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Full Length Research Paper

Possibilities of sweet potato [*Ipomoea batatas* (L.) Lam] value chain upgrading as revealed by physico-chemical composition of ten elites landraces of Benin

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Sweet potato is one of the most important food security promoted root crops in the world, especially in sub-Saharan Africa. Unfortunately, the crop is still neglected and underutilized in Benin Republic. To establish baseline data for its better utilization for upgrading its value chain, 10 selected local varieties (01 cream, 02 white, 03 yellow, and 04 orange flesh-colored) were compared for their macro-nutritional composition assessed using standard Association of Official Analytical Chemists (AOAC) procedures and spectrophotometry methods. The results indicate that sweet potato dry matter, protein, fiber, and ash content ranged from 46.11 to 25.9%, 4.09 to 1.97%, 1.81 to 3.00%, and 4.70 to 2.56%, respectively and orange flesh cultivars were found very rich. Pearson correlation analysis of variables revealed that dry mater content is positively correlated with carbohydrate and energy values, but negatively correlated with ash and fiber content, while starch content is strongly correlated with fiber content. Principal component analysis allowed us to classify the sweet potato varieties into 03 varietal groups among which Group 2 (05 varieties) exhibited rich fiber, ash, and protein contents and may be recommended for infant foods formulations. These results constitute important orientation for sweet potato processing chain organization in Benin and for the establishment of future nutrition and breeding programme.

Key words: Benin, sweet potato, *Ipomoea batatas*, nutritional composition, orange flesh cultivar, value chain.

INTRODUCTION

Sweet potato [*Ipomoea batatas* (L.) Lam.] is one of the major staple crops and the most important food security promoting root crops in the world, especially in sub-Saharan Africa (Low et al., 2009). Well adapted to the

tropical and subtropical regions, sweet potato has nutritional advantage for the rural and urban dwellers (Ingabire and Hilda, 2011). Sweet potato is an excellent source of energy (438 kJ/100 g edible portion) and can produce more edible energy per hectare per day than cereals, such as wheat and rice (Abu et al*.,* 2000) and has other advantages, such as versatility, high yield, hardiness, and wide ecological adaptability (Laurie et al., 2012).

Sweet potato roots are rich in starch, sugar, vitamin C, β-carotene, iron, and several other minerals (Laurie et al., 2012; Oloo et al., 2014). Despite its high carbohydrate content, sweet potato has a low glycemic index due to low digestibility of the starch making it suitable for diabetic or overweighed people (Ellong et al., 2014; Fetuga et al., 2014; ILSI, 2008; Ooi and Loke, 2013). The root is reported to usually have higher protein content than other roots and tubers, such as cassava and yams (Oloo et al., 2014). In addition, some varieties of sweet potatoes contain colored pigments, such as β-carotene, anthocyanin, and phenolic compounds. These pigments form the basis for classifying the foods as nutraceuticals (Oloo et al., 2014). Sweet potato's leaves are recognized to be rich in essential amino acids, such as lysine and tryptophan which are always limited in cereals. Hence, sweet potato can easily complement cereal based diets in the region (Mwanri et al., 2011; Oloo et al., 2014). Moreover, sweet potatoes have high technological potential and it is reported that it can be used for various products, such as drinks (wine, liquor, vinegar), sugar production, biscuits, flour, pasta, alcohol, etc. (Ellong et al., 2014).

Nowadays, several research programmes are focusing on orange-fleshed or vitamin A sweet potato with great potential to prevent and combat vitamin A deficiency for the crops value chain upgrading within the West African sub-region (Inaghe and Hilda, 2011). However, in Benin, cassava and yam appear as major roots and tubers crops and they are valorized through various food products across the country. According to Gnonlonfin (2008), cassava and yam processing into chips is common traditional activity in Benin. In addition, cassava is also largely consumed after processing into garri, traditional flour, lafun, and improved flour. However, the potential benefits of crop such as sweet potato are marginalized and are underutilized despite their technological potential which is well recognized and exploited elsewhere. In Benin, Sanoussi et al. (2015) reported the existence of high diversity of sweet potato that are poorly managed for proper value chain development as they are only eaten boiled, fried or as puree. Unfortunately, no reports on the nutritional (macronutrient) composition of Benin sweet potato cultivars is available to help in identifying landraces that have interesting nutritional and technological aptitude for more efficient utilization in diverse agro-food chain sector. In order to encourage diversification of utilization of the crop and upgrade sweet potato value chain in Benin Republic to fully exploit it potential, preliminary study focused on the study of existing diversity (Sanoussi et al., 2015). The objectives of the present study are to: evaluate the proximate composition of the selected varieties (including their total and reducing sugars contents) for better utilization through agro-food processing chain; examine the correlation between the chemical parameters to predict the probable link between the variable for future use or breeding purpose; clarify the relationship among the cultivars to predict the possibility of the crop efficient utilization and its value chain upgrading via sustainable food industry.

MATERIALS AND METHODS

Ten sweet potato landraces were selected for the chemical analysis. These were selected from the 108 accessions collected from the southern and central part of Benin Republic and maintained at the experimental farm of the Faculty of Sciences and Technology of Dassa. Selection of cultivars was based on their productivity and/or their predominant and secondary flesh color and peel color. Flesh color was considered as it is known to be related to vitamin A content which needs to be promoted for combating vitamin A deficiency and associated illness among the local population. Table 1 presents the ten selected cultivars description including their vernacular name, the name of the sites (village) of provenance, their flesh and peel colors as well as their estimated yield after plantation in the experimental farm.

Treatments

For the analysis, five medium size sweet potato roots, freshly harvested from the farm were thoroughly washed with potable tap water and cleaned using toweling paper. Cleaned roots were peeled manually with a stainless steel knife and cut into small pieces, wrapped into aluminum paper and kept into refrigerator for future analysis. Before the analysis, the samples of sweet potato flesh were dried in oven at 72°C for 72 h and ground using GRIND-MILL laboratory grinding machine for obtain flour (dried) samples for the analysis.

Proximate composition

The proximate composition analysis was focused on the evaluation of moisture, protein, fat, carbohydrate, ash, as well as fiber content of each of the ten varieties of the sweet potato and carried out

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Table 1. List and characteristics of the selected cultivars.

Accession number	Vernacular name	Collecting sites	Peel color	Predominant flesh color	Secondary flesh color	Estimated mean yield (t/ha) on Wet weight basis
E5	Wêli F	Sissèkpa	White	Cream	None	76.38
E1	Avoungokan vôvô	Sagon	Pink	White	None	40.80
E6	Gbagolo	Dasso	Purple	White	None	33.90
E10	Bombo wéwé	Sokan	Cream	Yellow	Pink	12.65
E4	Atanboué	Dasso	White	Yellow	None	18.53
E9	Carrotti	Bozoun	Orange	Dark orange	None	21.32
E3	Loki kpikpa	Pira	Pale orange	Pale orange	None	36.96
E8	Dokouin C	Kétou	Pink	Pale orange	Orange	36.35
E7	Mansawin	Sokan	Yellow	Pale orange	Yellow	18.51
E2	Bombo vôvô	Sokan	Dark purple	Yellow	Purple	38.88

according to the standard procedures of Official Method of Analysis of the Association of Official Analytical Chemists (AOAC, 2000). The determination of moisture was carried out by oven method, while ash content was determined by the sample ashing method in a muffle furnace. The protein (total nitrogen) content was determined by Kjeldahl method while the fat content was evaluated by extraction of 10 g of dried sample through Soxhlet apparatus using petrol ether as solvent. Crude fiber was determined from the residue of defatted sample by keeping 5 g of sample in a muffle furnace at temperature of 900°C for 6 h after extraction of insoluble matter following AOAC methods. The carbohydrate content was determined by simple differences (dry extract - ash+ lipids + proteins) following Ellong et al. (2014).

Total and reducing sugars as well as starch content were determined by Spectrophotometric method. Reducing sugars content was evaluated using dinitrosalycilic acid DNS as reagent (Eleazu and Eleazu, 2012) while for total sugars determination, phenol and sulfuric acid were used and the absorbance were read at 546 nanometers for these two parameters. Starch content of the sample was determined by reading solution floated after preparing the (hydrolysis of starch) medium using the different sweet potato flour sample, sodium hydroxide (1 M) and chlorhydric acid (1 M) at 580 nanometers using BioMate^{TM} 3 spectrophotometer.

Calorie value was estimated following Etong et al. (2014) using Atwater factors by multiplying the proportion of protein, fat, and carbohydrate by their respective physiological fuel values of 4, 9, and 4 kcal/g, respectively and taking the sum of the products.

Proximate analysis were carried out at Laboratory of Food Sciences and Technology of the Faculty of Agronomics Sciences (University of Abomey-Calavi), while starch, total, and reducing sugars analysis was proceeded at the Laboratory of Protein Biochemistry and Enzymology of the Faculty of Sciences and Technology (University of Abomey-Calavi).

Statistical analysis

The results obtained were analyzed using descriptive statistics (means and standard deviation) and Pearson correlation coefficient. The analysis of variance (ANOVA) and test of Newman-Keul were performed to assess the significance of differences between means of proximate analysis variable of the ten sweet potato cultivars at probability p=5%. Pearson correlation coefficient was calculated and correlation matrices were generated to assess the correlation between variables. Principal component analysis (PCA) was also performed to examine the relationship between the 10 cultivars by considering them as individuals and proximate composition parameters (dry matter, fat, protein, carbohydrate, ash, fiber, total sugars, reducing sugars, and starch contents as well as energy value) as variables. Apart from the PCA which was performed using XLSTAT software, MINITAB® software version 14 was used for all the statistics analysis.

RESULTS AND DISCUSSION

Proximate composition

The proximate composition including dry matter, protein, fat, carbohydrates, ash, and fibers of the selected ten cultivars as well as their energy value, their total and reducing sugars and starch content were evaluated.

The dry matter content of the ten selected cultivars ranged from 46.12 to 25.09%. These values are similar to the one reported by Ellong et al. (2014) in Martinique (29.56 to 39.32%), but higher than those reported by Laurie et al. (2012) in South Africa (18.5 to 30.5%). This result is an indication of the existence in Benin of high dry matter content sweet potato cultivars. The higher dry matter content was recorded with E4 (Table 2), a yellow flesh sweet potato cultivar while the lowest rate was obtained with E3, an orange flesh sweet potato (OFSP) variety. Tomlins et al. (2012) reported that OFSP varieties with high carotenoid content tend to have lower dry matter contents. Therefore, OFSP cultivars E3, E7 and E9 that exhibited lower dry matter content (Table 2) might present higher carotenoids content than E8(38.56%) and others colored (white, cream, yellow) flesh sweet potato cultivars. Dry matter content relates to good cooking qualities and extended storage lives (Eleazu and Eleazu, 2012; Eleazu and Ironua, 2015) and could contribute to increase the yield and the

Table 2. Proximate composition of roots of selected ten sweet potato cultivars from Southern and Central Benin.

Sample	Flesh color	Name and proximate composition parameters (g/100 g on dry weight basis) of sweet potato cultivars							
		Vernacular name	Dry matter (%)	Protein (%)	Fat (%)	Carbohydrates (%)	Ash (%)	Fiber (%)	Energy value (kcal)
E1	White	Avoungokan vôvô	27.79±1.01^{b}	3.40±0.00^{cd}	0.67±0.07^{a}	20.19±0.93^{b}	3.53±0.00^{d}	2.51±0.05^{e}	100.39±4.39^{a}
E6	White	Gbagolo	31.64±0.05^{C}	3.35±0.04^{cd}	8.88±0.30^{c}	15.71±0.23^{a}	3.70±0.00^{d}	2.35±0.01^{d}	156.18±1.71^{d}
E5	Cream	Wêli F	35.46±0.13^{d}	3.61±0.05^{cd}	1.45±0.21^{b}	27.39±0.48^{c}	3.00±0.10^{bc}	2.11±0.04b^{c}	137.06±0.15c
E2	Yellow	Bombo vôvô	30.33±1.08^{bc}	3.20±0.03^{c}	1.49±0.22^{b}	22.36±1.19^{b}	3.28±0.31^{cd}	2.56±0.05^{e}	115.68±6.68^{b}
E4	Yellow	Atanboué	46.12±0.17^{f}	4.09±0.01^{e}	0.54±0.13^{a}	38.92±0.04^{f}	2.56±0.01^{a}	1.81±0.05^{a}	176.96±1.33^{e}
E10	Yellow	Bombo wéwé	31.26±1.13^{C}	3.22±0.09^{c}	1.81±0.23^{b}	35.49±0.70^{e}	3.61±0.01^{d}	1.99±0.02^{b}	171.15±1.06^{e}
E3	Pale orange	Loki kpikpa	25.09±0.08^{a}	4.19±0.25^{e}	1.74±0.09^{b}	14.46±0.07^{a}	4.70±0.01^{e}	2.98±0.00^{f}	90.24±0.10^{a}
E7	Pale orange	Mansawin	30.15±0.45^{bc}	2.03±0.06^{a}	2.22±0.51^{b}	22.86±0.43^{b}	3.03±1.00^{bc}	3.00±0.04^{f}	119.54±1.92^{b}
E8	Pale orange	Dokoui C	38.56±0.41^{e}	2.82±0.05^{b}	2.05±0.23^{b}	30.97±0.73^{d}	2.71±0.04^{ab}	2.19±0.01^{c}	153.63±0.64^{d}
E9	Dark orange	Carroti	30.73±0.88^{bc}	3.71±0.04^{d}	2.04±0.24^{b}	21.50±1.10^{b}	3.07±0.02^{bc}	2.33±0.05^{d}	122.86±2.06^{b}

texture of derivative products (Mégnanou et al., 2009). Following Odebode et al. (2008), these high dry matter cultivars could be recommended to flour production industries for various products such as baking flour, composite flour, weaning formula, stabilizer in ice cream industry, etc. According to Eleazu and Ironua (2015), processing sweet potato roots into flour, is one way of minimizing post-harvest losses and increasing its utilization. However, future study can be suggested to understand the relationship between the flesh color of the varieties and their real storage life, since good storage shelf life is requested quality in sweet potatoes.

The protein content of cultivars studied ranged from 2.03 to 4.19% on dry weight basis (Table 2). These values are in the same range of 3.28 to 4.16% obtained by Ukom et al. (2009) in Nigeria, but higher than the values of 0.71 to 0.91% reported by Ingabire and Hilda (2011) in Rwanda. Among the cultivars studied, higher protein contents were recorded with some of the yellow (E4=4.09%) and the dark orange (E3=4.19%; E9=3.71%) flesh cultivars. This suggested that colored flesh (yellow and orange) sweet potato cultivars, in addition to their probable highest β-caroten content, could be good sources of protein than white flesh varieties. Therefore, they could be recommended as good food for alleviating protein-energetic malnutrition and vitamin A deficiency. In comparison with the leaves protein content (15.10 to 27.10%) as reported by Nkongo et al. (2014), the storage roots exhibited lower protein content, hence indicating the necessity of encouraging the promotion and the consumption of composite foods mixing sweet potato storage root and leaves for balanced diet. With regards to the recommended daily allowance of proteins for infants (9.1 to 13.5 g/day), children (13 to 19 g/day), adult women (34 to 46 g/day), and adult men (45 to 50 g/day) (Eleazu and Ironua, 2015), the consumption of only 100 g of the storage roots (dried) of the studied sweet potato cultivars is enough to help meet one third (1/3) to one fourth (1/4) protein requirements of infant and children and about one tenth (1/10) protein requirements of adults. Protein plays an important role in human body and have numerous benefits, such as provision of vital body constituents, maintenance of fluid balance, contribution to the immune function and formation of hormones and enzymes which control a variety of body functions, such as growth, repair and maintenance (Iheanacho and Udebuani, 2009; Eleazu and Ironua, 2013).

Lipids are very important in food substances since they are vital to the structure and biological function of cells and contribute significantly to the energy value of foods (Eleazu and Ironua, 2013). Cultivars E6 (Table 2) showed a very high fat content (8.88%) which did not fall in any range of sweet potato fat content reported in the literature, while the other cultivars have fat content of 0.54 to 2.22%. In previous morphological characterization (Sanoussi et al., 2015), E6 has been distinguished itself from others cultivars of the collection with its general leaves shape which is also different from leaves shape proposed in sweet potato descriptors. Therefore, future studies could be undertaken on this sweet potato landrace through

use of molecular tools to better understand the genes responsible for its characteristics. Knowing that vegetable oils provide essential fatty acids, it will be interesting to know the fatty acid profile of this sweet potato variety and especially its essential fatty acids content. Fats function in the increase of palatability of food by absorbing and retaining flavours (Antia et al., 2006) are well recognized. Thus, sweet potato cultivar E6, due to its exceptional fat content could be promoted as food flavoring. In addition, the utilization of this cultivar could be recommended in food diet for prevention of obesity and cardiovascular diseases as excess fat consumption is implicated in certain cardiovascular disorders such as atherosclerosis, cancer and aging. Diet providing 1 to 2% of its caloric of energy as fat is said to be sufficient to human beings (Antia et al., 2006).

Ash content varied from 2.56 to 4.70% and are higher than those (0.40 to 2.35%) generally presented in fresh weight basis in literature (Eleazu and Eleazu, 2012; Eleazu and Ironua, 2013; Ellong et al., 2014). Following Ukom et al. (2009), this high ash content indicates that cultivars studied are rich in some mineral salt. E3, pale orange flesh cultivar, exhibited the higher ash content (4.70% on dry weight basis) and could be promoted for preventing and curing hidden hunger especially among children, pregnant, and lactating women.

Dietary fiber content (Table 2) varied from 1.81 to 3.00% in dry weight basis. Orange flesh cultivars recorded higher fibers content indicating that they could be more digestible than the others. The values obtained are similar to the ones reported by Inghabire and Hilda (2011), higher than those of Ukom et al. (2009) in Nigeria but lower to the values (3.30 to 5.40%) reported by Ellong et al. (2014) in Martinique. According to Trinidad et al. (2013), dietary fibers are important in preventing cardiovascular diseases and diabetes mellitus and they are also efficient in reducing the incidences of colon cancer and certain digestive diseases (Ingabire and Hilda, 2011).

Carbohydrate is represented in higher quantities (14.86 to 38.92%) for all the ten selected sweet potato cultivars indicating that they are good source of energy. Apart from the light orange flesh cultivar E3 (14.86%), the cream, the yellow, and the light and dark orange fleshes seem to have higher carbohydrate content than white flesh cultivars. It is well recognized that foods with carbohydrates that break down quickly during digestion and release glucose rapidly into the bloodstream tend to have a high glycemic index and foods with carbohydrates that break down more slowly, releasing glucose more gradually into the bloodstream, tend to have a low glycemic index (Jenkins et al., 1981). The type of carbohydrate available in these local varieties and their effects on blood glycaemia needs to be more investigated for better understanding of the reported potential of sweet potato in diabetes management. According to Eleazu and Ironua (2015) significant amounts of carbohydrates in foods give them a role in human health and they can serve as substrates for the production of aromatic amino acids and phenolic compounds through the shikimic acid pathway. The shikimate pathway leads to essentials amino acids, such as tryptophan and phenylalanine synthesization.

