at the aqueous-organic interface where thay catalyse hhydrolytic reactions of triglycerides as well as esterification, transesterification, or interesterification in low-water environments (Nagarajan, 2012). New applied lipase technology, including using it in genetic engineering methods and diverse applications in the food, chemical, and pharmaceutical industry means the current global market for lipase is expanding (Salihu et al., 2012). In addition, there is great interest in lipase use for the treatment of high lipid-content effluents for the production of bioenergy (Cammarota and Freire, 2006;

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Alberton et al., 2010; Singh and Mukhopadhyay, 2012; Hama and Kondo, 2013; Guldhe et al., 2015).

An increasing number of publications and patent applications reflect a worldwide interest in the use of lipases for the synthesis of biodiesel. An extensive review was published by Christopher et al. (2014) covering many aspects of these studies, including the nature and properties of the enzyme catalyst. However, the use of commercial lipases for the transesterification of biodiesel, as well as for the hydrolysis of oil effluents, is not currently competitive with other processes. Although advantageous under the environmental point of view, at the moment, the main drawback is the production cost of these enzymes (Guldhe et al., 2015).

Commercial lipases are produced, primarily, by microorganisms such bacteria, yeasts, and filamentous fungi, due to their short generation time, ease of genetic manipulation, easily increased scalability, purification, specificity, and stability (Nagarajan, 2012).

Filamentous fungi are preferred sources of lipase production because they are easily extracted from fermentation processes, are considered safe and easy to hand, and can potentially be used as whole cells (Alberton et al., 2010; Singh and Mukhopadhyay, 2012; Andrade et al., 2014). Filamentous fungi have proven to be the most convenient biosystem for industrial applications because of their strong cell walls. *Rhizopus oryzae*, *Rhizopus chinensis*, *Aspergillus niger*, and *Mucor circinelloides* have all been studied as whole cell biocatalysts by several research groups (Fukuda et al., 2009; Andrade et al., 2014).

Filamentous fungi show favorable characteristics for the growth and lipase production in solid-state fermentation systems (Singh and Mukhopadhyay, 2012). Solid substrates are effective for the growth of filamentous fungi, due to the environmental similarity with their natural habitat, which result in higher enzyme production, lower demand for water and, energy, and easy aeration. This is a low-cost alternative, can potentially be used to clean modern oil residues or can be used by the agroindustry for the production of enzymes (Colen et al., 2006; Griebeler et al., 2011). In addition, they may be used in the form of whole-cells lipase, directly used in solid fermentation conditions (Fukuda et al., 2009; Li and Zong, 2010; Athalye et al., 2013; Talukder et al., 2013; Ferrarezi et al., 2014; Guldhe et al., 2015). The advantage of such an approach that it does not require an enzyme purification step, and the whole-cells and solid substrates can act as immobilizing agents for enzyme support (Kumar and Kanwar, 2012; Salihu et al., 2012; Farias et al., 2015; Carvalho et al. 2015a; Carvalho et al., 2015b).

Fungi are capable of producing several enzymes for their survival within a wide range of substrates. In view of the interesting applications of lipases, it could be of tremendous value to screen and identify fungi with the highest potential for the biodegradation of oils and fats.

There are two basic types of fungal lipases, the extracellular lipase, which is secreted into the liquid medium, and the intracellular lipase, which is inside the cell or linked to its membrane or cell wall, intracellular lipase, also called whole-cell lipase, is less expensive to use in full-scale. Therefore, the isolation and selection of new fungal strains with high levels of intracellular lipolytic activity for use as whole-cell lipase in wastewater treatment and biodiesel production is of great interest (Fukuda et al., 2009; Carvalho et al., 2015a).

Although, different screening strategies have been proposed for the determination of lipase extracellular activity, assays using agar plates are highly recommended, because it is an easier method with lower cost. However, agar plates are often insufficient or clear for quantifying intracellular lipase activity of various strains, and the process results in losses to the isolation and selection processes; therefore, these must be complemented with other methods (Gopinath et al., 2014).

The aim of this work was to isolate filamentous fungi from grease trap scum and to select favorable isolates based on their high lipase production and fungal biomass, production while in submerged fermentation. This work also assessed, the potential characteristics of the selected isolates for use in future research, in the form of whole-cell lipases, wastewater treatment, and biocatalysis of biodiesel from oil residues found in environmental sanitation.

MATERIALS AND METHODS

The aim of this study was to isolate and select efficient lipase-producing fungi for potential whole-cell lipase use in the hydrolysis of oily wastewater and synthesis of fatty acid esters from oil residues. Figure 1 shows the experimental steps of the work.

We proposed the concomitant use of submerged fermentation methods and solid-state fermentation to produce strains of filamentous fungi with high potential of lipase intracellular. Table 1 shows shows the main methods used, in accordance with the purposes of the research.

Isolation and identification of lipase-producing fungi

Collection and characterization of grease trap scum

The source of filamentous fungi lipase producers was scum that had for a period of 3 months, accumulated on the surface of, a grease trap in a restaurant at the Federal University of Espírito Santo, Brazil. Five 200 mL samples were collected from different surface points of the trap and stored in previously sterilized 500 mL Erlenmeyer flasks. The scum was quantified using the following parameters: volatile solids (VS), chemical oxygen demand (COD), content of oils and greases (O&G), and pH, in accordance with the "Standard Methods for the Examination of Water and Wastewater" (APHA, 2005). The values of the parameters were VS (mg L^{-1}) = 922110.45, COD (mg L^{-1}) = 1429001.83, O&G (mg L^{-1}) = 921936.67, and pH = 6.08, while those relating to ECG-RU were VS (mg L^{-1}) = 756472.75, COD (mg L^{-1}) = 922991.29, O&G (mg L^{-1}) = 595478.25, and pH = 5.60.

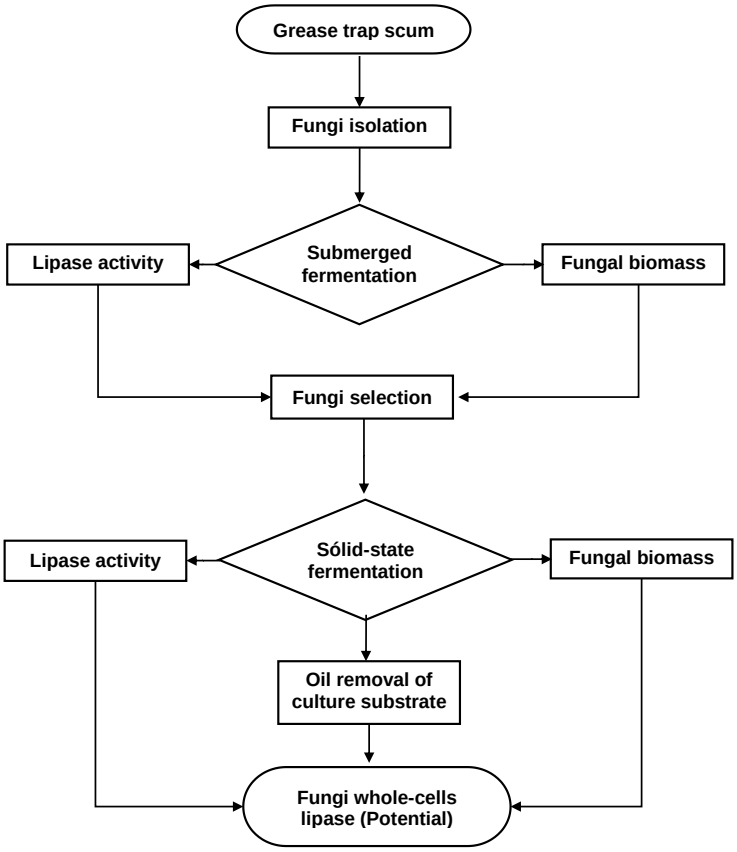

Figure 1. A flowchart of the main steps of this work, for the isolation and selection of lipase-producing fungi for potential whole-cell lipase use.

Table 1. Main parameters, units, methods and correspondent references in this work, for the isolation and selection of lipase-producing fungi for potential whole-cell lipase use.

Parameter	Unit	Method	References
pH	-	Potentiometric	APHA (2005)
COD	mg L^{-1}	Closed reflux colorimetric	APHA (2005)
O&G/SO removal	mg L^{-1}	Soxhlet extraction	APHA (2005)
Volatile solids	mg L^{-1}	Gravimetric	APHA (2005)
Fungal genus	-	Microculture method	Barnett and Hunter (1998)
Protein	mg	Bradford method	Bradford (1976)
Lipase activity	U mg^{-1}	Colorimetric	Winkler and Stuckmann (1979); Rowe and Howard (2002)
Ergosterol	µg	HPLC	Montgomery et al. (2000)
Fungal biomass	mg L^{-1}	Gravimetric	Colen et al. (2006)
	mg g^{-1}	HPLC	Montgomery et al. (2000)

pH, hydrogen ionic potential; COD, chemical oxygen demand; O&G, oils and greases; HPLC, high performance liquid chromatography; APHA, American Public Health Association.