On the basis of the total sugars content, the sweet potato cultivars studied can be classified into four groups of low (1.63 to 7.00 mg/g; E1, E3, E5, E7 and E8), intermediate (24.23 to 27.42; E2 and E6), high (42.64 mg/g; E4), and very high (242.68 and 684.63 mg/g; E9 and E10) total sugars content. Once again E6 distinguished itself from the others white flesh cultivars after been demarked for it specific fat content. E10 (yellow flesh) and E9 (dark orange flesh) could be recommended to intervene as natural sweeteners for food industry or for diabetics consumption since sweet potato has been reported as low index glycemic food (Ellong et al., 2014) that have natural nutraceutical capacity of controlling glycaemia (Ooi and Loke, 2013). In addition, the individuals of intermediate and high total sugar content groups could also be recommended as raw material in food industries aimed at limited sugar addition from others sources.

The reducing sugars content (Table 3) ranged from 0.38 to 12.11 mg/g and is lower than the range of the value 1.74 to 2.50% reported by Ingabire and Hilda (2011). Cultivars E2 (yellow flesh cultivar; 12.11 mg/g) and E9 (orange flesh cultivar; 10.86 mg/g) that exhibited relatively higher values of reducing sugars show promising potential for ethanol production. Apart from E4, all the yellow flesh cultivars exhibit higher reducing sugars than white and cream flesh cultivar. Similar results were obtained by Eleazu and Eleazu (2012), who stipulated that yellow cassava cultivars have higher quantities of reducing sugars than the white cassava cultivars. According to these authors, the difference in reducing sugars content among the cultivars may be due to the difference in genotype being analyzed and possibly to phenolic compound present in the yellow and orange flesh cultivar.

The starch content of sweet potato cultivars studied ranged from 70.56 to 326.73 mg/g. Apart from cultivar E4 which recorded the highest starch content (326.73 mg/g equal to 32.67%), all the sweet potato analyzed, present the starch content values lower than the values ranging from 20.30 to 31.05% as reported by Ellong et al. (2014) in Martinique. The differences may be due to genotypes variations or to a probable conversion of the starch of the sweet potato studied and only the resistant starch has been quantified. Iheagwara (2013) have mentioned the relatively low stability of sweet potato starch. In addition, Eleazu and Ironua (2015) reported high amylasic activities of the sweet potato protein. Starch has a wide application in both food and sugar industry since it is

Table 3. Sugars and starch content of the ten sweet potato cultivars.

Sample	Flesh color	Vernacular name	Parameter (mg/g)		
			Reducing sugars	Total sugars	Starch
E1	White	Avoungokan vôvô	2.70±0.18^{b}	7.00±0.35^{a}	70.56±1.96^{a}
E6	White	Gbagolo	1.22±0.06^{a}	27.42±0.90^{ab}	83.10±12.74^{ab}
E5	Cream	Wêlli F	1.14±0.19^{a}	1.63±0.07^{a}	144.65±3.53^{c}
E2	Yellow	Bombo vôvô	12.11±0.13^{f}	24.23±0.70^{ab}	172.87±3.14^{d}
E4	Yellow	Atanboué	0.38±0.00^{a}	42.64±1.50^{b}	326.73±3.00^{e}
E10	Yellow	Bombo wéwé	6.34±0.36^{d}	684.43±16.0^{d}	-
E3	Pale Orange	Loki kpikpa	3.98±0.09^{c}	4.40±0.20^{a}	169.34±4.30^{d}
E7	Pale Orange	Mansawin	5.73±0.30^{d}	6.00±0.01^{a}	143.08±4.31^{c}
E8	Pale Orange	Dokoui C	3.35±0.05^{bc}	6.10±0.03^{a}	78.40±1.96ab^{ab}
E9	Dark Orange	Carroti	10.86±0.75^{e}	242.68±14.0^{c}	141.12±7.25^{c}

Table 4. Pearson correlation matrice of the physico-chemical data.

Variable/Correlation	Dry matter	Protein	Fat	Carbohydrate	Ash	Fiber	Energy	Reducing sugars	Total sugars
Protein	0.122								
Fat	-0.146	-0.130							
Carbohydrate	0.792*	-0.016	-0.445						
Ash	-0.777*	0.322	0.238	-0.660*					
Fiber	-0.758*	-0.315	0.065	-0.774*	0.551				
Energy	0.769*	-0.020	0.226	0.767*	-0.530	-0.832*			
Reducing sugars	-0.420	-0.202	-0.194	-0.181	0.038	0.289	-0.337		
Total sugars	-0.089	0.006	-0.069	0.417	0.098	-0.390	0.417	0.307	
Starch	0.549	0.429	-0.404	0.580	-0.227	-0.361	0.378	-0.042	0.158

Values with * indicate a significant correlation.

used to influence or control foodstuffs characteristics, such as aesthetics, moisture, consistency and shelf stability. In addition, starch is widely used as a thickener, water binder, emulsion stabilizer, and gelling agent (Iheagwara, 2013; Eleazu and Ironua, 2015). From the results of this study, there is a need to improve the starch content of the cultivars through molecular biology tools, in a bid to upgrade sweet potato value chain through a supply of various nutrient (starch, sugars, dry maters, protein, ash) rich sweet potato cultivars for different industrial application in Benin as suggested by Bovell-Benjamin (2007).

The energy value of the cultivars ranged from 90.24 to 176.96 kcal/100 g (Table 2) with an average of 134.37 kcal/100 g equal to 561.67 kJ/100 g. Cultivars E4 and E10 with both yellow flesh sweet potato, provided about 2 times more energy than E3 cultivars (OFSP). Consumption of 1 kg of E4 and E10 dry matter will provide about 1769.60 and 1711.60 kcal of energy which is not too far from the minimum (2500 kcal) daily calorie requirement for adults (Onyeike and Oguike, 2003). Based on the average caloric value of the other roots and tubers reported by Lebot (2009), it can be deduced that sweet potato cultivars analyzed can provide lower energy than cassava (600 kJ/100 g) roots and higher energy than yam (440 kJ/100 g) and aroids (400 kJ/100 g) tubers.

Correlation of the variables and PCA of the physico-chemical data

Pearson correlations analysis of the variables (Table 4) showed that the dry mater content is positively correlated with carbohydrate content and energy value, while negatively correlated with ash and fiber contents. This suggested that the more a cultivar is rich in dry matter, the higher its carbohydrate content and energy value and the lower its ash and fiber content. Therefore, improvement of dry matter content of the orange flesh cultivars as suggested earlier, may lead to the loss of their high mineral content via ash content losses. Total

Table 5. Contribution of each parameter to the variability on the first four principal components of PCA.

Variable	PC1	PC2	PC3	PC4
Dry matter	-0.439	0.179	0.084	-0.213
Protein	-0.068	-0.245	0.703	0.166
Fat	0.113	0.561	0.002	0.464
Carbohydrate	-0.447	-0.150	-0.142	-0.034
Ash	0.339	-0.150	0.337	0.387
Fiber	0.424	-0.041	-0.055	-0.234
Energy	-0.413	0.214	-0.101	0.318
Reducing sugars	0.148	-0.475	-0.438	0.025
Total sugars	-0.135	-0.372	-0.278	0.627
Starch	-0.291	-0.369	0.289	-0.105
Eigen-value	4.39	1.62	1.49	1.36
Proportion	43.96	16.22	14.99	13.61
Cumulative	43.96	60.18	75.17	88.79

sugars content (Table 4) is positively correlated with reduced sugars, carbohydrate content, and energy value, while negatively correlated with fiber content. Starch and fiber content are negatively correlated. Improvement of total sugars content may lead to the increase of reducing sugar which is a required parameter for directing a cultivar for bio-ethanol production. Among the cultivars studied, the same sweet potato cultivar could not be simultaneously breed for starch and ethanol (reducing sugars) industries as it was found out that a breeding programme focusing on improved reducing sugar content in a variety will lead to an increase of it fiber content and therefore a lowering of its starch content.

PCA grouped the variables into nine components among which the first four are significant (Eigen value>1) and explained 88.79% of the total variability (Table 5). Principal Component 1 (PC1) is correlated with dry mater, carbohydrate, ash, fiber, and energy explains 43.96% of the total variability, while PC2 (16.22% of total variability) is associated with fat, starch, total, and reducing sugars (Table 5). Fat, reducing sugars, dry matter, and carbohydrate contents recorded the highest values for their coordinates on PC1 and PC2 and represent the most important factors of the variability between the sweet potatoes cultivars studied.

Projection of the cultivars on the factorial axis 1 and 2 allowed the classification of the sweet potato cultivars into three groups (Figure 1). Group 1 assembles cultivars (E4 -Atanboué, E5-Wêli F, E8-Dokoui C, and E10-Bombo wéwé) and is characterized by cultivars with high dry matter and high carbohydrate content with a subgroup of cultivars rich in starch and total sugars. These cultivars can be a good raw material for sweet potato flour and derivative (starch, noodle, confectionaries, baking, porridge, and sweeteners) production industry. Group 3 is represented by only one sweet potato cultivars (E6-Gbagolo) which is characterized by high fat content, while the group 2 combines five (05) sweet potato cultivars (E1-Avoungokan vôvô, E2-Bombo vôvô, E3-Loki kpikpa, E7-Mansawin, and E9-Carroti). Individual of group 2 recorded high protein content, high ash, and fiber content. The characteristics of this last group is quite interesting, because it can be used to combat malnutrition and this in two ways (helping in preventing or fighting against protein-energetic malnutrition and helping to alleviate hidden hunger through it mineral content via ash content). Individual of this last group is very important to be promoted as infant formula since FAO (2013) reported that apart from protein-energetic malnutrition, micronutrient deficiency is the most redoubtable form of malnutrition among children.

These last two analyses help to identify the most important variables that should be taken into account in breeding and selection program aimed at improving and creating varieties especially dedicated to serve as raw material in various food industries to upgrade the crop value chain. The results of the PCA seem particularly interesting, because the ten sweet potato local varieties can be directed to different applications at the processing chain and boosting the species' value chain.

As regards all the chemical parameters analyzed, cultivars “Bombo wéwé” and “Carroti” recorded the highest total sugars content, while “Atanboué” and “Bombo vôvô” on one hand and “Atanboué” and “Wêli C”, on the other hand, have the highest amount of starches and dry matter, respectively. These 05 varieties highly need to be promoted for the rapid implementation of sweet potato processing chain in Benin as it well recognized that dry matter, starch and total sugars contents are important parameters with large applications in agro-food processing and business. In terms of protein content cultivars, “Loki kpikpa” and “Atanboué” are the richest while “Loki kpikpa” and “Bombo wéwé” present the highest ash content. In addition, all of these local sweet potato varieties have yellow/orange flesh making them most useful for future orientation in vitamin A deficiencies alleviation programme.

However, in order to prevent the genetic erosion of the rest, an action should be undertaken over the ten cultivars for the *in situ* and *ex situ* conservation of their genetics materials on one hand. On the other hand, these sweet potato cultivars can be oriented as potentially good raw materials to implement food based approaches for combating malnutrition.

This study appears as very interesting for future applications in research and constitutes a significant contribution for better knowledge of Benin sweet potato genetic resources and for their future utilization in food and agribusiness development in Benin and around the world for more enhanced food nutrition and security level. However, due to the fact that chemical composition vary

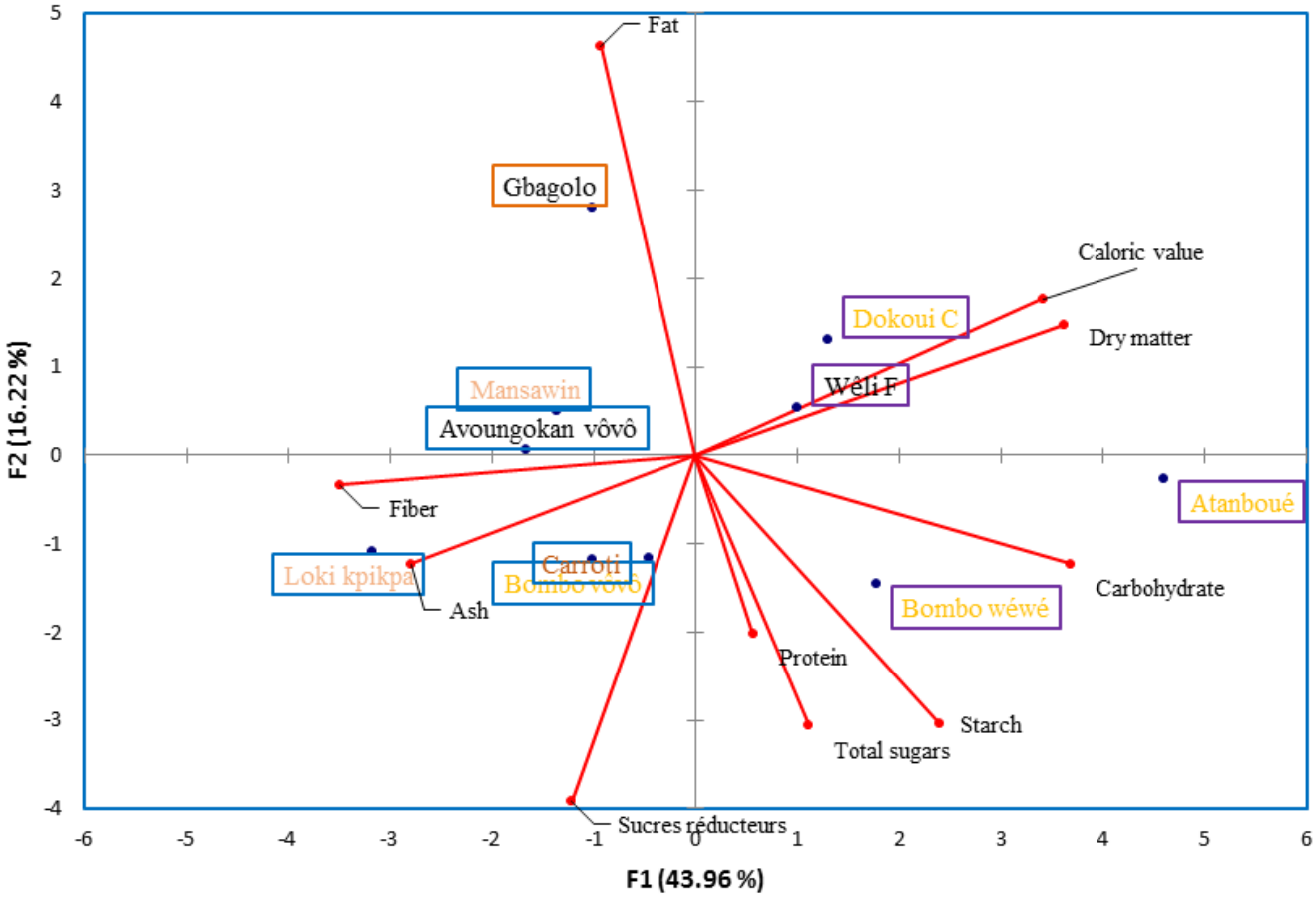

Figure 1. Projection of the sweet potato cultivars on the first and second component axes. Boxes represent the sweet potato cultivars name. Boxes with different colors indicate sweet potato cultivars that belong to different varietal groups. Group 1= Cultivars with indigo boxes; Group 2=Cultivars with blue boxes; Group 3: Cultivar with orange boxes.

greatly in crop depending on soil conditions, temperature and environment in which it grows; further analysis of samples planted at different regions of the country are recommended.

Conclusion

This study allows the establishment preliminary data on nutritional composition of sweet potatoes landraces of Benin Republic. The ten selected sweet potato cultivars of the study present interesting nutrient composition which varies significantly following the genotypes and dry matter, fat, reducing sugars, and carbohydrates contents represent the most important factors of variability among the cultivars studied.

The orange flesh sweet potatoes local varieties recorded the highest nutrient content for almost all the parameters analyzed. However, the study also shows the existence of important significant correlations between certain chemical analyses variables (dry matter content correlated with carbohydrate, ash and fiber contents) which can be useful in future breeding programme. In addition, the different varieties of sweet potatoes analyzed have been structured into groups with potential good technological aptitude to be promoted in different agro-food sectors for sweet potato value chain upgrading in Benin Republic and for orientation in future nutrition programme. The new trend for utilization of six promising local varieties (Atanboué, Carroti, Bombo wéwé, Dokoui C, Loki kpika and Bombo vôvô) which presented quite interesting values for the parameters analyzed out of the ten cultivars analyzed is highly whished. In addition, these six (06) cultivars can be promoted through different sector of the food chain in order to improve food security among both children and aged people.

Sweet potato local varieties mineral and vitamin content analysis could be the next step to validate the possibly potential of these varieties in sweet potato value chain upgrading in Benin as well food security insurance.

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Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of osmotic stress on *in vitro* propagation of *Musa* sp. (Malbhog variety)

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In vitro **propagation of banana preferably use sword sucker as explant source where microbial contamination poses a great problem in establishment of aseptic cultures. This study demonstrates up to 36% reduced microbial contamination in aseptic culture establishment and subsequent micropropagation due to osmotic stress induction in the banana suckers. Osmotic stress was induced by keeping the freshly collected suckers in shade and measuring fresh weight at 0, 7, 14, 21, and 28 days interval to ascertain loss of moisture. Stress induced for 21 days showed 58.85% moisture loss showing lowest contamination upto 40% against 76% for fresh suckers. Micropropagation of *Musa* sp. (Malbhog variety) through shoot tip culture of stressed suckers was carried out in Murashige and Skoog (MS) medium supplemented with 1 to 2 mg/l 6-benzyl-aminopurine (BAP), 2 to 4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l α-naphthalene acetic acid (NAA). Multiplication of plantlets was observed till 6th passage. From 19 aseptically established explants of 21 days stressed sucker sub cultured in MS medium fortified with 1.0 mg/l BAP and 0.5 mg/l NAA, for 6th passage produced 5122 plantlets. Regenerated micro shoots were rooted in MS medium fortified with 0.5 mg/l IAA. The plantlets were hardened in polybag containing soil and seasoned cow dung.**

Key words: Osmotic stress, shoot tip culture, *in vitro*, *Musa,* Malbhog.

INTRODUCTION

Banana is an important group of plants that provides millions of livelihood. India has a rich genetic diversity of banana with more than 90 distinct clones. Banana is grown under diverse conditions and production systems and hence selection of varieties is based on needs and situation. Around 20 cultivars viz. Dwarf Cavendish, Robusta, Monthan, Poovan, Nendran, Red banana, `Safed Velchi, Basrai, Ardhapuri, Rasthali, Karpurvalli, Karthali, Grande Naine, Malbhog etc. are commercially cultivated. Malbhog is a Musa cultivar of AAB genomic group under subgroup of silk type having reference accession number TRY0077 (Daniells et al., 2001) commonly found in Assam, India. Malbhog banana is very popular and has high commercial value in Assam due to its sweet aroma, taste and high post-harvest life. However, shortage of quality planting materials remains the bottleneck for the banana farmers of the state. Tissue culture techniques have been employed for large scale banana planting material production. Many regeneration protocols dealing with different *Musa*

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species has been reported for *in vitro* propagation of banana and plantains (El-Saghir,1997; Kodym and Zapata-Arias, 2001; Vidhya and Nair, 2002; Madhulatha et al., 2004; Sebastian and Mathew, 2004; Anilkumar and Sajeevan, 2005; El-Dougdoug et al., 2006; Kulkarni et al., 2006; Darvari et al., 2010; North et al., 2012; Ngomuo et al., 2014; Qamar et al., 2015). However establishment of aseptic culture remain a great challenge for banana micropropagation. Endo-bacterial contamination is one of the major problems for aseptic establishment of banana where the contaminants may survive in the plant material for several subculture cycles and it may over extend the period of time without expressing symptoms in the tissue or visible signs in the medium (Van Den Houwe and Swennen, 2000). The use of antibiotic, commercial fungicide, thermotherapy, warm water treatment and ultrasonic treatment are some of the commonly used methods to control *in vitro* contamination (Cole, 1996). In this report, osmotic stress induction of banana suckers as a technique to reduce microbial contamination and its subsequent influence on multiplication is emphasized.

MATERIALS AND METHODS

Stress induction

Two months old sword suckers of banana variety Malbhog were collected from farmer's field of Assam, India. Sucker were washed thoroughly under running tap water for half an hour and stress was induced by keeping the cleaned suckers in shade and fresh weight were recorded at 0, 7, 14, 21, 28 days interval to ascertain loss of moisture. Loss of moisture was calculated as follows:

Moisture loss (%): a-b/a x 100

where, a = initial fresh weight of suckers; b = fresh weight of suckers after stress for d.

Establishment of explants in aseptic media

After each interval of stress induction, 25 suckers were washed under running tap water for 30 min and trimmed into square block ranging from 5 to 8 cm in sizes and dipped in fungicide solution (2 gm/l Bavistin and 100 mg/l ascorbic acid) for 30 min followed by washing with liquid detergent (Extran, Merck, 0.05 ml/l) for 20 min and 4 to 5 times rinsed with clean water. Thereafter, sucker cubes were treated with Savlon (Johnsons and Johnsons) for 30 min and brought under laminar hood. Surface sterilization was carried out with 0.1 mg/l $HgCl_2$ fortified with 2 to 3 drops of Tween 20 (Merck) for 10 to 12 min and the disinfectant was removed by 4 to 6 rinse with sterile distilled water. After surface sterilization the cut ends of explants were trimmed with a sterile surgical blade and the shoot tips of 8 to 10 mm from decapitated shoot apexes of suckers are carefully inoculated in pre sterilized MS medium (Murashige and Skoog, 1962) supplemented with 1 to 2 mg/l 6-Benzyl-aminopurine (BAP), 2-4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l α-naphthalene acetic acid (NAA), 30 g /l sucrose, 2.2 g /l Gelrite. The pH of the medium was adjusted to 5.8 using 0.1 M NaOH before autoclaving for 20 min at 121°C at 15 lbs psi pressure. The inoculated media with explants were incubated in the growth room at 25±2°C with 16 h illuminations. The influence of stress on contamination reduction was calculated as follows:

Contamination % = c-d/c*100

where c = contamination of explant of fresh suckers d = contamination of sucker explants stressed for d.

Plant multiplication

Aseptically established explants were subcultured into basal medium (MS) with varying combination of 0.5 to 1 mg/l of BAP, 0.5 mg/l NAA and 2 to 4 mg/l 2, 4-D. Number of plantlets from each explant was recorded for subsequent subculture till 6th passage. Subculture was carried out at every 3 weeks interval.

Root induction and hardening

Microshoots of 4 to 5 cm length were separated from the cluster and inoculated into MS medium supplemented with 0.5 mg/l IAA. Rooted plantlets of 6 to 8 cm length were taken out from culture bottle and washed under running tap water for 10 min to remove media attached to root system. Thereafter plantlets were treated with 1 g/l Bavistin for 5 min and transferred to polybag (size 12 × 18 cm) containing river sand and kept inside the polyhouse for 21 days. Subsequently, these plantlets were transferred to larger polybag (size 15 × 21 cm) containing soil, seasoned cow dung at 1:1 and kept under agro shade net house with provision of 50% penetration of natural light. Irrigation was ensured depending on the requirements.

RESULTS

Stress induction and impact on contamination reduction

Fresh weight of banana suckers decreased with increasing storage duration due to loss of moisture and is expressed as induced osmotic stress. The moisture loss ranged between 15 to 70% during 7 to 28 days of shade storage (Figure 1a, b). Bacterial contamination was visible within 3 days of the inoculation. The lowest contamination (36%) was recorded in 21 days of shade storage where the sucker has lost 58% of moisture (Figure 1c, d). Increasing moisture loss has negative relation with microbial contamination till 21 days (Table 1). This could be attributed to the microbial load present at the periphery of suckers reduced due to drying of suckers.

Influence of pre induced stress on regeneration potential

Initially, the aseptic shoot tip cultures swell at base and developed green colour irrespective of growth hormone supplementation after 3 weeks of inoculation. In the subsequent week, it formed shoot with swollen corm. Corms after dissection into halves and subcultured in MS medium produced microshoots from the base. Regeneration of microshoots in MS basal medium increased from 69 microshoots for fresh suckers to 586 for 21 days stored

Figure 1a. Fresh collected Malbhog sucker.

Figure 1b. Malbhog suckers stored for 21d in shade.

sucker after six passages. Effect of different combination of BAP, NAA and 2,4-D were studied for *in vitro* multiplication of the stress induced explants. Among the various combinations, the effective results were obtained on the MS medium supplemented with BAP and NAA. The shoot proliferation was found to be best in MS basal medium supplemented with BAP (1.0 mg/l) and NAA (0.5 mg/l). Subculturing of the *in vitro* raised microshoots for multiplication on the same medium induced multiple shoots. Shoot multiplication and clump formation at the base was observed after two to three subcultures. After attaining vigorous growth and proliferation, the clumps were divided into smaller clumps and transferred to the same medium for further multiplication. In this combination, the micro shoot regeneration increased to 5221 after 6th passage (Table 2 and Figure 1e, f). The fully grown healthy plantlets were aseptically transferred to the MS basal medium supplemented with 0.5 mg/l IAA for in

Figure 1c. Malbhog explant from stressed sucker ready for surface sterilization.

Figure 1d. Aseptically established Malbhog explant.

Table 1. Effect of different storage duration on moisture loss of Banana Malbhog suckers and its impact on contamination reduction during *in vitro* culture.

Parameters	Stress duration (days)				
	0	**7**	**14**	**21**	**28**
Average weight loss (%) ± SE	0	15.65 ± 0.10	37.79 ± 0.19	59.36 ± 0.21	72.98 ± 0.22
Contamination (%)	76	72	48	40	56

Contamination recorded 7 days after inoculation. 25 explants were used for each treatment.

Table 2. Effect of different storage duration of Banana Malbhog suckers on multiplication efficiency.

MS + PGR (mg./l)			Stress duration (days)	Number of explants aseptically established	Number of microshoots after each passage					
BA	NAA	2,4-D			1	2	3	4	5	6
-	-	-		6	6	10	16	25	57	69
2				6	10	17	38	55	78	95
1	-	2	0	4	7	16	48	71	156	210
1	-	4		8	15	36	66	82	187	402
1	0.5	-		9	16	47	70	133	228	562
-	-	-		8	12	26	40	58	73	108
2				9	16	52	106	218	345	569
1	-	2	7	8	16	43	94	238	457	734
1	-	4		11	24	50	114	190	558	1132
1	0.5	-		13	37	63	124	218	772	1296
-	-	-		13	20	45	68	109	203	269
2				13	24	50	106	243	746	1154
1	-	2	14	12	23	65	124	462	773	1344
1	-	4		14	40	64	213	616	1044	2256
1	0.5	-		16	43	70	228	697	1349	2416
-	-	-		15	36	69	113	302	445	586
2				15	40	74	173	398	774	1119
1	-	2	21	16	42	80	190	452	827	1240
1	-	4		18	56	117	360	1159	3012	5170
1	0.5	-		19	62	150	457	1327	3610	5221
-	-	-		11	20	39	62	120	196	287
2				13	28	46	111	236	686	774
1	-	2	28	10	26	47	92	467	570	1093
1	-	4		15	30	55	109	480	910	1215
1	0.5	-		16	41	60	94	551	1012	2312

Explants inoculated for each treatment are 25.

Figure 1e. Micro shoot cluster of 21d stress induced sucker in MS supplemented with 1mgl BAP and0.5mgl NAA.

Figure 1f. Shoots ready for root induction.

vitro root induction and recorded 9 numbers of healthy roots per shoot within 9 days of culture. The *in vitro* rooted plantlets were washed thoroughly in running tape water and planted in sterile potting mixture and hardened in a agro shade house under 50% penetration of natural light and 70% relative humidity (RH) for two weeks. After four weeks of hardening, the established plants were transferred to the field. This clearly indicates that pre inoculation stress induction to suckers was beneficial for reduction of microbial contamination and subsequent microshoot production.

DISCUSSION

Banana is cultivated in humus and organic matter rich soil where microbial load in rhizosphere region is very high and poses challenge to establish aseptic culture. Various techniques to contain contamination begin with pretreatment of donor plants to reduce contamination (Holdgate and Zandvoort, 1997) with antibiotics and fungicides (Kritzinger et al., 1997) as well as anti-microbial formulations, such as plant protection mixture (PPM) (Guri and Patel, 1998). The explants are also culture indexed for contamination by standard microbiological techniques, which are occasionally supplemented with tests based on molecular biology or other techniques (George, 1993; Leifert and Woodward, 1998; Leifert and Cassells, 2001). Since plants do not have an immune system to antibiotics and as such many of the antibiotics, that are effective against bacteria, fungi, *and* phytoplasmas, are toxic to plants as well. Use of antibiotics is not full proof or the desired method to get rid of microbial contamination (Pierik, 1989). Islam and Zobayed (2000) reported microbial contamination in banana using sugar free medium. We report inexpensive and effective mode of microbial contamination reduction due to stress induction in suckers. Besides reducing contamination, this technique also has the advantage of stretching inoculation period after material collection.

Plants cell experience severe strain and stresses during multiplication of plantlets under artificial medium depending on the hormonal combination. Thus, synergistic effect of BAP and auxin on shoot proliferation has already been reported in banana tissue culture (El-Saghir, 1997; Nauyen and Kozai, 2001; Kagera et al., 2004; Kalimuthu et al., 2007; Sheidai et al., 2008; Qamar et al., 2015). In the present experiment, plants tissue were exposed to osmotic stress during pre-inoculation stage and its subsequent exposure to hormonal combination in the artificial medium responded exceptionally well for microbial contamination reduction and multiplication of microshoots. This is the first report to study the influence of stress on contamination free *in vitro* establishment and multiplication of microshoots (Tables 1 and 2). Root induction is one of the important steps in plant tissue culture. In case of banana, rooting occurs simultaneously for 4 weeks old cultures. However, micro shoots when subcultured in MS medium fortified with 0.5 mg/l IAA induced 9 numbers of healthy roots per shoot within 9 days of culture. Rooting of microshoot in IBA supplemented media has been reported (Haq and Dahot, 2007).

Hardening of plantlets in two stages was found beneficial. Initially, plantlets were transplanted into river sand with intermittent watering for 21 days in polyhouse followed by subsequent transplanting into larger polybag with river sand and seasoned cow dung in agroshade resulted to satisfactory survival and growth.

Conclusion

Microbial contamination in tissue culture could be curtailed for sucker based banana explant by inducing stress for 21 days storage in shade. This technique also increases the multiplication efficiency of microshoots in the subsequent subculture. The present protocol with 21 days osmotic stress induction of banana suckers with MS medium fortified with BAP (1.0 mg/l) and NAA (0.5 mg/l) for *in vitro* establishment, shoot initiation, mass scale multiplication, rooting and two step hardening could be used for mass scale production of banana planting material.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Development of putative transgenic lines of cassava variety H-226

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Cassava production in India is drastically affected by cassava mosaic disease (CMD) caused by the Indian cassava mosaic virus (ICMV) and Sri Lankan cassava mosaic virus (SLCMV). An attempt was done to develop transgenic cassava lines resistant to SLCMV through RNAi vector targeting a conserved 440 bp of 5' end of SLCMV *Rep* (AC1) gene which also overlaps with part of AC4 gene, and functions as a viral RNAi suppressor protein. The partial *Rep* gene of SLCMV was cloned in sense and anti-sense orientations in the RNAi intermediate vector, pHANNIBAL and finally mobilised into binary vector pART27, to construct pSCR1 which contains the kanamycin-resistance gene as a plant selectable marker. In order to use hygromycin as selection agent in cassava genetic transformation, Rep-RNAi gene cassettes of SLCMV was cloned into pCAMBIA1305.2 and the constructs was named pSCR2. *Agrobacterium* mediated cocultivation of cassava embryogenic calli was done with the developed RNAi constructs using two different explants namely, immature leaf lobes and somatic cotyledons. In total, 48 putative transgenic cassava shoots were regenerated on a regeneration medium containing 30 mgl/l hygromycin, of which 2 putative transgenic plants were transferred for hardening. All the putative transgenic cassava plants were PCR-positive for *hph* gene and *Rep* gene indicating integration of transgenes of interest.

Key words: RNAi constructs, cocultivation, cassava mosaic disease (CMD).

INTRODUCTION

Cassava (*Manihot esculenta*) is a major tuber crop cultivated in 13 states of India. Cassava is grown in an area of 2.34 mha in India, with Kerala ranking first in area (1.04 mha) followed by Tamil Nadu (0.95 mha) (FAOSTAT, 2010). Cassava production is affected by a combination of biotic and abiotic stresses, among them; cassava mosaic disease (CMD) caused by cassava mosaic geminivirus (CMG) limit the productivity of cassava (Pita and Fauquet, 2001). CMD had not been reported in India before 1966 and has become more prevalent in recent years in Southern India especially Salem, Dharmapuri districts of Tamilnadu and almost all

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Abbreviations: SEIM, Somatic embryo induction medium.

Table 1. Primers designed for amplification of sense and Antisense of Rep gene of SLCMV used for RNAi vector construction.

S/N	Particulars	Primer (SLCMV)	Primer Position
1	SLCMV Sense FP	5'CCC***CTCGAG*** CCCACAAACCCAAAATTCA 3'	112 –131
2	SLCMV Sense RP	5'CCC***GGTACC*** ATACGGAGGTGGTGGTGTT 3'	521- 540
3	SLCMV Antisense FP	5'CGC***GGATCC*** CCCACAAACCCAAAATTCA 3'	112 –131
4	SLCMV Antisense RP	5'CCC***AAGCTT*** ATACGGAGGTGGTGGTGTT 3'	521- 540

parts of Kerala and causes severe yield loss ranging from 25-80%. The main reason for the fast spread of the disease is due to the indiscriminate use of the infected planting material. Most of the popular varieties grown in Tamil Nadu are either susceptible (such as H-226, SreeHarsha) or tolerant (for example, H165, Co-1, Co-2 and MDV2) to the disease. None of the varieties grown in Tamil Nadu are resistant to the disease. Conventional cassava breeding is not successful in developing resistant varieties, which is seriously limited due to long growth cycle, highly heterozygous nature, poor seed set and viability.

Cassava Mosaic Disease (CMD) in India is caused by two species of virus namely, Indian Cassava Mosaic Virus (ICMV) and Sri Lankan Cassava Mosaic Virus (SLCMV) (Patil et al., 2005). Both the DNA viruses belonging to the bipartite begomovirus genus of the family *Geminiviridae*, and are transmitted by whitefly *Bemisia tabaci* Genn. Mixed infection of ICMV and SLCMV is often involved in causing CMD in India (Patil et al., 2005). It is reported that Geminivirus species simultaneously infecting the same plant can lead to very severe yield reductions and even crop failure (Pita et al., 2001; Calvert and Thresh, 2002). Transformation and regeneration of transgenic cassava plants expressing kanamycin resistant gene as selectable marker has been reported by several laboratories (Sarria et al., 1993, 1995, 1997; Li et al., 1996; Raemakers et al., 1996; Schopke et al., 1996; Gonzalez et al., 1998; Taylor et al., 2004; Zhang et al., 2005; Vanderschuren et al., 2007; Bull et al., 2010). However, there is no report so far available for the regeneration as well as stable genetic transformation for Indian cassava varieties.

Several reports have shown that RNA interference (RNAi) is more potent in controlling plant virus than the sense or antisense expression of the viral genes (References). It is now well established that both RNA and DNA viruses can be controlled by RNAi approach. The RNA viruses are effectively controlled by silencing the coat protein gene (Patil et al., 2011; Yadav et al., 2011), whereas, the DNA viruses are effectively controlled by silencing the *Rep* gene, which is indispensable for DNA replication of virus (Pooggin et al., 2003). As a proof of the concept that RNAi can be engineered to effectively target DNA virus namely, Mung Bean Yellow Mosaic Virus (MYMV-Vig) was demonstrated by Pooggin et al. (2003). Furthermore, a PTGS based strategy to control DNA virus replication was demonstrated when plant cells simultaneously transfected with African Cassava Mosaic Virus (ACMV) and with a synthetic siRNA designed to target the AC1 gene of the virus showed a reduction in the accumulation levels of AC1 mRNA by more than 90% and viral DNA by 70% compared with controls (Vanitharani et al., 2003). The DNA-A of the CMV codes for the AC1 gene, the replication-associated protein gene (or *Rep* gene), which is indispensable for the replication of virus and disease development. This is the first attempt to develop transgenic cassava resistant to CMG using RNAi approach in India.

MATERIALS AND METHODS

Construction of gene silencing vectors with *npt* and *hph* selectable marker genes

The hairpin binary vectors are made using CSIRO RNAi vector construction steps using pKANNIBAL vector. A conserved sequence of size 440 bp from the 5' region of the Rep gene sequence of SLCMV were identified and primers were designed to amplify 440 bp Rep gene of ICMV covering 112 – 540 nt region of Rep gene (Table 1). The restriction enzymes, *Xho*I and *Kpn*I were appended with sense forward and reverse primer and *BamH*I and *Hind*III were appended with antisense forward and reverse primer of SLCMV respectively. The PCR product is then cloned sequentially on either side of the pdk intron to become the two arms of the hairpin. The complete hairpin RNAi gene cassette was released by digestion with *Not*I restriction enzyme and cloned into pART27, plant transformation binary vector from CSIRO Plant Industry, Australia. The constructed pART27 vectors with RNAi-SLCMV Rep cassette was designated as pSCR1. Also, the RNAi cassette of SLCMV was further cloned into the *Not*I site of pBLUESCRIPT (SK+) vector and then the insert was released with *Sal*I and *Sac*I restriction digestion and cloned into pCAMBIA1305.2 which has *hph* selectable marker gene, hygromycin B (Hy) phosphotransferase-encoding gene. The recombinant clones were identified by restriction analysis with *Sal*I and *Sac*I enzyme. The recombinant plasmids were named pSCR2 (Figure 1).