Isolation of lipase-producing fungi

For the proliferation of microorganisms, 40 mL of each each sample was poured into a 250 mL Erlenmeyer, along with 50 mL of minimum mineral culture medium (MM) and 10 mL of soybean oil (Liza), emulsified with 0.1% Tween 80 (MMSO). The MM consisted of (g L^{-1}): $(NH_4)_2 SO_4$, 5.0 g; KH_2PO_4, 0.9 g; NaCl, 1.0 g; $MgSO_4$. $7H_2O$, 0.3; Na_2HPO_4, 6.2 g; soybean oil 10% and 1 mL micronutrients solution ($FeCl_3$. $6H_2O$, 2000 mg; $ZnCl_2$, 50 mg; $CuCl_2$. $2H_2O$, 30 mg; $MnCl_2$. $2H_2O$, 500 mg; $(NH_4)_6.Mo_7O_{24}$.$4H_2O$,

50 mg; $AlCl_3$, 50 mg; $CoCl_3$. $6H_2O$, 2000 mg).

The flasks with enriched samples stirred at 120 rpm in an orbital shaker (Benchtop Shaker Incubator NT 712, Novatécnica) at 30°C for 96 h. Then, 1 mL aliquots of enriched medium were mixed with MM plus 10% commercial soybean oil (Liza) and 0.1% Tween 80 (MMSO), and 1.7% agar, and were poured in previously autoclaved 90 mm diameter Petri dishes supplemented with 50 ppm of streptomycin and 50 ppm chloramphenicol and incubated for 120 h (DBO-Biotec BT424) at 28°C. After the incubation period, colonies of the filamentous fungi were successively transfered to new Petri dishes, and all strains obtained were successively transferred to new Petri dishes, and all strains obtained were transferred to culture tubes containing BDA (potato-dextrose-agar) and stored at 5°C.

Identification of lipase-producing fungi

The previously isolated fungi were identified by microcultivation technique to distinguish which samples were promising for lipase production. The fungi samples were individually inoculated on a slice of agar laid on a sterile glass slide and covered by a sterile coverslip. The slide was then placed in a Petri dish, and the setup was incubated for 96 h at 25°C. Then, the coverslip with the adhered hyphae was removed and stained with a cotton blue dye. The same procedure was adopted to examine spores and hyphae bound to the slide. The identification of different fungi genera was based on the macroscopic morphology of the colonies and the fructification structures of the strains, following the key of investigation of genera proposed by Barnett and Hunter (1998).

Selection of lipase-producing fungi in submerged fermentation

The filamentous fungi, obtained in the previous step, were evaluated for lipase activity and fungal biomass production on submerged fermentation. The submerged fermentation was performed with a completely randomized design with 20 fungi strains and three replications, and the means were compared by the Tukey test at 5% probability. This step of the work permitted the selection of two fungal isolates for subsequent work step, one of them by highest value displayed of the lipase activity and the other by the highest value of fungal biomass production.

Lipase activity determination

The experiments were conducted using 125 mL Erlenmeyer flasks, containing 50 mL MM (sterile) with an additional 10% soybean oil and 0.1% Tween 80 (v/v) as sole carbon sources. In each flask, the medium was inoculated with 1 mL of an aqueous suspension of fungus at a concentration of 10^7 spores mL^{-1} followed by an incubation of 120 h on an orbital shaker at 30°C and 150 rpm. After incubation, the content of each Erlenmeyer flask was vacuum-filtered and three 1 mL aliquots of liquid fraction were transferred to sterile Eppendorf tubes and centrifuged at 15000 rpm for 20 min at 4°C (Hettich Mikro 22R, Andreas Hettich GmbH Co. KG &, D-78532 Tuttlingen). The resulting supernatant was evaluated for lipase activity using p-nitrophenyl palmitate (pNPP, Sigma) as a substrate, as stated by Winkler and Stuckmann (1979) and Rowe and Howard (2002).

Samples of 0.1 mL of the supernatant were mixed with 0.9 mL substrate solution of the following composition: 3 mg of pNPP dissolved in 1 mL propanol diluted in 9 mL of tris-HCl pH 8.0 and containing 40 mg of Triton X-100 and 10 mg of arabic gum. After 30 min of incubation at 37°C, absorbance at 410 nm was measured by spectrophotometry (Ultrospec 1000, Pharmacia Biotech), against a control without enzyme. A lipase activity unit (U) was defined as the amount of enzyme to release one mol of p-nitrophenol min^{-1}. The protein measurements were conducted by the method of Bradford (Bradford, 1976).

Fungal biomass determination

For quantification by gravimetry, the fungal biomass was separated from the supernatant by filtration with Whatman filter paper nº 1, followed by acetone cleaning and successive washes with milli-Q water. The biomass was then transferred to filter papers previously weighed and dried at 80°C, and then the biomass was dried until the weight remained constant (meaning that all the water evaporated). The dry biomass was calculated by determining the difference between the measurements at the beginning and end of the previous step, with and without the presence of biomasses as studied by Colen et al. (2006).