Bacterial strains and culture conditions

E. coli conjugative helper strain, DH5α harbouring pRK2013 were grown separately on LB agar plates containing Spectinomycin (100 mg/l) and Kanamycin (50 mg/l) respectively. The *Agrobacterium* recipient strain, LBA4404 was grown on a YEP agar (10 g/l peptone, 5 g/l NaCl, 10 g/l yeast extract, 15 g/l agar, pH 7.0) plate containing Rifampicin 10 mg/l. The RNAi-Rep vector was mobilized into Agrobacterium strain LAB4404 using triparental method.

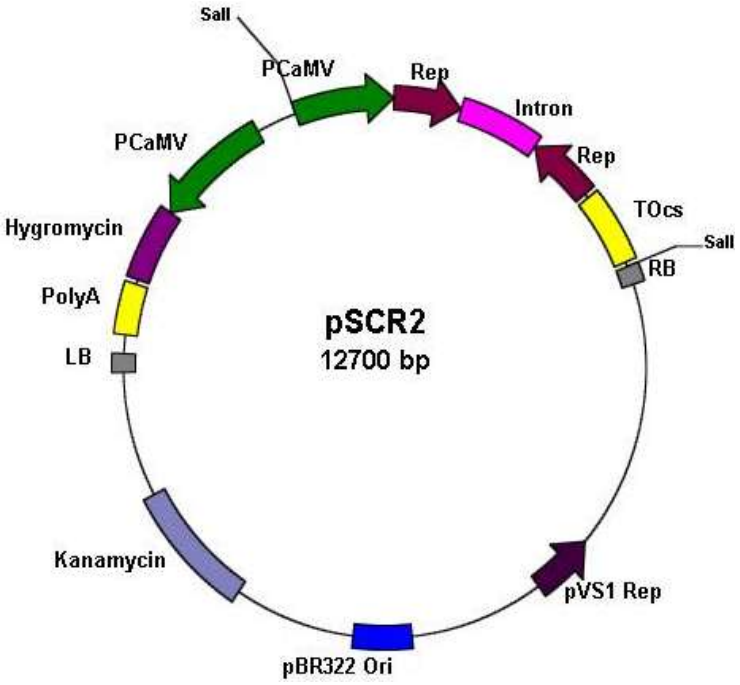

Figure 1. Physical map of RNAi vector pSCR2.

Cocultivation

Young immature leaf lobes of 0.5 to 1 cm in size just near to the apical meristem of cassava and green coloured individual mature somatic cotyledons of 1.0 cm in size separated from cluster of germinated somatic embryos (This stage is obtained 20 to 30 days after transfer of somatic embryo into SEIM) were used for cocultivation. These explants were precultured on SEIM for 2 days in dark at 28°CA donor *E. coli* strain DH5α harbouring the recombinant plasmid, pSCR2 The *Agrobacterium* strain *LBA4404* harbouring the pSCR2 vector was inoculated in 50 ml culture and shaken overnight at 250 rpm in LB medium at 26°C until the OD560 was 1.0. The *Agrobacterium* culture was then pelleted at 4000 rpm, supernatant discarded and the pellet was resuspended in AAM broth supplemented with 100 µm acetosyringone. Explants (both leaflobes and green cotyledons) were dipped in *Agrobacterium* solution for 10 min and thoroughly blot dried using sterile Whatmann filter paper. After Agrobacterium infection, the explants were transferred to Cocultivation medium containing MS salts, B5 vitamin, 12 mg/l Picloram, 30 g/l Maltose, 4 g/l Phytagel, 100 µm Acetosyringone, and pH-5.8 and incubated for 48 h in darkness at 26°C. After cocultivation, explants were washed twice with sterile distilled water and once with ½ MS basal salts and vitamins containing 300 mg/l Cefotaxime. Explants were then blot dried on filter paper and transferred to callus induction medium containing MS salts, B5 vitamins, 30 g/l maltose, 12 mg/l Picloram, 300 mg/l Cefotaxime and 30 mg/l Hygromycin as selection agent.

Selection and plant regeneration

After 48 h of cocultivation, the treated explants were kept in SEIM with hygromycin (30 mg/l) and cefatoxime (300 mg/l). The cultures were incubated in the dark at 26°C. Two rounds of selection were done to obtain transformed tissue. The sub culturing was done at 20 days interval. The somatic embryos developed at the end of second subculture in SEIM were transferred to somatic embryo maturation medium. After 20 days on maturation media, mature somatic embryos were transferred to regeneration medium (MS basal salts, MS vitamin, 3% sucrose, 0.4% Phytagel) containing plant growth regulators 0.1 mg/l of BAP, 0.02 mg/l of α-naphthalene acetic acid (NAA) and 0.2 mg/l GA_3). The somatic embryos cultured in regeneration medium were incubated under light with 16/8 photoperiod. Subculture was done every two weeks in fresh regeneration medium and incubated under light. Two to three subcultures were done for shoot regeneration.

Molecular analysis of putative transgenic lines of cassava

Crude DNA was extracted from small leaf bits (2-3 cm) which were ground in a 1.5 ml Microfuge tube containing 300 µl of extraction buffer (200 mM Tris-HCl pH 7.5, 200 mM NaCl, 25 mM EDTA and 0.5% SDS) and acid-washed sand using a pestle. The homogenate was centrifuged at 12,000 rpm for 10 min. Equal volume of isopropanol was added to the supernatant and kept at - 20°C for 20-30 min. after centrifugation at 12,000 rpm for 10 min, pellets were air dried at room temperature and dissolved in 30 µl of 0.1X TE buffer (1.0 mMTris-HCl pH 8.0 and 0.1 mM EDTA pH 8.0; Proebski et al., 1997). The PCR analysis was carried out using 100 ng of genomic DNA in a 20 µl reaction mixture containing 2.0 µl of 10X PCR buffer (50 mMTris-HCl pH 8.8, 50 mMKCl and 1.5 mM $MgCl_2$), 200 µM of each dNTPs, 1 µl of each primer (forward and reverse) and 2 units of Taq DNA polymerase. The primer sequences used for amplification of *hph* gene are: Forward primer (H1 - 5'GATCTCCAATCTGCGGGATC3') and Reverse primer (H3-5'ACTCACCGCGACGTCTGTCG3'). The reactions are carried out in PTC-100 minicycler (MJ Research, USA) with following temperature conditions, pre-incubation period at 94°C for 3 min, leading to 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and synthesis at 72°C for 1 min, followed by extension at 72°C for 5 min. Amplified PCR product (10 µl) was

Table 2. *Agrobacterium* mediated *genetic* transformation of cassava for SLCMV resistance.

Explant	No of explants cocultivated	No of embryos survived in 1st selection	No of embryos survived in 2nd selection	No of embryos germinated	No of embryos regenerated	No of lines hardened	Transformation efficiency (%)
Somatic cotyledons	500	85	24	14	8	-	1.6
Immature leaf lobes	3000	133	106	64	40	2	1.3

subjected to electrophoresis on a 0.8% agarose gel and visualized under UV light. The SLCMV Rep gene in putative transgenic plants were amplified as using the SLCMV Rep gene specific primers with the following temperature conditions, pre-incubation period at 94 °C for 5 min, leading to 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and synthesis at 72°C for 1 min, followed by extension at 72°C for 5 min.

RESULTS

Genetic transformation of cassava variety H-226 for SLCMV resistance

Selection and plant regeneration

Two explants immature leaf lobes and somatic cotyledons were tried for genetic transformation using agrobacterium strain LBA4404 containing the RNAi-SLCMV Rep gene cassette in pSCR2 with *hph* as gene selectable marker. Out of 3000 immature leaf lobes co cultivated, 106 calli survived two rounds of Hygromycin (30 mg/l) selection. The resistant embryos when transferred to somatic embryo maturation medium resulted in germination of 64 embryos and further to regeneration medium resulted in regeneration of 40 lines to give a transformation efficiency of 1.6% (Table 2). Out of 500 somatic cotyledons (derived from primary somatic embryos) cocultivated, 24 survived two rounds of Hygromycin (30 mg/l) selection. The resistant embryos when transferred to somatic embryo maturation medium resulted in germination of 18 embryos. The germinated embryos when transferred to regeneration medium resulted in regeneration of 6 shoots giving a transformation efficiency of 1.2% (Table 2). In total, out of the 48 transgenic shoots developed two shoots developed well and were hardened in greenhouse (Figures 2 and 3).

Molecular analysis of putative transgenic cassava plants by PCR

The transgenic plants integrated with the SLCMV Rep-RNAi constructs were analysed using PCR amplification of the selectable marker genes (hygromycin and kanamycin primers) and Rep gene specific primers. The PCR analysis with *hph* gene specific primers amplified an expected size of 620 bp in all the 8 analyzed putative transgenic lines of cassava variety H-226 (Figure 4B). The Rep gene was amplified by using the Rep reverse and *ocs* terminator reverse primers. As the *ocs* gene specific primer will not amplify the Rep gene of infected plants, hence it may be considered that the amplification from these primer combinations is due to true integration of transgene in the transgenic plants. The PCR analysis of transgenic lines with SLCMV *Rep* gene specific reverse primer and *Ocs* terminator reverse primer amplified an expected size of 540 bp in all the 8 analyzed transgenic lines (Figure 4A). The putative transgenic plants transformed with SLCMV specific gene silencing construct are showing normal growth and development at *in vitro* conditions. Further assays on the transgenic plants are under progress.

DISCUSSION

Cassava (*Manihot esculenta)* is a staple food for 600 million people in the tropical and subtropical belt, as well as a feedstock for numerous industrial applications, including food, feed and starch. Cassava production in India is seriously hampered by the occurrence of two strains of cassava mosaic virus, ICMV and SLCMV leading to a serious decline of the crop and drastic yield reduction (Reference?). Screening the cassava germplasm for natural resistance and conventional breeding were some of the initial attempt to obtain CMD resistance. However, most of the popular elite cultivars grown in India are either susceptible (includes H-226, SreeHarsha) or moderately tolerant (includes H-165, Co-1, Co-2 and MVD2) to CMG. The high heterozygosity and inbreeding depression complicates conventional breeding in cassava. However, to enhance the efficiency of cassava breeding a number of resources and molecular tools have been developed during the recent years. This include the construction of genetic maps using RFLP, isoenzymes, microsatellite markers (Fregene et al., 1997; Mba et al., 2001) that have already allowed the identification of a variety of QTLs and a major gene (CMD2) for CMD resistance (Jorge et al., 2000, 2001; Akano et al., 2002; Okogbenin and Fregene, 2002). However, such markers are limited in their application to

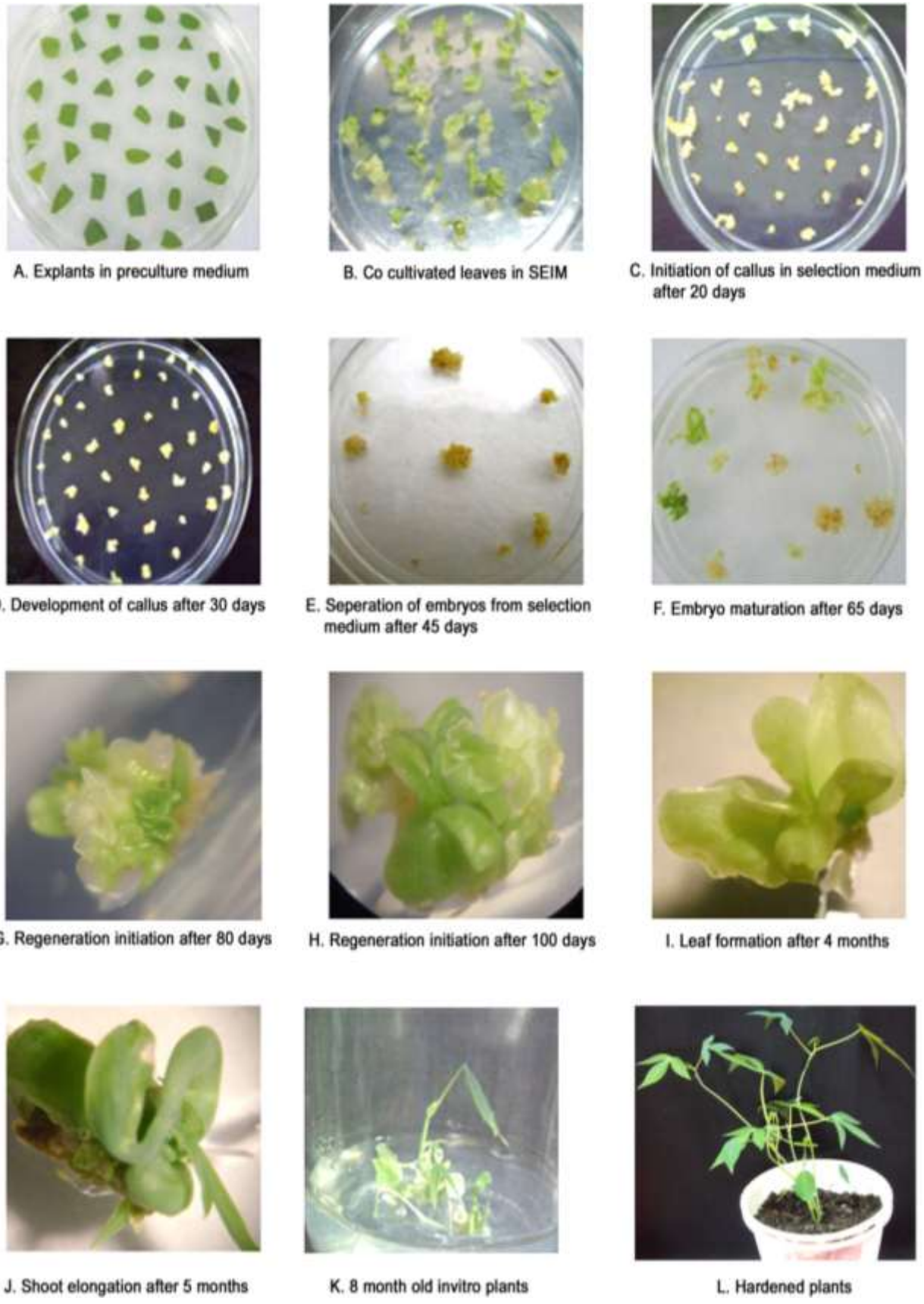

Figure 2. Agrobacterium mediated transformation of cassava immature leaflobes.

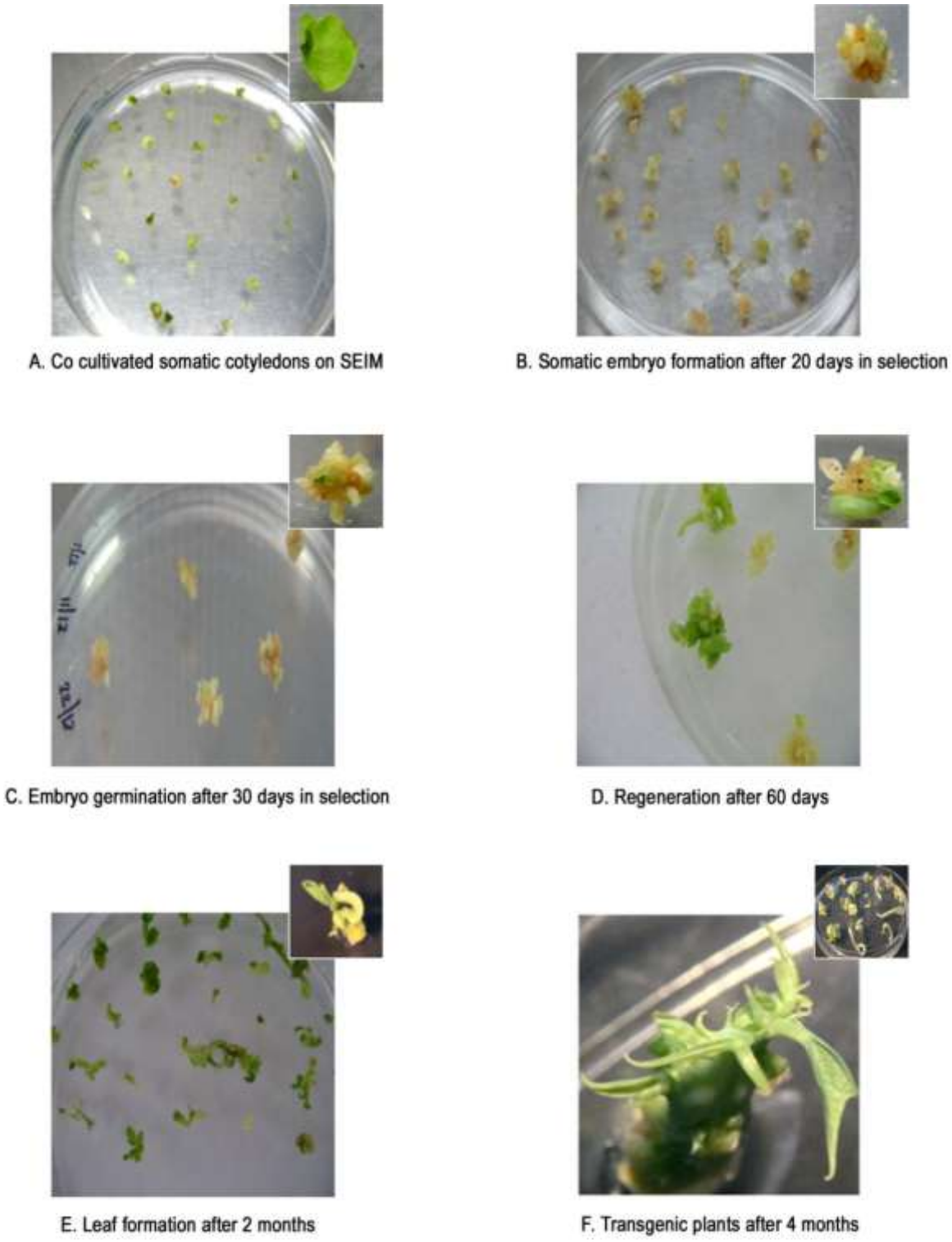

Figure 3. Agrobacterium mediated transformation of somatic cotyledons.

breeding, and a more precise approach to gene mapping using candidate genes is required.