Lipase production in solid-state fermentation

Fungal biomass production and removal of the soybean oil of cultive substrate by *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18 (selected in the previous step, respectively, by lipase activity and fungal biomass values), in intervals of 24 h, and ≥ 120 h, in solid-state fermentation conditions were assessed for lipase activity. The cultive substrate used was prepared from at mixture of 50% sand and 50% vermiculite (v/v) previously washed and completely dried at 100°C. The mixture (40 g) was added to 250 mL Erlenmeyer flasks capped with hydrophobic cotton. Mineral medium minimum (MM) was added to the sand-vermiculite mixture in a sufficient volume to achieve 50% of field capacity. This mixture was autoclaved at 121°C for 20 min, the pH was adjusted to 4.5 with H_2SO_4 1.5 mol L^{-1}, and 10% soybean oil (Liza) was added as the exclusive carbon source and inducer for lipase production. Each Erlenmeyer was inoculated with 1 mL of suspension of 10^7 spores mL^{-1} and incubated at 30°C for 120 h. Three replications were used for each parameter evaluated and for each assessment time as follows: 0 (initial time), 24, 48, 72, 96, and 120 h of incubation.

Lipase activity determination

The method for the lipase extraction of fermented solids was as the same as that used by Alberton et al. (2010), except agitation time, which was reduced from 1 h to 30 min. Each fermented solid (10 g) was transferred to a 250 mL Erlenmeyer, 100 mL of a 2% NaCl solution was added, and then the mixture was agitated on a rotary shaker for 30 min at 200 rpm and 30°C. The resulting suspension was filtered through cheesecloth, and the excess liquid was manually squeezed out. The extract was centrifuged for 10 min at 12500 rpm. The quantification of lipase activity proceeded as described in the previous method.

Oil removal of culture substrate

Substrate soybean oil content was quantified using the method of continuous extraction apparatus Soxhlet type, according to the "Standard Methods for the Examination of Water and Wastewater" (APHA, 2005), which is based on the gravimetric quantification of material extracted with hexane. One (1) g samples analysed were collected at 0 (date of installation of the experiment), 24, 48, 72, 96 and 120 h, and on occasions, were modified with HCl to reach a pH of 2.0. The samples were stored in a freezer at - 25°C, until used in analyses, when the sample concentrations were expressed in mg g^{-1} substrate dry weight (dried at 80°C).

Fungal biomass determination

Fungal biomass was indirectly quantified, through the determination from ergosterol content, via high performance liquid chromatography (HPLC), as observed in Montgomery et al. (2000), with some modifications. For the extraction of sample ergosterol, 1 g of substrate was put in a test tube, 5 mL of methanol was added and the sample was vortexed for 1 min. After resting for 10 min, the supernatant was transferred to a 1.5 mL Eppendorf and centrifuged for 10 min at 10000 rpm at 22°C. Then, the supernatants were filtered using 0.22 µ filter paper and were stored at - 25°C for later use.

The binary solvent system HPLC equipment (Shimadzu, model LC-20 AD/T, Japan) had a 100 × 2.1 mm C18 column, attached to a filter with a porosity of 0.5 × 0.004 µm. The mobile phase T was isocractic with methanol (HPLC grade) and water (Milli-Q) 95:5 (v/v), filtered and degassed for 15 min, with a flow rate of 0.5 mL min^{-1}. The race lasted approximately 7 min and was identified a peak integration of ergosterol through the comparison of retention times of the standard and the sample, was verified by the purity of the absorbance spectra obtained at the beginning, peak, and end of peak, at 282 nm. For the construction of the linear standard curve (outside standardization), the point at which it crossed the origin and covered the range of concentration of the samples, ergosterol was used with the minimum purity of 95% and 7 points for reading, whose concentrations ranged from 5.0 to 500 ug mL^{-1}.

To measure the content of ergosterol in a pure biomass sample of *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18, the fungi were grown in 250 mL Erlenmeyer flasks with MMOS, for 30 days in a 150 rpm shaker at 30°C. At 10, 20 and 30 days, their biomass was collected through filtration, followed by acetone cleaning and successive washes with milli-Q water. These samples were then transferred to filter papers previously weighed and dried at 80°C, and then the biomass was dried until the wight remained constant (meaning that all the water evaporated). The dry biomass was calculated by determining the difference between the measurements at the beginning and end of the previous steps, with and without the presence of biomasses as studied by Colen et al. (2006).

Samples of 1 g of dry biomass were frozen using liquid nitrogen, ground in a mortar, mixed with 5 mL methanol, and then the material was collected and transferred to culture tubes. We then followed he previously decribed preparation of material for chromatographic analysis. Based on the reasons, ergosterol-dry biomass obtained for both isolated in the collection dates, arrived to the fungal biomass values. After conversion, the average (n = 3), values of ergosterol content in mg g^{-1} fungal biomass in *Penicillium* sp. ECGF02 were 1.28 (10 days), 1.57 (20 days), and 1.49 (30 days), and in *Rhizomucor* sp. ECGF18 they were 1.55 (10 days), 1.52 (20 days), and 1.54 (30 days). The finals results of fungal biomass were expressed as mg g^{-1} cultive substrate that had been subjected to a temperature of 80°C until they reached a constant weight.

Statistical analysis

The analysis of variance followed by the Tukey test was applied for the statistical analyses of the obtained data. The data were analyzed with the aid of Statistica 6.0 (Statsoft Inc., Tulsa, OK, USA). All analyses were performed considering a level of 95% confidence ($p < 0.05$).

RESULTS AND DISCUSSION

Isolation and identification of lipase-producing fungi

In this work, it was possible to obtain 20 filamentous fungi isolates from grease trap scum, belonging to the genera *Aspergillus*, *Beauveria*, *Botrytis*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Geotrichum*, *Penicillium*, *Rhizomucor* and *Verticillium*, after successively growing them on MMSO plus bacterial antibiotics. Their taxonomic identification was based on macroscopic structures and as the colonies grew in BDA on Petri dishes, but was also based on microscopic structures and comparisons to specific literature descriptions (Barnett and Hunter, 1998). The 20 isolates were stored in BDA at 4°C, and allowed to multiply until it was time to evaluate their lipase activity and fungal biomass production, which occurred after incubation for 120 h in MMSO at 30°C, while shaking at 150 rpm, on submerged fermentation (Table 2).

Selection of lipase-producing fungi in submerged fermentation

The enzymatic activity and biomass production of the 20 previously filamentous fungi isolated and placed in submerdeg fermentation can be seen in Table 2. The results indicate that the fungi significantly influence ($p \leq 0.05$) the enzymatic degradation reaction of the p-nitrophenyl palmitate.

The lipase activity evaluated on supernatant (extracellular activity) ranged from 0.13 ± 0.03 U mg^{-1} protein of *Rhizomucor* sp. ECGF18 to 18.06 ± 0.36 U mg^{-1} protein of *Penicillium* sp. ECGF02. The dry weight biomass ranged from 7.82 ± 0.13 mg mL^{-1} of culture liquid medium for *Cladosporium* sp. ECGF19 to 12.68 ± 0.15 mg mL^{-1} culture liquid medium for *Rhizomucor* sp. ECGF18. Overall, statistically significant differences for lipase activity and fungal biomass were observed between the values obtained for various isolates belonging to different taxonomic genera and between isolates of the same genus (Table 2).

Using a similar methodology to quantify but a longer incubation time, Baron et al. (2005) observed a lipase activity of 11.82 ± 1.35 U mg^{-1} protein for *P. coryophilum* IOC 4211, after 144 h of incubation at 29°C and 120 rpm. Using a distinct method of quantification, Carvalho et al. (2005) obtained a lipase activity of 13.0 U mL^{-1} and a biomass of 14.2 mg mL^{-1} for *P.restrictum*, and a lipase activity of 10.5 U mL^{-1} and biomass of 6.56 mg mL^{-1} for *P. solitum*.

The following species of the *Penicillium* genus were previously reported as good producers of extracellular lipase activity: *P. citrinum, P. cyclopium, P. simplicissimum, P. caseicolum, P. restrictum, P. expansum, P. corylophilum, P. chrysogenum, P. roqueforti, P. camembertii, P. crustosum* and *P. abeanum*, among others (Baron et al., 2005; Carvalho et al., 2005; Li and Zong, 2010; Rigo et al., 2012). However, it is necessary to consider that the experimental results of lipase activity and growth of biomass will likely be different due to natural, diverse, baseline activities

Table 2. Taxonomic classification, lipase activity (LA) and fungal biomass production (FB) on mineral minimal culture media and 10% soybean oil (MMSO) in submerged fermentation (SF).