The number of identified and studied Geminivirus related R genes are very less and these genes are yet to be cloned and used for cassava genetic improvement. The lack of availability of natural resistant genes in the germplasm makes the pathogen derived resistance as one of the useful method for achieving viral resistance in

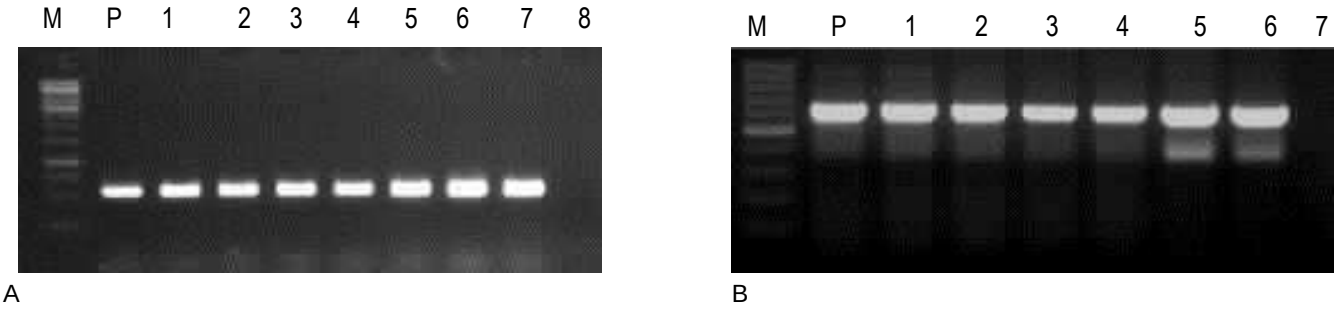

Figure 4. PCR analysis of putative transgenic lines of Cassava transformed with pSCR2 construct. **A.** Amplification of 540 bp SLCMV Rep gene from putative transgenic lines. M, Marker. P, Positive control. Lane 1 to 7, Transgenic plants with pSCR2 construct. Lane 8 –Untransformed cassava. B. Amplification of 640 bp hph plant selectable marker gene from putative transgenic lines. M, Marker. P, positive control . Lane 1 to 6, transgenic plants with pSCR2 construct; Lane 7, untransformed cassava.

cassava. Currently, there are two basic molecular mechanisms by which PDR is thought to operate; protein mediated resistance in which the expression of an unmodified or modified viral gene product (includes genes for coat protein, movement protein and replicase protein) interferes with the viral infection cycle and secondly, RNA mediated resistance, which does not involve the expression of a protein product. The RNA based resistance mainly includes antisense RNA technology and RNA interference. RNA interference (in plants Post Transcriptional Gene Silencing) describes one of the powerful innovations which can be directly applied to evolve crops resistant to stress caused by virus (Pooggin et al., 2003). To overcome CMV disease problem, genetic engineering approach provides scope for imparting CMD resistance in Cassava.

Agrobacterium-mediated transformations of cassava with developed RNAi constructs were done using the immature leaf lobes and green somatic cotyledon explants. SLCMV specific RNAi-Rep gene cassette was cloned into two binary vectors, one containing the kanamycin resistant gene (*nptII*) and other containing the hygromycin resistant gene *(hph*). Initially one of the vectors was used for cocultivation studies. Even though the explant somatic cotyledons were able to survive the Agrobacterium infection those transgenic plants failed during greenhouse hardening. In case of Immature leaf lobes, 50% of the explant turned yellow and dried after Agrobacterium infection, whereas, more than 90% of the cotyledonous explants showed normal growth after infection. The transformation efficiency was more in the case of somatic cotyledons but transgenic recovery were poor. The use of immature leaf lobes for transformation is a laborious process hence ways are under progress to develop friable embryogenic calli which can be preserved for long purpose.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Determining water content and other impurities in *Siparuna guianensis* Aublet essential oil using differential scanning calorimetry

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Siparuna guianensis **Aublet is a predominant species in the Brazilian Cerrado. Some studies found that its essential oil has properties that could be useful for manufacturing new products. Its quality depends on physicochemical properties, since its degradation, as well as water content and other volatile materials may cause several changes in its features. The differential scanning calorimetry (DSC) was used to determine the water content in the *S. guianensis* essential oil in order to evaluate its thermal parameters. The method is based on the hypothesis that the desolvation enthalpy (ΔH_{desol}) needed for removing n water moles from the essential oil is approximately the enthalpy needed for melting the same n water moles ($n\Delta H_f$). Thus, the current study considers the dissociation enthalpy (ΔH_{diss}) to be negligible. The total number of moles was calculated from the molecular mass value of the main essential oil components mentioned in the literature. The DSC curve in nitrogen atmosphere indicated the melting temperature of 1.6°C (ΔH=18.95 Jg^{-1}). Based on the dehydration enthalpies demonstrated in the DSC curve, it was possible to infer that the calculations on the number of water moles and other constant volatile materials found in the *S. guianensis* essential oil were compatible with Karl Fischer Titration (KFT) method. This method is commonly used to determine the water content in substances.**

Key words: *Siparuna guianensis,* differential scanning calorimetry, thermogravimetric analyses.

INTRODUCTION

The physicochemical properties of the essential oils are crucial in the development of new products. Featuring the active ingredient may optimize quality parameters, ensure effectiveness and decrease the presence of water, impurities and/or degradation compounds. They may lead to undesired effects on the oil's composition. Water is a polar molecule and it may strongly interact with ions or polar molecules of ion-dipole or dipole-dipole interactions, respectively. Since the hydrogen bonding energy between water molecules in solid state does not significantly differ from the bonding energy of hydrogen molecules of water in liquid state water molecules grouping may occur on the ice surface even before a complete monolayer is formed (Brown, 2005). Thermal

analysis refers to a group of techniques in which a property of the sample is measured against time or temperature, whereas the temperature of the sample, in a specified atmosphere, is heated or cooled at fixed rate of temperature change or hold at constant temperature (Giron, 2002). The differential scanning calorimetry (DSC) and the thermogravimetric analysis (TG) are the most used analytical techniques in the development of different studies on a wide variety of pharmaceutical materials (Souza et al., 2012). Thermal analysis methods, especially DSC and TG, have been used to feature the study, development and quality control of medications in general (Shamsipur, 2013).

This work applied these techniques to feature moisture contents in the study on the essential oil from *Siparuna guianensis*. It is a plant widely used in the Northern and Northeastern Brazilian regions due to its ethnobotanical properties such as antipyretic and decongestant and to its importance to healthcare. DSC and TG techniques are mainly applied to feature the molecules used in the development of bioproducts (Shen et al., 2010). Therefore, determining moisture content and testing the stability and decomposition kinetics of some valuable parameters regarding the thermal behavior of drugs and medication compounds, such as decomposition rates, possible mechanisms, and values of some thermokinetic and thermodynamic parameters, became necessary (Santos, 2004; Dantas, 2006).

Using DSC to determine essential oil moisture has some advantages in comparison to other methods, such as the relatively short analysis time and the use of small amounts of sample (Sandor, 2005). Thus, the DSC technique allows detecting a fraction in the total amount of a given substance, such as water molecules. It also allows quantifying the components from other volatile materials according to the dehydration and vaporization enthalpies (Khankari et al., 1992). Therefore, the current study aimed to determine the thermal featuring parameters and the number of water moles per mole in the *S. guianensis* essential oil.

Calculation methods

The hydrodistillation method used to extract essential oil is able to modify the water content in the samples. Terpenic and sesquiterpenic compounds easily oxidize under the influence of air, light and humidity due to their unsaturated character (Ganlim et al., 2002). Thus, it is necessary to accurately measure the water content in the tested samples before analyzing the pure essential oils. It is done in order to obtain conclusive results. The current study suggests using an oil dehydration process similar to the hydrate desolvation performed by Khankari et al. (1992). In that case, the endothermic desolvation included steps that begin with the breaking up of solvate bonds up to solvent vaporization. The crystal structure reorganizes itself by breaking up and forming intermolecular forces after the desolvation. The structural rearrangement is part of the process (Chadha et al., 2012). According to the current study, the dehydration process is given by:

$$D_{oil}.n\,A_{solid} \longrightarrow D_{oil} + n A_{liquid}\ \Delta H_{desol} \quad (1)$$

ΔH_{desol} corresponds to the enthalpy of desolvation (J / g hydrate) obtained from the area in DSC. The whole process can be divided into the following steps:

$$.n\,A_{solid} \longrightarrow D_{oil} + nA_{solid} \quad \Delta H_{diss} \quad (2)$$

$$A_{solid} \longrightarrow A_{liquid} \quad \Delta H_f \quad (3)$$

With

$$\Delta H_{desol} = \Delta H_{diss} + n\Delta H_f \quad (4)$$

ΔH_{diss} is the water/oil dissociation heat . ΔH_f is the water fusion heat after its dissociation, and is similar to the vaporization hypothesis by Khankari et al. (1992), the current study considers ΔH_{diss} negligible.

$$\Delta H_{diss} \approx 0 \quad (5)$$

which results in

$$\Delta H_{desolv} \approx n\Delta H_f \quad (6)$$

The equation does not apply to the desolvation process in which the ΔH_{diss} dissociation heat is not negligible. The calculation performed to determine the number of water moles (n) per mole of anhydride is based on the premise that if one does not know the hydrate molecular weight value, it is necessary to determine the enthalpy change per mass unit (Δh_f) instead of per

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Abbreviations: DSC, Differential scanning calorimetry; **KFT,** Karl Fischer titration; **TG,** thermogravimetric analysis.

mole (ΔH_f)

$$\Delta H_{desolv} = \Delta h_{desolv}.(M_s + nM_W) \quad 7$$

$$\Delta H_f = \Delta h_f.M_W \quad 8$$

Δh_f is the melting enthalpy of water (333 J/g, Stark and Wallace, 1976); M_s is the average molecular weight of the major components of *S. guianensis* essential oil identified by the CG-MS (120.77±12 $gmol^{-1}$); and M_w is the molecular weight of water (18.016 $gmol^{-1}$). Equation 9 was formed by grouping Equations 5, 6 and 7:

$$n = \frac{\Delta h_d M_s}{(\Delta h_l - \Delta h_d)M_W} \quad 9$$

The stoichiometric number, n, represents the number of water moles per oil mole.

MATERIALS AND METHODS

Oil extraction

The harvesting of *S. guianensis* Aublet leaves was performed in a Cerrado fragment in Formoso do Araguaia, in Northern Brazil, Latitude: 11° 47' 48" S Longitude: 49° 31' 44" W. The botanical material exsiccates were deposited at Universidade Federal do Tocantins - Campus Porto Nacional (register 10.298). The samples were ground, and their mass was analyzed (25 ± 5 g) and stored at 4°C for later use. Subsequently, they were placed in a volumetric flask containing 500 ml distilled water in each extraction. The adjusted methodology defined the ideal time to extract the essential oil in 80 min distillation (after the first sign of boiling). The essential oil was extracted according to the steam distillation method - using Clevenger type apparatus - and it was expressed based on its fresh mass (Sandor, 2005). All experiments were performed in triplicate and standard deviations were calculated.

Differential scanning calorimetry (DSC)

A 25.78 mg essential oil sample extracted from *S. guianensis* leaves was subjected to DSC analysis using Netzsch apparatus, model STA 449 F3 Jupiter, in N_2 atmosphere at the flow of 50 ml / min under temperature ranging from -30 to 40°C, with heating rate $\beta = 10\ °C/min$. The sample mass (%) and temperature (°C) were measured at 1.2 s intervals for 36 min.

Simultaneous thermal analysis (DSC-TG)

Essential oil samples of approximately 32.3 mg extracted from *S. guianensis* leaves were subjected to thermal analysis and the thermogravimetric curves (TG) were obtained using Netzsch apparatus, model STA 449 F3 Jupiter, in N_2 atmosphere, at the flow of 50 ml / min under temperature ranging from 39.15 to 400°C, with heating rate β = 2, 3 and 5°C/min. The sample mass (%) and temperature (°C) were measured at 1.2 s intervals for 36 min.

Analysis in Karl Fischer titrator

Karl Fischer method

The water content of the essential oil was determined according to Karl Fischer method in order to compare the results; Equation (10):

$$\%H_2O = (100\ E.V) / m \quad 10$$

Where E = Water equivalent to the Karl Fischer reagent (g/mL); V = volume of Karl Fischer's solution spent in the oil sample titration (mL); and m = Sample weight (g).

The measurements were performed in a Mettler Toledo Karl Fischer titrator, DL31 model. The principle of this method is the titration of a conventional Karl Fischer reagent in an anhydrous methanol solvent in the presence of *S. guianensis* essential oil. The same procedure was carried out in triplicate. Regarding this titration test, the oil samples were measured on an analytical scale, using specific glazing. The values were expressed as % of humidity and converted into moles.

RESULTS AND DISCUSSION

Thermoanalytical featuring of the *S. guianensis* essential oil

Figure 1 shows the DSC curve. As it can be seen, the mass loss, due to water sublimation in *S. guianensis* essential oil, happened at the temperature interval from -3.1 to 5.9°C, with maximum peak at 1.9°C, thus totaling the area of 18.95 jg^{-1}. Many studies use these methodologies as alternatives in the quality control of pharmaceutical materials (Atkins, 2002; ASTM, 2003; Sandor, 2005).

Using DSC to determine the water content and other impurities in the *S. guianensis* essential oil

The non-linearity theory explains the determination of water content and other impurities by means of DSC. In other words, the area where the event normally starts is derived from processes such as the vaporization, oxidation or the decomposition of the oil itself as well as the impurities themselves. The endothermic transition of approximately 1.6°C may be evidence that the *S. guianensis* essential oil presents water of hydration in its composition. The stoichiometry of water molecules may be calculated at each stage by using the vaporization hypothesis. The results are listed in Table 1. As for the analysis in the Karl Fischer titrator, the essential oil was titrated in triplicate and presented mean moisture of 0.352 ± 0.005 water moles per anhydrous mole. The presence of moisture in oils may negatively influence the transesterification process (for the production of bioproducts) by disabling the basic catalysts and releasing water molecules, thus reducing yield (Simões et al., 2004). Table 2 shows the values obtained during the analyses of samples with mass almost identical to that of

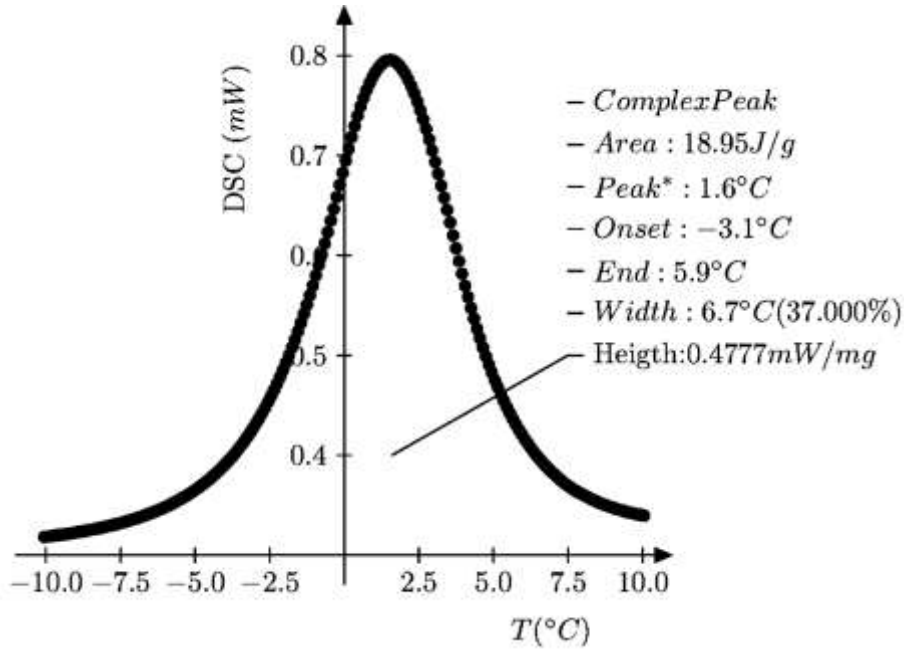

Figure 1. DSC curves of *S. guianensis* essential oil obtained in N_2 atmosphere (50 mL min^{-1}) at 10 °C min^{-1}.

Table 1. Water distribution between both endothermic dissolutions of *S. guianensis* essential oil.

Peak temperature (°C)	ΔH of the desolvation endotherm (J/g)	Water in the energy state determined by using DSC in the vaporization hypothesis, Eq. 9 (moles/mole of anhydrous ± EP)	Water moles from Karl Fischer titration (KFT± EP)
1.6	18.95	0.404 ± 0.04	
Total water moles		0.404 ± 0.04	0.352 ± 0.005

Table 2. Measurements and parameters used from DTA curves.

β (C min^{-1})	T_M (K)	1/T (K^{-1})	ln ($β/T^2_M$)
2	367.64	0.002720	-11.1211
3	375.15	0.002666	-10.7560
5	390.47	0.002561	-10.3253

the *S. guianensis* essential oil, at three different heating rates (β): 2, 3 and 5°C min^{-1} (Figure 2). The values mentioned in the T_M column are, in this case, those read in the respective maximum peaks of DTA curves, as they were determined by the method developed by Kissinger (Kissinger, 1957). Figure 3 shows the values presented in graphic form, with the linear regression, the straight-line equation and the correlation coefficient R^2. The straight-line equation (Equation 11) is

$$Y=-4885.5x + 2.207 \quad (11)$$

The Activation Energy value E_a, may be calculated using parameters such as angular coefficient of 4885.5 and gas constant of 8.314 Kj mol^{-1},

$$E_a = -(-4885.5 \times 8.314)/1000 = 40.61 \text{ kJ mol}^{-1} \quad (12)$$

The E_a calculated for the *S. guianensis* essential oil was 40.61 kJ mol^{-1}. From the linear coefficient (Equations 13-16):

$$2.207 = \ln (AR/E_a) \quad (13)$$

$$e^{2.207} = A.8.314/40.61 \times 10^3 \quad (14)$$

$$A = e^{2.207} \times 40.61 \times 10^3/8.314 \quad (15)$$

$$A = 4.44\ 10^4 \text{ min}^{-1} \quad (16)$$

Therefore, based on the data analysis by DTG, it is

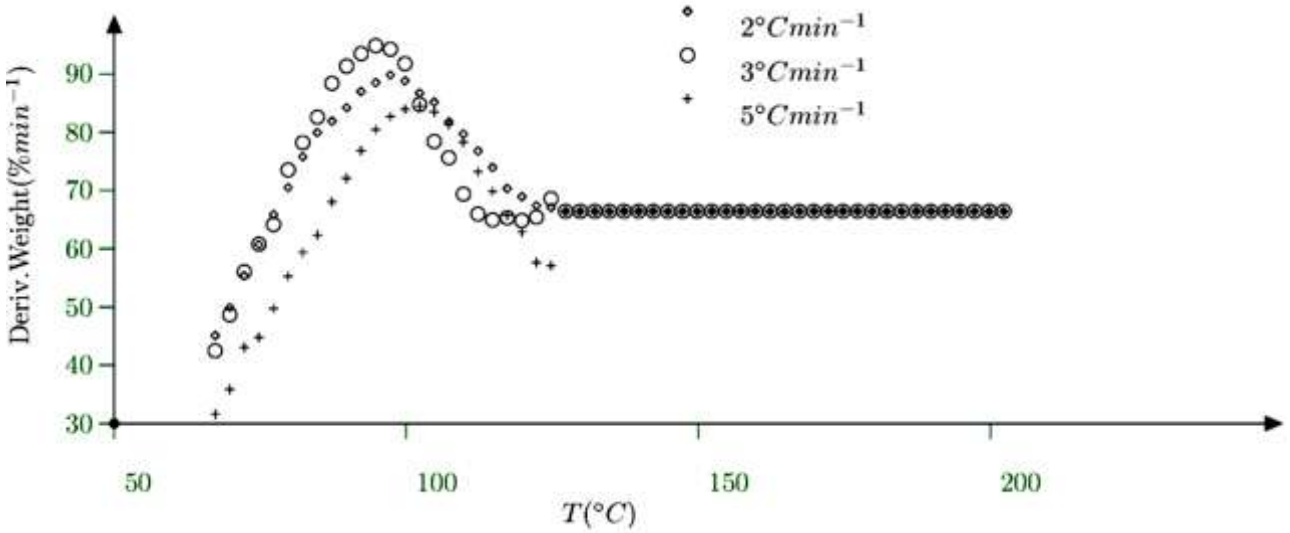

Figure 2. DTG curves obtained from the analyses of 25.78 mg essential oil, at different heating rates.