Isolate	Taxonomic classification	LA* (U mg^{-1} protein)	FB* (mg mL^{-1} MMSO)
ECGF02	*Penicillium*	18.06 ± 0.36^{a}	10.19 ± 0.13^{cdef}
ECGF09	*Geotrichum*	14.25 ± 0.31^{b}	9.23 ± 0.18^{h}
ECGF01	*Beauveria*	9.13 ± 0.23^{c}	9.62 ± 0.14^{g}
ECGF19	*Cladosporium*	6.10 ± 0.22^{d}	7.61 ± 0.15^{j}
ECGF15	*Cladosporium*	4.85 ± 0.16^{e}	7.82 ± 0.13^{j}
ECGF03	*Colletotrichum*	3.79 ± 0.07^{f}	9.71 ± 0.16^{g}
ECGF20	*Penicillium*	2.74 ± 0.17^{g}	10.12 ± 0.16^{ef}
ECGF13	*Penicillium*	2.56 ± 0.05^{g}	8.77 ± 0.12^{i}
ECGF12	*Colletotrichum*	2.49 ± 0.25^{g}	9.05 ± 0.18^{hi}
ECGF16	*Colletotrichum*	2.36 ± 0.39^{g}	9.02 ± 0.13^{hi}
ECGF11	*Aspergillus*	1.53 ± 0.25^{h}	10.45 ± 0.11^{cde}
ECGF14	*Botrytis*	1.44 ± 0.11^{h}	11.89 ± 0.10^{b}
ECGF17	*Geotrichum*	0.51 ± 0.10^{i}	9.07 ± 0.15^{hi}
ECGF05	*Beauveria*	0.47 ± 0.01^{i}	10.74 ± 0.19^{c}
ECGF04	*Fusarium*	0.39 ± 0.04^{i}	10.27 ± 0.12^{cde}
ECGF10	*Fusarium*	0.28 ± 0.08^{i}	10.25 ± 0.80^{cde}
ECGF06	*Rhizomucor*	0.25 ± 0.01^{i}	9.91 ± 0.12^{fg}
ECGF07	*Verticillium*	0.18 ± 0.01^{i}	10.50 ± 0.15^{cd}
ECGF08	*Aspergillus*	0.18 ± 0.05^{i}	9.87 ± 0.21f^{g}
ECGF18	*Rhizomucor*	0.13 ± 0.03^{i}	12.68 ± 0.15^{a}

* = Means (n = 3) followed by same letters, do not differ statistically by Tukey test (p = 0.05).

between species and within the same species, but there is more likely an influence of factors such as the composition of the culture media, temperature, pH, agitation and forms of quantification, as reviewed by Turky (2013) and reported by Talukder et al. (2013) and Bueno et al. (2014).

The genus *Rhizomucor*, mainly represented by the species *R. mihei*, stands out as a top producer of lipase, and as such, it is currently one of the most marketed extracellular lipase producers; it was the first for whose lipase structure was reported, having its activation interface well elucidated and resulting in its basic use for enzyme modeling studies. An extensive review was recently published stating the main uses of LRM (*Rhizomucor mihei* lipase) and featuring some of the most relevant aspects in its use for the processing of oils and fats, including its use in the hydrolysis of glycerides, transesterification, esterification, acidolyses, and intersterification (Rodriguez and Fernando-Lafuente, 2010).

In this results suggest that the more effective lipase activity of *Penicillium* sp. ECGF02 (18.06 ± 0.36 U mg^{-1} protein) was mainly intercellular. In contrast, the value of the extracellular lipase activity of *Rhizomucor* sp. ECGF18 (0.13 ± 0.03 U mg^{-1} protein) did not express its lipolytic potential, because this isolate showed greater biomass production (12.68 ± 0.15 mg mL^{-1}) in the liquid medium (Table 2). For *Rhizomucor* sp. ECGF18, the results suggest that the greater lipolytic potential was intracellular (membrane-bound or cell-wall lipase). Thus, *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18 were selected, in solid-state fermentation, for evaluation of lipase activity, fungal biomass production, and removal of soybean oil in culture substrate, to confirm their lipolytic profile and their potential use as whole-cell lipases in solid culture medium.

Lipase production in solid-state fermentation

Figure 2 shows the mean results from solid-state fermentation (SSF) for *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18, for lipase activity, fungal biomass production, and soybean oil removal of culture substrate (120 h growth).

Culture substrate soybean oil removal was quantified through the widely used method in the area of environmental sanitation for the determination of the content of oil and grease in wastewater, solid waste, and sludge (APHA, 2005). This methodological tool was used to indirectly estimate the hydrolytic capacity of two fungal isolates evaluated in solid-state fermentation. The activity and the fungal biomass (120 h) for *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18 were better than in submerged fermentation. These results are similar to those reported by Alberton et al. (2010) and Rigo et al.

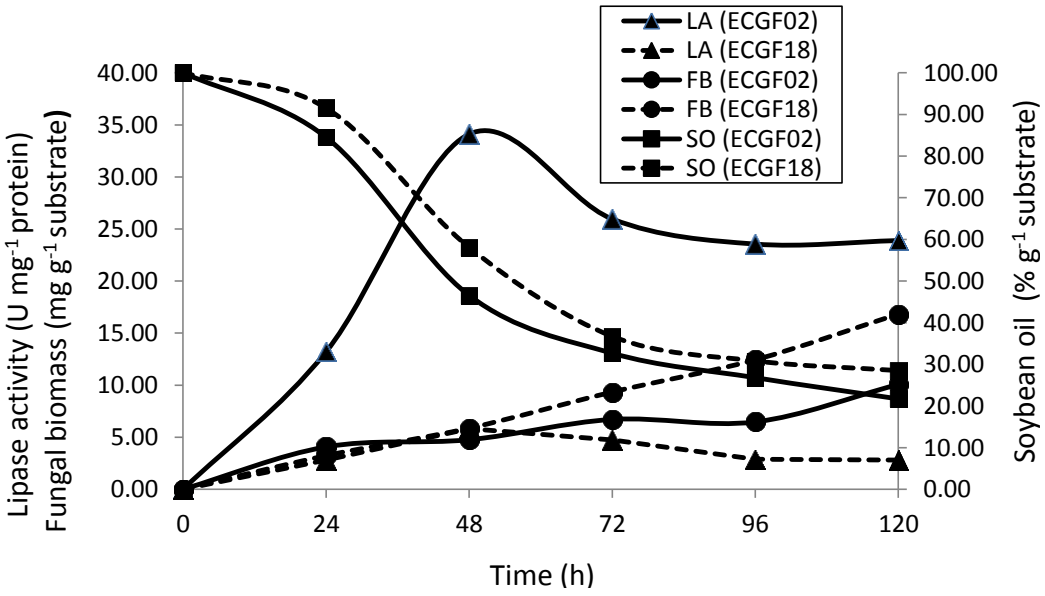

Figure 2. Lipase activity (LA), fungal biomass (FB) and soybean oil content (SO) in the culture substrate of *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18 in solid-state fermentation.

Table 3. Correlation and linear regression coefficients alongside values obtained from lipase activity (LA), fungal biomass (FB), and reduction of soybean oil (rOS) of substrate cultivation of *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18 in solid-state fermentation (FES).

Interactions	Correlations and linear regression coefficients	
	ECGF02	**ECGF18**
LA × FB	Correlation = 0.1313 Y = 0.04109X + 5.4279 R^2 = 0.01725 p Value = 0.64083	Correlation = 0.3351 Y = - 1.27831X + 14.4042 R^2 = 0.11228 p Value = 0.22213
LA × rSO	Correlation = 0.542807 Y = 1.85664X + 12.6804 R^2 = 0.29464 p Value = 0.03654	Correlation = 0.08288 Y = - 1.6991X + 60.241 R^2 = 0.00687 p Value = 0.76897
FB × rSO	Correlation = 0.81253 Y = 8.8829X + 0.48021 R^2 = 0.66021 p Value = 0.00023	Correlation = 0.901646 Y = 4.84411X + 7.5517 R^2 = 0.812967 p Value = 0.0000044

(2012) and are the physical conditions closest to those of the natural habitat of the filamentous fungi.

Because the soybean oil was the oily component of the substrate as well as the exclusive source of carbon for fungal growth, we inferred the potential lipase activity through its removal (consumption), as implicated in its transformation to biomass production by the growing fungi isolates. Soybean oil removal of 79.30 ± 0.43% by *Penicillium* sp. ECGF02 and of 71.50 ± 0.32% by *Rhizomucor* sp. ECGF18, allows a similar interpretation to that of submerged fermentation that assigned the production of intracellular lipase, indirectly estimated, the significant responsibility for lipolytic profile presented by *Rhizomucor* sp. ECGF18.

The results of the analyses of correlation and linear regression between lipase activity, fungal biomass production and soybean oil removal, by *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18, in conditions of solid-state fermentation, were presented in Table 3. The obtained statistical values were attributed to variations in the lipase activity (growing and decreasing) throughout the 120 h, with peaks (48 h) of 34.11 ± 0.62 U mg^{-1}

protein for *Penicillium* sp. ECGF02 and 5.81 ± 0.25 U mg^{-1} protein for *Rhizomucor* sp. ECGF18 (Figure 2). The fact that the values obtained for lipase activity depended on the method used to quantify it, expressed that extracellular lipase activity could be directly measured, but intracellular activity could not. Satisfactory linearity was verified with a correlation between fungal biomass and soybean oil removal parameters, for *Penicillium* sp. ECGF02 (R = 0.81) and *Rhizomucor* sp. ECGF18 (R = 0.90), these were strong and positive for both, with a greater statistical significance of the latter (Table 3).