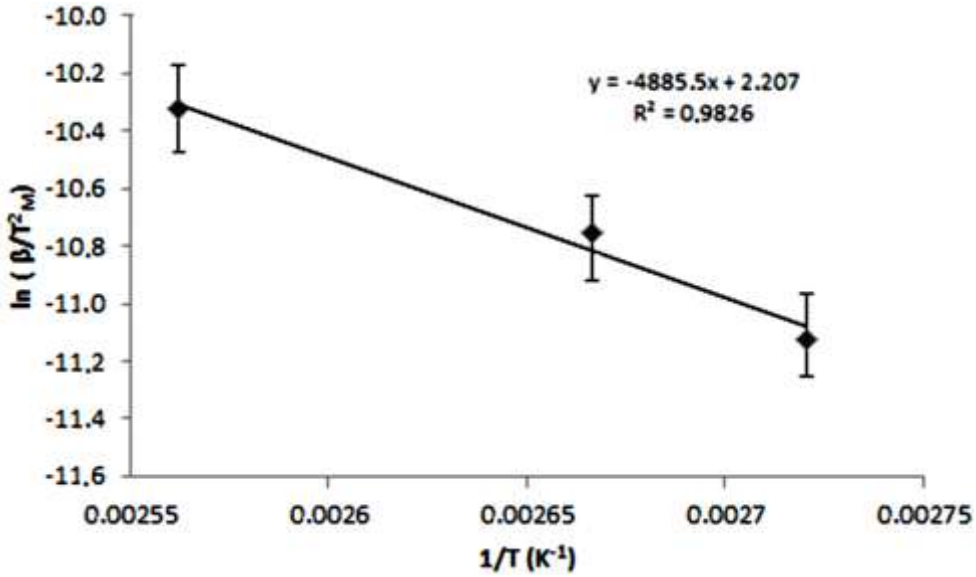

Figure 3. Determination of kinetic parameters according to Kissinger's method, using DTA data.

possible to highlight that the Arrhenius Equation (Equation 17) for the overall conversion of the *S. guianensis* essential oil can be described by:

$$K(T) = A\, e^{\frac{-E_a}{RT}} \quad (17)$$

$$K = 4.44 \times 10^4 e^{\frac{-40.61KJ}{RT}} \quad (18)$$

Equation 17 adjusts itself to the experimental data at temperature ranging from 35 to 400°C. It highlights the hypothesis that melting is the prevalent phenomenon, although there are parallel ones, since the activation energy was relatively low (Martins, 2008).

Kinetic study of *S. guianensis* decomposition

The stability (in days) of the *S. guianensis* essential oil was calculated based on Equation (18), using 35°C, (lower limit of the analyzed range). The estimated thermal

stability time of approximately 33 days or 1 month was obtained starting from the analysis date. Guimaraes et al. (2008) used other analysis techniques and found 50% myrcene concentration loss in 120 days. Myrcene is the major component found in *S. guianensis* essential oil.

Conclusion

The thermoanalytical techniques used in the current study provided the parameters of the *S. guianensis* essential oil. They are important to determine water content and other impurities during the quality control of this oil. The DSC found water content similar to that found by Karl Fischer technique. DSC was considered to be a faster and more reliable technique because the oil melting is not followed by material decomposition.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

Genetic diversity and gene flow revealed by microsatellite DNA markers in some accessions of African Plum (*Dacryodes edulis*) in Cameroon

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***Dacryodes edulis* is a multipurpose tree integrated in the cropping system of Central African region still dominated by subsistence agriculture. Some populations grown are wild which can provide information on the domestication process, and could also represent a potential source of gene flow. Leaves samples for DNA extraction were collected from wild forms in Mbakwa supe region and from cultivated forms in Yaounde and Santchou region. Six microsatellites DNA markers were employed in genotyping to analyze population structure and gene flow. Amplification rate was high and genotyping revealed high level of genetic variation. The overall polymorphic level at the six loci was also high with average expected heterozygosity of 0.53; polymorphism of 0.46; mean allelic diversity of 0.5 and mean allele number of 8.33. There were no clear differences with only 1% variation among the three populations and 6% variation among individuals within populations. In contrast, the rate of heterozygosis was high in all the three populations. Both the number of migrant per generation (Nm=20) and the Wright's F-statistics (F_{ST}=0.012) suggest that there was substantial gene flow among the populations. These findings indicate that *D. edulis* possess a great potential of pollen dispersal and dominant cross-pollination within populations. Most of the loci with private alleles (45%) were found in wild individuals which could be a source of pollen for crossing their cultivated relatives.**

Key words: *Dacryodes edulis*, domestication, genetic diversity and structure.

INTRODUCTION

The knowledge of trees diversity is very important for the planning of conservation programs. Genetic diversity is fundamental for the evolution of species and it made the plants to be adapted to environment changes since

thousand years. Fire, forest destruction and factors like exploitation, affect continuously agro forestry genetic resources (Achard et al., 2002). Most of them are still under-utilised, meanwhile sustainability use of genetic diversity of agroforest contributes to the achievement of the new challenges and the promotion of economic, social and cultural values as well as services and environmental incomes (FAO, 2010). Unfortunately, advances in the fields of genomics and molecular genetics of agroforestry's species are not remarkable although biochemical and DNA markers are developed for an increasing number of tropical species (Baird et al., 1996; Mhameed et al., 1997; Ude et al., 2006). These tools increases knowledge on forests tree and allows for better plan of the domestication process.

The characterisation of many groups of agroforestry plants is now well established. Among them, is *Dacryodes edulis* (Don.), a multipurpose tree known for its potential dietary uses and economic properties with a great potential of industrial applications. The plant is a source of edible oils and nutritious fruits (Vivien and Faure, 1996; Ajibesin, 2011). It has been classified as priority species for domestication by the International Centre for Research in Agroforestry (ICRAF) (Tchoundjeu et al., 2002). In central Africa, cultivated forms are integrated into the cropping systems in a region still dominated by subsistence agriculture and is the centre of diversity for the species (Mbeuyo et al., 2013). The ecological and cultural conditions under which traditional agriculture is carried out have helped to preserve large amounts of their diversity (Kengue, 2002). The cultivated forms consist of heterogeneous genetic material, resulting from empirical selections in different ecological regions (Youmbi et al., 2010). Actually, the selection pressure especially among Cameroonian populations, for the choice of plant material by farmers is high and may leads to the erosion of the genetic base of the species. Some populations grown are wild but the species has evolved very little over time (Okafor, 1983; Kengue, 2002). The species is essentially allogamous; pollination is entomophilous and achieved by insects (Fohouo et al. 2002). The pollen can be spread over a long distance (Kengue, 1990). The wild genetic materials could have many important influences on their cultivated relatives, and store great amounts of genetic variation which may be of interest for future crop improvement programs (Kengue, 2002). In addition, they can provide information on the domestication process, and could also represent a potential source of gene flow with their domesticated materials. Thus there is need to enhance genetic richness of this important crop. This will help in identification, germplasm collection, improvement and conservation. The aim of this study was to assess genetic diversity, population structure and gene flow of *D. edulis*.

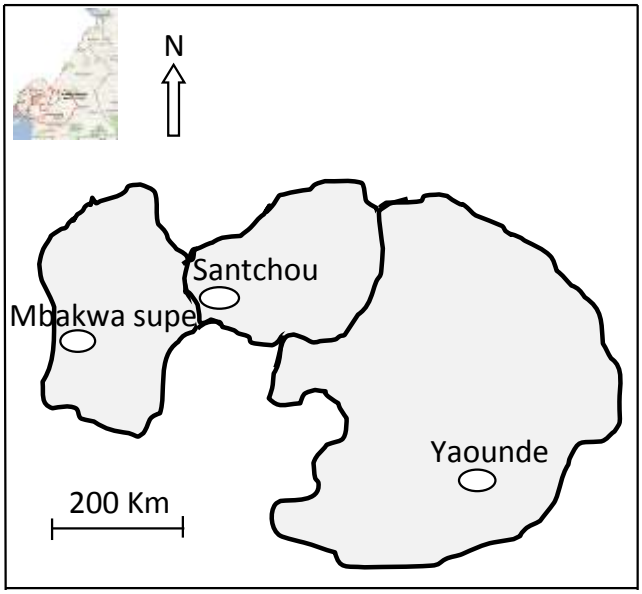

Figure 1. Location sites of the 3 *Dacryodes edulis* populations in Cameroon.

MATERIALS AND METHODS

Study area and sample collection

The plant material was collected in three localities: Yaounde (Latitude 11° 31 ′ 12 ″ N, Longitude 3° 52 ′ 12 ″ E) in the central region of Cameroon, approximately 813 m a.s.l; Santchou (Latitude 4° 37' N, Longitude 9° 50' E, rainfall 1500 mm, altitude 320 m) in the western region of Cameroon and Mbakwa Supe (Latitude 5° 1' 59" N, Longitude 9° 25' 0" E, altitude 425 m) in the south west region of Cameroon (Figure 1). The choice of localities was based on preliminaries survey to know the origin and estimated age of cultivated material and be sure that cultivated populations were not established from the natural stand sampled. Santchou and Yaounde are one of the most important regions respectively for production and commercialization of *D. edulis*. In these localities, populations of *D. edulis* are integrated into cropping systems in gardens among food crops, in cocoa based agroforests and in fallow (Mbeuyo et al., 2013).

Three populations consisting of wild forms at Mbakwa supe and cultivated forms at Yaounde and Sancthou were assessed. 25 samples' leaves were collected in duplicate per population for a total of 75 samples. The plant material was dried in silica gel and kept for short time in container until DNA extraction.

DNA extraction and microsatellite loci

The total genomic DNA was extracted from dried leaves with a modified Saghai-Maroof et al. (1984) protocol. Approximately 0.4 g of tissue was macerated in 15 mL tubes in a tabletop vortexer.

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Table 1. General information for the six microsatellite loci applied in *D. edulis*.

Loci	ASR	GN	AN	GD	He	PIC	IC
CB09	0.45	25	12	0.73	0.73	0.71	0.0042
CC01	0.78	7	5	0.37	0.39	0.35	-0.0511
CE09	0.39	31	14	0.79	0.92	0.78	-0.1474
CG11	0.77	6	5	0.37	0.44	0.34	-0.1708
LB12	0.92	9	7	0.14	0.11	0.15	0.2782
LD06	0.49	10	7	0.56	0.55	0.47	0.0388
Mean	0.63	14.67	8.33	0.50	0.53	0.46	-0.0448

ASR: Allele size range, GN: genotype number, AN: allele number, GD: genetic diversity, He: expected heterozygosity, PIC: polymorphism index content and IC: inbreeding coefficient.

Tissue was then incubated at 65°C in extraction buffer (2% CTAB; 100 mM Tris-HCl pH 7.5; 700 mM NaCl; 50 mM EDTA pH 8.0; 2% PVP and 140 mM β-mercaptoethanol just prior to use). Approximately 50 ng of genomic DNA was used in PCR reactions under standard conditions.

Six microsatellites loci (CB09, CC01, CE09, CG11, LB12 and LD06) previously developed by Benoit et al. (2011) were employed. The allele size ranged from 125 to 375 and the repeats motifs concerned were guanine-adenine (GA) and cytonine-thymine (CT). PCR was carried out in two thermocyclers (Ependort and Corbet) in 0.2 mL individual tube and 96 well plates. It started with an initial step of 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 1 min at 57°C and 30 s at 72°C. Final elongation was carried out at 72°C for 20 min. The products were separated by electrophoresis in 1.8% agarose gels with a running time of 40 min at 100 V in 0.5X TBE buffer. A 50 bp DNA ladder (Invitrogen) was used as molecular size marker. Gels were stained with Gelred and the DNA bands were visualized under ultraviolet light; digital images were recorded using the software Image Aide, version 3.06.04.r®.

Genotyping by capillary gels

For each genotyping sample, 1 µL of PCR product was diluted with 9 µL mixture of 1 mL deionized Formamide and 15 µL ROX-labeled y internal sizing standard with a total reaction volume of 10 µL. The fragments of the DNA were denatured (3 min at 95°C with thermocycler and immediately cooled in ice bath) and size fractioned using capillary electrophoresis on an ABI 3730 automatic DNA sequencer. The GeneMapper software Version 4.1 was applied to size peak patterns, using the internal GS500LIZ 3730 size standard and for allele calling.

Statistical analysis

For each loci site, the number of alleles, expected heterozygot (He), polymorphism index content (PIC) and imbreeding coefficient were calculated using Popgen32 software (Yeh et al., 2000). The genotype frequency within samples was tested for agreement with Hardy-Wemberg expectation by Chi square test to compare observed versus expected outcomes.

Wright's F-statistics (1965) were calculated by analysis of molecular variance (AMOVA) to evaluate the level of population structure. Values F_{ST}, F_{IS} and F_{IT} were estimated through variance among populations; variance among and within individuals.

The rate of inter-population gene flow among all populations was estimate using the procedure based on Wright's F_{ST} , where $F_{ST} = [(1/4N_m) + 1]$ (Wright,1965). This method estimated N_m which is the average number of migrants into a population per generation. The calculation of genotype frequency, Wright's F-statistics and the rate of inter-population gene flow were done by GenAlEx_6.4 (Peakall and Smouse, 2006).

RESULTS

Microsatellite variability and heterozygosity

Level of genetic variation and related parameters at each locus for the three populations are summarized in Table 1. Of the six loci sites used, a total of 50 alleles ranging from 5 to 14 with an average of 8.33 were detected in the populations. Apart from LB12, each locus was revealed to be polymorphic. The expected He ranged from 0.11 to 0.92, with an average of 0.53. High rate of polymorphism was confirmed with calculation of PIC ranging from 0.15 to 0.78, with an average of 0.46.

Population variability and heterozygosity

Allele's frequency for each population is given in Table 2. In terms of individual, Mbakwa supe population was the least polymorphic with an average of 4.83 alleles per locus (Table 2) and unbiased expected heterozygosity (He) of 0.49 (Table 3). The population of Yaounde and Santchou were the most polymorphic respectively with an average of 5.67 and 5.50 alleles per locus and unbiased expected heterozygosity of 0.53 and 0.51.

For 3 populations at 6 loci, 10 out of 18 cases had a single dominant allele with frequency exceeding 0.5 (Table 2): 1 population (Yaounde) at CB09 and the 3 populations (Yaounde, Santchou and Mbakwa supe) at CC01, CG11 and LB12. Otherwise, all the populations had at least 4 alleles at each locus and the frequencies were largely spread out among all alleles. Shanon index confirmed a great level of genetic diversity with the highest values in cultivated forms (Yaounde and Santchou) (Table 3).

Hardy-Weinberg expectation

Genotype frequency was compared at 6 loci with Hardy-

Table 2. Allele frequencies at six microsatellite loci in Cameroon *Dacryodes edulis* populations.

Loci	Allele (bp)	Sample locality and size (n)		
		Mbs (n=18)	Stc (n=21)	Yde (n=23)
	158			0.022
	166		0.024	0.022
	176	0.056	0.024	0.043
	178	0.417	0.333	0.522
	180	0.083	0.071	0.022
CB09	182	0.222	0.167	0.174
	184			
	186	0.056	0.143	0.022
	188	0.083	0.143	0.130
	190	0.028	0.071	0.022
	194	0,056	0.024	0.022
	346	0.056		
	354	0.083	0.048	0.087
CC01	356	0.806	0.738	0.717
	358	0.056	0.214	0.174
	360			0.022
	127	0.056	0.119	0.087
	129	0.111	0.119	0.087
	137		0.024	
	139	0.083	0.071	0.109
	141	0.250	0.095	0.174
	143	0.028	0.048	0.022
CE09	145		0.048	0.022
	151			
	153	0.028		0.065
	155		0.024	
	157	0.056		
	175	0.361	0.452	0.435
	199	0.028		
	175	0.083	0.095	0.370
	177	0.028		
CG11	179	0.028	0.048	0.022
	181	0.861	0.833	0.609
	183		0.024	
	208	1.000	0.905	0.870
	214			0.022
	216			0.022
LB12	218		0.095	0.022
	220			0.022
	222			0.043
	153	0.472	0.429	0.435
	155			0.022
	159	0.472	0.452	0.543
LD06	171		0.024	
	173	0.056	0.048	
	175		0.024	
	177		0.024	
Av. Al		4.83	5.50	5.67

Mbs: Mbakwa supe; Stc: Santchou; Yde: Yaounde. Av al.: Average allele no.

Table 3. Different allelic patterns across populations.

Populations	Mbakwa supe	Santchou	Yaounde
Na	6.00	5.67	5.67
Ne	2.11	2.65	2.67
SI	0.78	1.03	1.05
Np	1.167	0.667	0.833
Nc	0.83	0.67	0.83
He	0.49	0.51	0.53

Na: Number of different alleles, Ne: Number of effective allele, Np: Number of private allele, Nc: Number of common alleles, SI: Shannon's index and He: unbiased heterozygosity.

Weinberg expectation using Chi square test. Considering 18 combinations of the three populations over 6 loci, significant departures ($P < 0.05$) were found in 5 cases, all showing deficiencies in heterozygotes ($P < 0.05$). The distribution of the 5 cases was clustered by 5 loci and all belong to the cultivated populations. Two of these deficiencies were found in Santchou population for CC01 and LB12 loci while 3 of each were found in Yaounde populations for CB09, CE09 and CG11 loci.

Genetic population differentiation and gene flow

Wright's F-statistics expressed by F_{IS} F_{ST} and F_{IT} were used to assess population structure by analysis of molecular variance. The probability (F_{IS}) that two alleles in an individual are identical by descent (relative to the subpopulation from which they are drawn) was equal to 0.058 and showed that there was a high average heterozygotes in each *D. edulis* population. The probability (F_{IT}) that two alleles in an individual are identical by descent (relative to the combined population) was equal to 0.069 and showed that there was a high average heterozygotes in a 3 groups of populations. The last probability (F_{ST}) for which two alleles drawn at random are identical by descent (relative to the combined populations) was equal to 0.012 and showed that the degree of gene differentiation among populations in terms of allele frequencies was low. Otherwise, there were no clear differences with only 1% variation among the three populations, 6% variation among individuals within populations and 93% within individual of the populations. The level of gene flow was estimated by the number of migrant. Both the number of migrant/generation $(N_m = 20)$ and the $(F_{ST} = 0.012)$ values suggest that there was substantial gene flow among the populations.