Emphasis has been given to studies on the production and application of intracellular lipase produced by fungi as *Rhizopus oryzae* (Athalye et al., 2013), *Rhizopus microsporus* (Alberton et al., 2010), *Mucor circinelloides* (Andrade et al., 2014), *Thermomucor indicae* (Ferrarezi et al., 2014) among others, on which extensive reviews have been published, in regards to its applications as biocatalysts, whether, in the form of whole cells or lipolytic biomass, with the potential for use in industrial processes, sanitation and bioenergy production (Fukuda et al., 2009). As an application the aimed to hydrolyze sanitary sewage of dairy industry oil, with level of oils and greases above 1300 mg L^{-1}, Alberton et al. (2010) tested a solid-fermented lipase of *Rhizopus microsporus*, and found that reduced oil and grease levels to below 300 mg L^{-1}. Rigo et al. (2012) have proposed that noncommercial lipase preparation is more suitable for the hydrolysis of meat industry effluents due to its higher hydrolytic rates. Furthermore, its cost is low because it can be crudely produced from enzymatic preparations of agro-industrial residues. On the other hand, aiming at the great interest in generating biodiesel from used oil, n,n-bis(2-hydroxyethyl) - ethanol, Andrade et al. (2014) selected the fungus *Mucor circinelloides* URM 4182 as an integral cell biocatalyst with the greatest potential to be used in the process, resulting in an 83.22% yield in the conversion process.

Fungal lipases are mostly extracellular, and their production is greatly influenced by nutritional and physicochemical factors such as temperature, pH, nitrogen and carbon sources, the presence of lipids, inorganic salts, the intensity and extend of agitation, and dissolved oxygen concentration. Because the major factor for the expression of lipase activity usually studied as the carbon source, these enzymes are generally produced in the presence of a lipid such as oil or any inducer, such as fatty acids, triglycerols, hydrolysable esters, bile salts, tweens, and glycerol, though few authors have produced good yields in the absence of fats and oils (Singh and Mukhopadhyay, 2012; Turki, 2013).

In this work, the soybean oil, at a concentration of 10%, served as a sole source of carbon in the culture substrate as well as a lipase inducer for the 20 strains of filamentous fungi in submerged fermentation, and for *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18, in sequence, in solid-state fermentation. Such a concentration allows a greater selective pressure on lipase-producing strains and differs from that reported by most authors, who use oil concentrations of or lower than the 2%, they considered sufficient for enzyme-induction. Usually, the carbon concentration must be greater than that of nitrogen to favor the pathways for lipase biosynthesis in these microorganisms (Bueno et al., 2014, Ramos-Sánchez et al., 2015).

The use of the mixture of sand and vermiculite to the natural soil simulation, proved convenient as a substrate for the solid-state fermentation conditions adopted in this study. These inert minerals were not considered a source of nutrients to the fungi evaluated. Therefore, the growth and lipolytic activity of *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18 were associated with simple factors, best defined as consistent, with simple nutritional composition and low cost. Thus, we believe that these factors contribute to the ease in reproducing these experiments and reliability of the results obtained.

Conclusion

Here it was possible to obtain 20 filamentous fungi isolates from grease trap scum found in a restaurant at Federal University of Espírito Santo, Brazil. The 20 fungi belong to the genera *Aspergillus*, *Beauveria*, *Botrytis*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Geotrichum*, *Penicillium*, *Rhizomucor*, and *Verticillium*. In submerged fermentation, *Penicillium* sp. ECGF02 showed greater lipase extracellular activity, while *Rhizomucor* sp. ECGF18 showed less, despite having the highest biomass production among all isolates evaluated. In solid-state fermentation, lipase production, biomass production, and soybean oil removal from culture substrate, confirmed the high extracellular lipase activity of *Penicillium* sp. ECG02 and the high intracellular activity by *Rhizomucor* sp. ECGF18. The methodology used in this work proved to be efficient for the characterization of the lipolytic activity of the isolates evaluated. This was an important factor to define before determining its potential use in the production and use of fungal lipases, because the main idea was to lessen the technical and economic burdens of lipase production. This work takes an initial step by isolating new intracellular lipase-producers for commercial interest a whole-cells lipase use. Thus, because of their high lipase intracellular activity, *Rhizomucor* sp. ECG18, showed the potential for use in future research, as whole-cell lipases, are potentially useful in wastewater treatment and biocatalysts in the production of biodiesel from oily residues found in the modern human environment.

Conflict of Interests

The authors have not declared any conflict of interests.

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