Private alleles were assessed within individuals of the 3 populations. Private alleles in the context of this study are alleles unique to a single population; all the other alleles totaled were shared between them. These alleles were found in 17 individuals of the three populations (8 for Mbakwa supe, 4 for Santchou and 5 for Yaounde). The total number of loci (9) and loci sites (5) in wild forms with private allele was highest as compared to those of the two cultivated forms which were (10) and (5), respectively.

DISCUSSION

The main areas where microsatellite markers are being applied in forest trees include studies of genetic diversity in natural and breeding populations, particularly in species with low levels of isozyme variation, gene flow, pollen and/or seed dispersal and mating systems. As these parameters are relevant to the conservation of forest genetic resources, microsatellites are being used to monitor genetic impacts of forest management practices and of fragmentation. It is the case of *Eucalyptus sieberie* (Glaubitz et al., 1999) and *Pinus radiate* (Echt et al., 1999). In the present study, the number of alleles was high and the loci sites were polymorphic. The calculation of polymorphism index contain suggest that we can get a reliable results of the population genetic estimate using the six SSRs.

There was not structure in the populations. This cannot be understood without considering that there is no genetic drift occurring in some of its subpopulations. In addition, migration might be uniform throughout the population, or mating is random throughout the population. A population's structure might affect the extent of genetic variation and its patterns of distribution

A high degree of polymorphism was observed within all the groups and particularly in wild forms. In terms of genetic resource, this suggests that wild forms present a significant genetic diversity. In addition, most of the species like *D. edulis* have a greater genetic diversity among wild population in terms of number of alleles (Saghai-Maroof et al., 1984). Both the number of migrant/generation ($N_m=20$) and the $F_{ST}=0.012$ values suggest that there was substantial gene flow among the populations. Our very low F_{ST} estimates are lower than the average reported for outcrossing seeds plants (Hamrick, 1989; Vigouroux et al., 2008). These results can be explained by great potential of pollen dispersal and dominant cross-pollination within populations. In addition, in a separate study regarding growing of *D. edulis* under different cropping conditions in Makenene region of Cameroon, it was found that the majority of *D. edulis* plants (88%) are planted from seeds derived from adult individuals, while 12% are from conventional breeding techniques (aerial layering). The seeds used are selected from trees bearing large, nonacid fruits that are rich in oils (Mbeuyo et al., 2013). Meanwhile, gene flow between cultivated plants and their wild relatives may have increased likelihood of rapid decrease of wild relatives. In fact, during this surveys, populations in the field reported their decreasing and coexistence with

cultivated form in certain regions. However, studies that examine the extent of introgression between wild and cultivated populations are necessary.

There was no significant difference ($p < 0.01$) between expected and observed genotype frequency in wild's population. For these reasons, Hardy-Wemberg equilibrium was respected, assuming no unbalanced external factor is affecting wild's population. In contrast, 05 cases of deficiencies in heterozygotes were mentioned in cultivated forms. In the domestication process of *D. edulis*, farmers grow plants requiring only agronomic and food traits (Kengue, 2002). These cultural practices increased the selection pressure for the choice of planting material by farmers and might progressively lead to the erosion of the genetic base of the species from where deficiencies in heterozygotes is observed in some cultivated individuals.

It is noted from the concluding remarks that there was no difference in the three population's structure. In contrast, variability and heterozygosis were very high within the populations. There could be substantial gene flow among the populations and the greatest number of private alleles was found in wild's individuals. For breeding programs, self-pollinations of selected individuals followed by crossing should be useful to produce hybrids of economic and agronomic interest such as size, taste and oil content of fruits. Some wild individuals could be a source of pollen for crossing their cultivated relatives.

Conflict of interests

The authors have not declared any conflict of interests.

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Abbreviations

EDTA, Ethylenediaminetetraacetic acid; **PVP,** polyvinylpyrrolidone; **DNA,** deoxyribonucleic acid; **PCR,** polymerase chain reaction; **He,** expected heterozygoty; **PIC,** polymorphism index content.

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Full Length Research Paper

Estimate of genetic diversity in cassutinga (*Croton heliotropiifolius*) based on molecular markers

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The *Croton heliotropiifolius* (Euphorbiaceae family) is a shrubby plant that has attracted attention both by the need for conservation as for their pharmacological potential. The present study aimed to characterize the diversity and structure genetic of a population of *C. heliotropiifolius* present in a forest fragment in the city of Itapetinga, Bahia. Young leaves were collected from 41 individuals after DNA extraction were genotyped with 18 RAPD primers and 15 ISSR primers. Frequentist and Bayesian statistical methods were used to estimate the diversity and genetic structure, being observed a total of 164 polymorphic markers (mean 4 and 4.3 bands obtained with the use of RAPD and ISSR primers, respectively). Genetic diversity ranging between 0.12 and 0.48 and Bayesian method indicated by the existence of three probable gene pools (K = 3). Indications of association between spatial distribution of plants and the genetic structure were also observed, being likely that the dynamics of the seed carriage performed by the ants and the pollination by insects are related with the results observed. These results allow the beginning of a discussion about the diversity and genetics patterns of the species, since there are no studies of this nature for *C. heliotropiifolius*.

Key words: Caatinga, conservation genetics, genetic variability, inter simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD).

INTRODUCTION

The genus *Croton* consists of approximately 1300 species that are mostly shrubs and subshrubs (Ziroldo, 2007). Several species of *Croton* spp. are known to have antioxidant and antimicrobial properties in potential, or for being used routinely as phytotherapy by local or regional populations (in Caatinga biome) (Abreu et al., 2001). Among the species of croton that are used as medicinal plants, it is possible to cite the *Croton heliotropiifolius*, a species popularly known in northeastern Brazil as "cassutinga". This species is used to relieve

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stomachaches in general, vomiting and diarrhea, apart from relieve fever (Randau, 2001), and also has important constituents for pharmacological and phytochemical studies, highlighting the presence of alkaloids and reducing sugars (Randau et al., 2004).

Despite its potential use as a medicinal plant and its wide dispersion in the Caatinga biome, genetic studies in *C. heliotropiifolius* are limited to testing methods for DNA extraction (Scaldaferri et al., 2013) and preliminary studies of genetic diversity (Scaldaferri et al., 2014). In contrast, there has been a growth in the number of associated studies with molecular techniques in plants, especially for genetic studies aimed at characterizing the diversity (Cerqueira-Silva et al., 2014). Genetic diversity consists in the existence of different phenotypic characteristics, as well as in molecular differences (enzymes, proteins and DNA sequences), present in individuals of a population (Frankham et al., 2008). In this context, Futuyma (2002) highlights that it is essential to carry out genetic studies to understand the different aspects of a natural population, such as diversity and genetic structure that contribute to an extensive discussion about the ecology of species.

The use of molecular markers in genetic characterization is routine in many laboratories and various molecular markers are available, however, despite the advancement and popularization of many molecular techniques in recent decades, Cerqueira-Silva et al. (2014) argue that the availability of resources and background information are still determining factors in the choice of markers used in genetic researches.

Considering the importance of genetic information, as well as the absence of molecular genetic diversity estimates for *C. heliotropiifolius*, the objective of this study was to characterize using RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeat) markers, the diversity and structure genetic existent among 41 wild genotypes of this species that were collected in a fragment of native forest in Itapetinga city, Bahia, Brazil.

MATERIALS AND METHODS

Sample collection and genomic DNA extraction

The collection was carried out in a small hill in the city of Itapetinga, Bahia, Brazil, located in the southwestern region of the state. The vegetation observed in this area is typical of deciduous and semi-deciduous forest (Radam Brasil, 1981). In total were collected leaves of 41 wild genotypes of *C. heliotropiifolius*, representing three distinct regions along of the small hill (-15.256456 lat, -40.257637 long), as follows: 12 genotypes representative of the top, 16 genotypes representative of the middle region and 13 genotypes representative of the valley of the hill. Sample of this species was identified in the herbarium of the Universidade Estadual de Feira de Santana (UEFS, BA, Brazil) and its excicatas have been properly deposited in this herbarium (under the codes A1-HUEFS189022, A2-HUEFS189023, A3-HUEFS189024, B1-HUEFS189025, B2- HUEFS189026, B3-HUEFS189027, C1-HUEFS189028, C2-HUEFS189029, C3-HUEFS189030, D1-HUEFS189031, D2-HUEFS189032, D3-HUEFS189033).

The genomic DNA was isolated from fresh leaves using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990) with modifications previously tested for *Croton* by Scaldaferri et al. (2013). The quality of the DNA samples was assessed in agarose gel 1% (w/v) by electrophoresis (in a 90 V for 100 min) and visualized with a gel red staining buffer (Invitrogen), according to the manufacturer's specifications. In order to quantify the DNA concentration (ng/µL) we adopted an intact Lambda molecular weight marker as standard (undigested Lambda DNA).

Genotyping of samples

The DNA samples of the 41 genotypes were tested with 20 RAPD primers and 23 primers ISSR, adopting routines used by the research group in assays with the genus *Croton* in the Applied Molecular Genetics Laboratory of UESB (Scaldaferri et al., 2013, 2014). From these primers were selected those with better quality in the patterns of bands and better genetic repeatability.

The amplifications with both types of primers were conducted in a total volume of 15 µL containing 15 ng of the DNA, PCR buffer 1X (20 mM Tris HCl [pH 8.4] and 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 µM of each dNTP, 1 µM primer and 1 U of Taq DNA polymerase (Invitrogen, Carlsbad, California, USA). The amplification program adopted for PCR reactions were: 94°C for 5 min, followed by 34 cycles [94°C for 50 s, 48°C (ISSR reaction) or 34°C (RAPD reaction) for 50 s and 72°C for 1 min], with the final extension at 72°C for 5 min. The amplification products were separated by electrophoresis in 2% agarose gel (w/v) in TBE 1X running buffer at a constant voltage of 120 V for approximately 2 h.

Analysis of molecular data

Analysis was performed in duplicate and only patterns obtained clearly twice were scored. Presence or absence of fragments were recorded as 1 or 0 (respectively), and treated as binary characters. Resulting matrices of molecular data for all primers were subjected to multivariate statistical analyses, such as: estimate on the complement of genetic similarity ($dg_{ij} = 1 - sg_{ij}$; similarity = sg_{ij} and dissimilarity = dg_{ij}) based on the coefficient of Dice (1945), and clustering of genotypes by using the neighbor joining method. The statistical analyses were carried out with the assistance of the Genes software, Windows version (Cruz, 2006) and DarWin software (Perrier and Jacquemoud-Collet, 2006).

Analyses of population structure were performed with the Bayesian method using the STRUCTURE software, version 2.3.4 (Falush et al., 2003; Pritchard et al., 2000). We used an admixture model, and the burn-in period and replication numbers were set to 100,000 and 1,000,000, respectively, for each run. The number of groups (*K*) was systematically varied from 1 to 10, and 10 simulations were performed to estimate each *K*. We used the Δ*K ad hoc* method described by Evanno et al. (2005) and implemented in the online tool Structure Harvester (Earl and Vonholdt, 2012) to estimate the most likely *K* in each set.

After estimating the most likely K, we used the "greedy algorithm" implemented in CLUMPP software, version 1.1.1 (Jakobsson and Rosenberg, 2007) with a random input order and 1000 permutations to align the runs. The results were visualized using DISTRUCT software, version 1.1 (Rosenberg, 2004). Based on the posterior probability of membership (*q*) of a given individual belonging to a given group compared to the total number of groups (*K*), we classified individuals with $q > 0.60$ as a member of a given cluster, whereas for clusters with membership (*q*) values ≤ 0.60, the individuals was classified as admixed.

Table 1. Primers used for obtaining RAPD markers with number total of bands (Nº bands), number of polymorphic bands (Nº PB) and percentage of polymorphism for 41 wild genotypes of *Croton heliotropiifolius*.

Primer*	Sequence (5'-3')	Nº bands	Nº PB	Polymorphism (%)
OPD-01	ACCGCGAAGG	4	4	100
OPD-02	GGAACCCAACC	4	4	100
OPD-03	GTCGCCGTCA	5	4	80
OPD-05	TGAGCGGACA	6	6	100
OPD-08	GTGTGCCCCA	4	4	100
OPD-10	GGTCTACACC	5	5	100
OPD-13	GGGGTGACGA	3	3	100
OPD-15	CATCCGTGCT	4	4	100
OPD-16	AGGGCGTAAG	3	3	100
OPD-18	GAGAGCCAAC	4	4	100
OPD-19	CTGGGGACTT	3	3	100
OPD-20	ACCCGGTCAC	4	4	100
OPE-01	CCCAAGGTCC	5	5	100
OPE-02	GGTGCGGGAA	5	5	100
OPE-03	CCAGATGAC	4	4	100
OPE-04	GTGACATGCC	3	3	100
OPE-05	TCAGGGAGGT	3	2	80
OPE-06	AAGACCCCTC	4	4	100

*Operon Technologies (http://www.operon.com).

RESULTS AND DISCUSSION

There were selected 18 RAPD and 15 ISSR primers that showed the better quality in the patterns of bands and genetic repeatability. Together, these 33 primers generated a total of 137 bands. The amplification reactions carried out with RAPD primers produced a total of 73 bands with an average number of four bands per primer. The extreme values oscillated from three bands (with use of the primers OPD-13, OPD-16, OPD-19, OPE-04 and OPE-05) to six bands (with use of the primer OPD-05) (Table 1). In turn, the amplification reactions carried out with ISSR primers produced a total of 64 bands with an average number of 4.3 bands per primer. The extreme values oscillated from two bands (with use of the primer TriCAC 5CY) to seven bands (with use of the primer DiCA 3G) (Table 2).

Although studies of this nature are still incipient to the genus *Croton,* and inexistent for *C. heliotropiifolius*, similar results were observed by Angelo et al. (2006) in *C. cajucara*. In this study, the authors used 10 RAPD primers and observed 71 bands, of which 66 were polymorphic. To species of others genus the results observed with RAPD and ISSR markers are very variable. As examples are the genetic diversity studies conducted by Ansari et al. (2012) using five ISSR primers to characterize 29 genotypes of *Tectona grandis*, being obtained 43 polymorphic bands and by Souza et al. (2008) from five ISSR primers to characterize 269 genotypes in 12 populations of *Zabrotes subfasciatus*, being obtained a total of 51 polymorphic bands.

The average distance revealed for the wild genotypes of *C. heliotropiifolius* on base in the molecular markers was dg_{ij} = 0.26, and maximum and minimum distances were dg_{ij} = 0.48 (among the genotypes located at the top and in the middle region of the hill) and dg_{ij} = 0.12 (among the genotypes located at the middle region and in the valley of the hill), respectively. This diversity can be considered relatively high, in view that for different accesses of *C. cajucara* the genetic diversity observed ranged from 0.07 to 0.25 (Angelo et al., 2006).

The dendrogram based on neighbor joining method showed the formation of different groups among the genotypes located at the top of the hill and the genotypes located in the valley of the hill (Figure 1). Genotypes collected in the middle region of the hill were grouped both among the genotypes of the top and among the genotypes of the valley of the hill, being possible that middle region of the hill being an convergence point for the genetic flow from the extreme portions of the hill. This hypothesis is supported by greater genetic diversity observed in the middle region (dg_{ij} in middle = 0.28) in relation to diversity found at the extremes of the hill (dg_{ij} in top = 0.24 and dg_{ij} in valley = 0.22).

In the Bayesian estimates (based on Delta K values) was observed the existence of three genetic pools as the most suitable structure for distribution and grouping of the 41 wild genotypes of *C. heliotropiifolius* evaluated (Figure

Table 2. Primers used for obtaining ISSR markers with number total of bands (Nº bands), number of polymorphic bands (Nº PB) and percentage of polymorphism for 41 wild genotypes of *Croton heliotropiifolius*.

Primer*	Sequence (5'-3')	Nº bands	Nº PB	Polymorphism (%)
DiCA 3G	CACACACACACACAG	7	7	100
DiCA 3RG	CACACACACACACARG	5	4	80
DiGA 3C	GAGAGAGAGAGAGAG	3	3	100
DiGA 3RC	GAGAGAGAGAGAGAGARC	4	4	100
DiGA 3T	GAGAGAGAGAGAGAGAT	4	3	80
TriCAC 3RC	CACCACCACCACCACRC	3	3	100
TriCAC 5CY	CACCACCACCACCACCY	2	2	100
TriCAG 3RC	CAGCAGCAGCAGCAGCAGRC	4	3	80
TriTGT 3YC	TGTTGTTGTTGTTGTYC	5	5	100
TriAAR 3RC	AACAACAACAACAACRC	4	4	100
TriAAG 3RC	AAGAAGAAGAAGAAGRC	5	5	100
TriACG 3RC	ACGACGACGACGACGRC	4	3	80
TriAGA 3RC	AGAAGAAGAAGAAGARC	5	5	100
TriCGC 3RC	CGCCGCCGCCGCCGCRC	5	5	100
TriGAC 3RC	GACGACGACGACGACRC	4	4	100

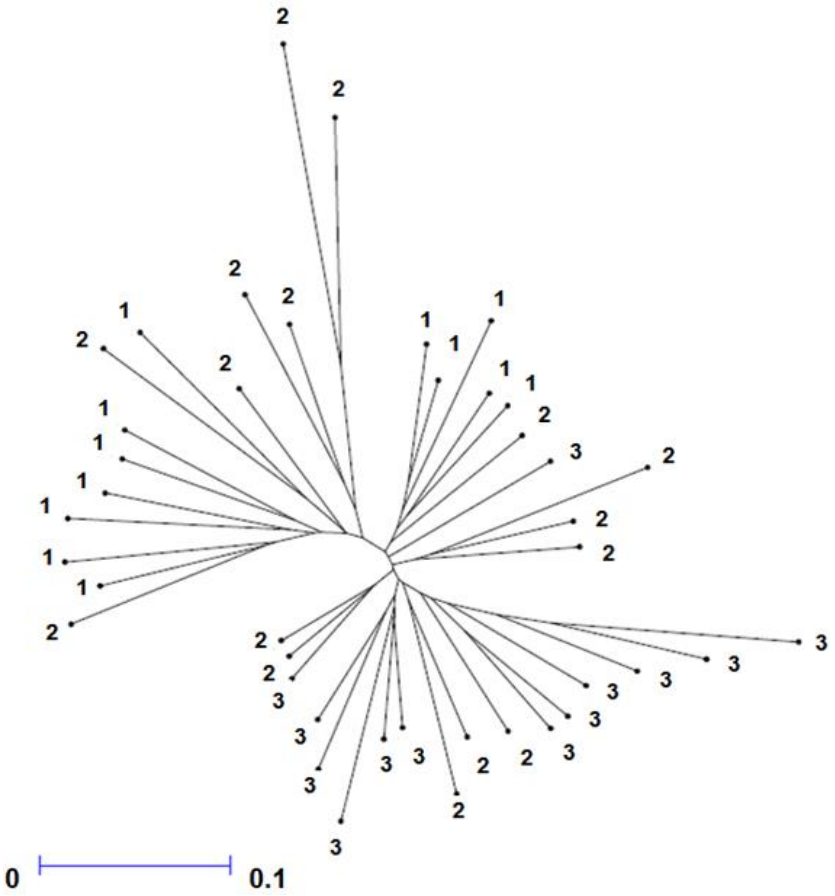

Figure 1. The neighbor-joining tree constructed using the coefficient of Dice (1945), from the genotyping data carried out with RAPD and ISSR markers in a population of 41 wild genotypes of *Croton heliotropiifolius*. The numbers indicate the collection region of each genotype along hill (1 = top, 2 = middle, and 3 = valley of the hill).

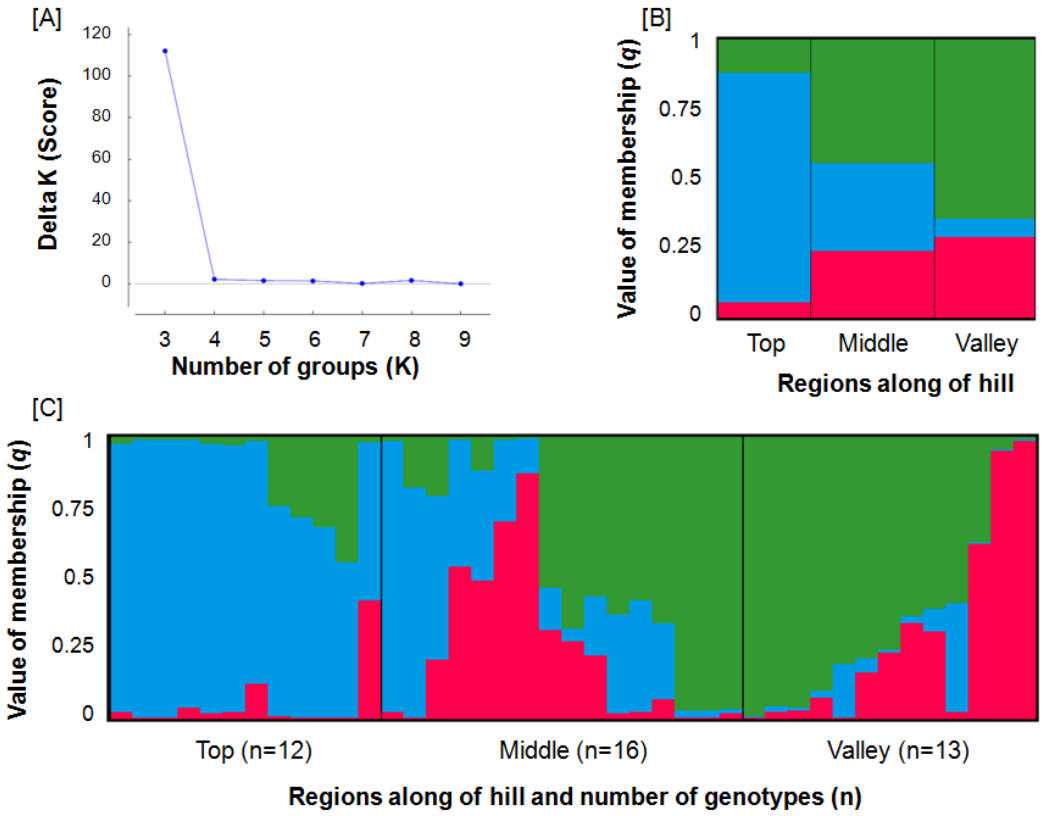

Figure 2. Numbers of genetic pool (clusters) inferred based on Bayesian analyses considering the most probable number of groups (K) estimated with the method described by Evanno et al. (2006) [A], well as histograms of the distribution of gene pools in the three regions of the hill [B] and in each of wild genotypes of *Croton heliotropiifolius* investigated [C]. Each column (histogram) represents the genotyping data (consensus) from each region of the hill [B] or from each wild genotype [C], and the colors used in the histograms represent the most likely ancestry of the cluster from which the accessions were derived.

2A). The histogram (Figure 2B and C) and dendrogram (Figure 1) indicate the existence of genetic structure among genotypes of *C. heliotropiifolius* along of the hill (*Serra da Torre*), corroborate with the hypothesis of occurrence of three genetic pools. At the top, and in the valley of the hill, the gene pools represented by the blue and green colors (respectively) are more predominant than the third gene pool represented by the red color (Figure 2B and C). In turn, in the middle region of the hill was observed a great mixture of the three gene pools, being evidenced in this region.

From the analysis, it can be inferred the occurrence of a barrier that prevent cross between the individuals of the top with those of the lowland region. However, a gene flow between individuals of the extremes with those who are in middle region seems to occur, which may be caused by pollinators and seed dispersors. Therefore, it is important to understand the pollination and seed dispersal mechanisms. In this context, in a study carried out by Leal et al. (2003) it was observed that for most species of the family Euphorbiaceae occurring in Caatinga the seed dispersal is carried out by ants. In this study, it was found that the species *C. campestris* St-Hil, *C. argyophyllus* Kunth., and *C. blanchetianus* Baill., have diasporas with a structure rich in lipidic compounds that have an important role to attract ants. Also, Passos and Ferreira (1996) studied the seed dispersal in *C. priscus* Croizad and found about 11 different species of ants interacting with the seeds of the species.

Study with *C. sellowi*, another caatinga shurb, indicated a total of 19 species of floral visitors, represented mostly by insects, predominantly bees (Pimentel and Castro, 2009). In this context, *C. heliotropiifolius* is important in the diet of bees, as this species visited the flowers very often (Silva et al., 2014). Similar results were found in a study by Dominguez and Bullock (1989) for *C. suberosus*, where it was observed that the insects represent the main species of floral visitors.

Addtionally, *C. heliotropiifolius* has a structure called elaiosome in its seeds that is rich in lipid and acts as an attractive for ants (Leal et al., 2003). Thus, once the plant seeds fall, ants feed on the elaiosome and the seed became exposed, sometimes close to the mother plant, which can contribute to a spatial structure (Bertagna,

2007). For *Mabea fistulifera* that also has elaiosome, ants of the genera *Atta* and *Acromyrmex*, carry the seeds for up to 4 m (Peternelli et al., 2004). This fact confirms the genetic structure observed in this study for the different regions of the hill (*Serra da Torre*).

Conclusion

The population of cassutinga (*C. heliotropiifolius*) considered in this study has its genetic diversity structure in three gene pools that are correlated with the spatial distribution of the species along of the sampled area (Itapetinga, Bahia, Brazil). Although further studies are needed to understand the factors responsible for this structure, it is likely that the dynamics of the seed carriage performed by the ants and the pollination by insects, especially the bees, are directly related.

Conflict of Interests

The authors have not declared any conflict of interests.

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Antioxidant activity, phenolic and flavonoid contents of some wild medicinal plants in southeastern Algeria

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This study reported on five plants known for their use in traditional medicine in southeastern Algeria, namely: *Artemisia campestris, Asteriscus pygmaeus, Pituranthos chlorantus, Pallenis spinosa* and *Aizoon hispanicum.* The work aimed to determine the antioxidant activity, phenol and flavonoid contents of their aqueous and methanolic extracts. Flavonoids and phenol contents varied according to the nature of the extract and the nature of the plant. Total phenols varied from 30.33 ± 2.03 µg EAG/mg of plant in aqueous extract of *Aizoon hispanicum* to 280 ± 5.46 µg EAG/mg plant extract in methanolic extract of *A. campestris*. Flavonoid contents were between 0.071 ± 0.0008 µg QE/mg extract in *A. hispanicum* aqueous extract and 29.68 ± 0.32 µg QE/mg extract in *A. campestris* methanolic extract. The aqueous extracts showed the lowest values of flavonoid contents while the methanol extracts showed the highest ones. The antioxidant activities expressed as IC_{50} values varied from 8.66±1.52 µg/ml for *Artemisia* aqueous extract, the most active to 325.7±5.50 µg/mL of DPPH solution to the less active *Aizoon* aqueous extract. The radical scavenging activity decreased in the following order: *A. campestris > P. spinosa> P. chlorantus > A. pygmaeus > A. hispanicum*.

Key words: Antioxidant, flavonoids, phenols, plants.

INTRODUCTION

Free radicals play a major part in the development of chronic and degenerative ailments such as cancer, autoimmune disorders, rheumatoid arthritis, cataract, aging, cardiovascular, neurodegenerative diseases and diabetes mellitus (Willcox et al., 2004; Pham-Huy et al., 2008). Oxidation process is one of the most important means for producing free radicals in food, drugs and even living systems. Catalase and hydroperoxidase enzymes convert hydrogen peroxide and hydroperoxides to non-radical forms, and function as natural antioxidants in human body. Due to depletion of immune system natural antioxidants in different maladies, consuming antioxidants as free radical scavengers may be necessary (Halliwell, 1994; Kuhnan, 1976; Kumpulainen and Salonen, 1999; Younes, 1981).

Recently, more attention has been given to medicinal plants of therapeutic potentials as antioxidants in reducing free radical induced tissue injury. Many plants have been investigated in the search for novel antioxidants (Bol'shakova et al., 1998; Erdemoglu et al., 2006). The synthetic antioxidants have restriction for use, as they are suspected to be carcinogenic. Therefore, the

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importance of searching for and exploiting natural antioxidants has increased greatly in present years (Mervat et al., 2009).

Medicinal plants have been used for centuries as remedies for human diseases because they contain chemical components of therapeutic value (Nostro et al., 2000). According to the World Health Organization (WHO) in 2008, more than 80% of the world's population relies on traditional medicine for their primary healthcare needs (Pierangeli and Windell, 2009).

The genus Artemisia, widespread over the world, growing wild over the Northern Hemisphere belongs to the Asteraceae family. Eleven species of Artemisia can be found in Algerian flora (Quezel and Santa, 1963; Salido et al., 2004). *Artemisia campestris* L., known in Algeria as “dgouft” grows wild on the steppe and desert (Dob et al., 2005). In Arab folk medicine, *Artemisia campestris* L. has been used as Stomach and liver diseases Hypoglycemic, (Hammiche et al., 2006). cholagogue, choleretic, digestive, depurative, antilithiasic, obesity and cholesterol (Sijelmassi, 1993; Hmamouchi, 1999).

The genre Pituranthos has over twenty species, some of which are specific to North Africa (Quezel and Sanata, 1963; Kaabeche, 1990) and are often encountered in arid or desert regions. The species *Pituranthos chlorantus* in folk medicine is generally used as Fever, diabetes asthma, rheumatism (Hammiche et al., 2006; Vérité et al., 2004).

The genre Pallenis is a typical Mediterranean type, occurrence in the desert and coastal habitats southern Europe, northern Africa, the Canary Islands and the Middle East (Ozenda, 1991; Quezel and Santa, 1963). *Pallenis spinosa* used in folk medicine for treat Eczema, anti Rheumatism, muscular contraction, tire, vomiting for the new one born, diabetes, headaches, disinfecting (Bouabdelli et al., 2012).

The species *Asteriscus pygmaeus* is an annual plant. It is a species recognized Sahara-Sindian but also encountered in the Arabian Desert (Meyers, 1888) According Bellakhdar, the infusion of this plant is mainly used in the Sahara, for calming the stomach pain (Bellakhdar, 1997).

Aizoon hispanicum is a taxonomically isolated species, distributed in southern Mediterranean habitats from SE Spain, N Africa and S Italy to Crete (Pignatti, 1982; Greuter et al., 1984; Gonçalves, 1990). This plant is used in veterinary medicine for stimulate the milk.

The purpose of the present study was to investigate the antioxidant activity, phenol and flavonoid content of some wild plants, representative of different types of soil were collected from different regions of the southeastern algeria (Table 1).

MATERIALS AND METHODS

The aerial parts of *P. chloranthus* Benth and Hook. (Apiaceae), *A. hispanicum* L. (Aizoaceae), *A. campestris* L. (Asteraceae), *P. spinosa* (L.) Cass. (Asteraceae) and *A. pygmaeus* (DC.) Coss. and Dsev. (Asteraceae) were collected just before the flowering period in Barika region (southeastern Algeria) and identified by Dr. Sarri Djamel of M'sila university. Plant materials were dried at room temperature and powdered.

Extracts preparation

Aqueous extracts (AqE)

200 g of each powdered plant were infused in 2 L boiling distilled water set aside for 30 min and filtered. After filtration, extracts were concentrated under vacuum below 40°C and the extracts were freeze-dried.

Methanol extracts (MeE)

20 g of each plant powder was extracted in 200 ml of methanol by maceration (48 h). The solvent was removed under the vacuum at temperature below 40°C.

Determination of total phenol content

Phenolic contents were determined by Folin-Ciocalteau method (Chen et al., 2007); an acquisition of 100 µL of the diluted extract was placed in the presence of 2 ml of a solution of sodium carbonate (2%) and then, the mixture was stirred with a vortex and let to stand for 2 min. Then 100 µL of an aqueous solution of 50% Folin-Ciocalteu (Merck Co. (Germany)) was added. The mixture was stirred again with the vortex and kept at rest in the darkness at room temperature (22-25°C/30 min). Finally, reading the absorbance was performed with a wavelength of 760 nm with a spectrophotometer UV/VIS SHIMADZU 1700. The levels of total phenolic were determined graphically from a standard curve of gallic acid (Merck Co. Germany).) representing the change in absorbance measured under the same conditions as the extracts, according to a range of concentrations of gallic acid in the prepared distilled water. The results are expressed in µg gallic acid equivalent per-mg of plant extract (µg EAG /mg plant extract).

Total flavonoids determination

Aluminum chloride colorimetric method was used for flavonoids determination (Bahorun et al., 1996). Each plant extracts (1 ml of 1:1 $mg.ml^{-1}$) in methanol for the MeE, and in water for AqE were separately mixed with 1 ml of 2% aluminum chloride. They remained at room temperature for 10 min. The absorbance of the reaction mixture was measured at 430 nm with a spectrophotometer (UV/VIS SHIMADZU 1700). The calibration curve was determined by preparing quercetin (Sigma Chemical Co. (St., Louis, USA).) solutions at concentrations of 0-35 µg/ml in methanol. The concentrations of flavonoids in the test samples were calculated from the calibration plot and expressed as µg Quercetin equivalent/mg of extract.

Antioxidant activity

The antioxidant activity was determined on the basis of their scavenging activity of the stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical. DPPH is a stable nitrogen-centered free radical, the color of which changes from violet to yellow upon

Table 1. Ethnobotanical data of the investigated wild vegetal species of the regions of the southeastern Algeria.

Scientific name	Local name	Used parts	Preparation	Ethnomedical use	References
Artemisia campestris (Asteraceae)	Dgouft	Aerial parts, leaves, flowers	Infusion Decoction	Stomach and liver diseases; Hypoglycemic, cholagogue, choleretic, digestive, depurative, antilithiasic, obesity and cholesterol	Hammiche et al. (2006), Sijelmassi (1993), Hmamouchi (1999)
Pituranthos chlorantus (Apiaceae)	Guezzeh	Aerial Parts	Infusion	Fever, diabetes, asthma and rheumatism	Hammiche et al. (2006), Vérité et al. (2004)
Asteriscus pygmaeus (Asteraceae)	Rose of Jericho Nougd	Leaves	Infusion	calming the stomach pain	Bellakhdar (1997)
Pallenis spinosa (Asteraceae)	Nougd	Leaves	Infusion	Eczema, anti-rheumatism, muscular contraction, tire, vomiting for the new one born, diabetes, headaches, disinfecting	Bouabdelli et al. (2012)
Aizoon hispanicum L. *(*Aizoaceae)	Melah	Aerial parts		Leaves and stems are used raw or cooked. Can be used as a spinach substitute. Leaves have an acid flavor; they are thick and succulent with a slightly salty tang. Plant ash yields soda which is used in making soap and glass; Used in veterinary medicine for stimulate the milk	Phillips and Rix, (1995) Facciola (1990)

reduction by either the process of hydrogen- or electron donation (Dehpour et al., 2009). The procedure adopted by Wong et al. (2006) and modified by Akrout et al. (2012) was used. Different concentrations of extracts (0.125 to 5 mg/ml) and standard [ascorbic acid (20 to 100 µg/ml) (Merck Co. (Germany)) were prepared (MeE for methanol extract, and AqE for distilled water extract). 50 µL of each prepared solution were mixed with 2 mL of methanol and DPPH (Sigma Chemical Co. (St., Louis, USA)) solution (4 mg of DPPH in 100 mL of methanol) and kept for 30 min at room temperature and in darkness. Then, the measurement of absorbance at 517 nm was performed after adjusting the zero absorbance with methanol realized by using a spectrophotometer (UV/VIS SHIMADZU 1700).

The antioxidant activity of the standard (ascorbic acid) or extracts was expressed as the concentration of the extract or the standard providing 50% inhibition (IC_{50}). This concentration was determined graphically by plotting the curve showing the percentage inhibition against the extract concentration in µg plant extract/ml of DPPH solution. The percentage of inhibition (I %) was calculated using the following formula:

$$I\ (\%) = 100 \times [(A_0 - A) / A_0]$$

Where A_0 is the absorbance of the control solution and A is the absorbance of sample solution or standard.

RESULTS AND DISCUSSION

Total phenol and flavonoid contents

It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process (Kessler et al., 2003; Cook and Samman, 1996). Phenolic compounds are a class of antioxidant agents which act as free radical terminators (Shahidi and Wanasundara, 1992).

Total phenol, total flavonoid content and the antioxidant activity of different plant extracts are shown in Table 1. Phenolic contents are expressed as µg gallic acid equivalent (GAE)/mg of plant extract, with reference to standard curve ($y = 0.0036x-0.029$, $R^2 = 0.985$). Flavonoid contents are reported as µg quercetin equivalent/mg of extract, with reference to standard curve ($y = 0.0501x + 0.0167$, $R^2 = 0.998$). It is observed that phenol and flavonoid contents in different plants differ.

Table 2. Total phenol, flavonoids contents and DPPH scavenging activities in the studied plant extracts.

Plant species	Flavonoid (µg Q E/mg extract)		Total phenol µg EAG /mg extract		Antioxidant activity IC_{50} (µg/ml)	
	AqE	MeE	AqE	MeE	AqE	MeE
Artemisia campestris	17.21±0.45	29.68±0.32	192.28±8.59	280.4±5.46	8.66±1.52	20.67±1.52
Pituranthos chlorantus	12.76±0.36	12.34±0.21	91.03±4.41	77.59±2.88	56.67±3.51	71.67±3.05
Asteriscus pygmaeus	3.93±0.18	7.63±0.39	72.59±4.72	40.71±3.09	40.67±2.08	74.33±4.72
Pallenis spinosa	5.45±0.21	25.43/0.11	125.71±4.96	71.59±4.44	19.67±3.05	49.33±3.21
Aizoon hispanicum L.	0.071±0.0008	0.17±0.007	30.33±2.03	40.39±3.21	325.7±5.50	292.3±7.02

IC_{50} of ascorbic acid = 1.38 ± 0.2 µg/mL.

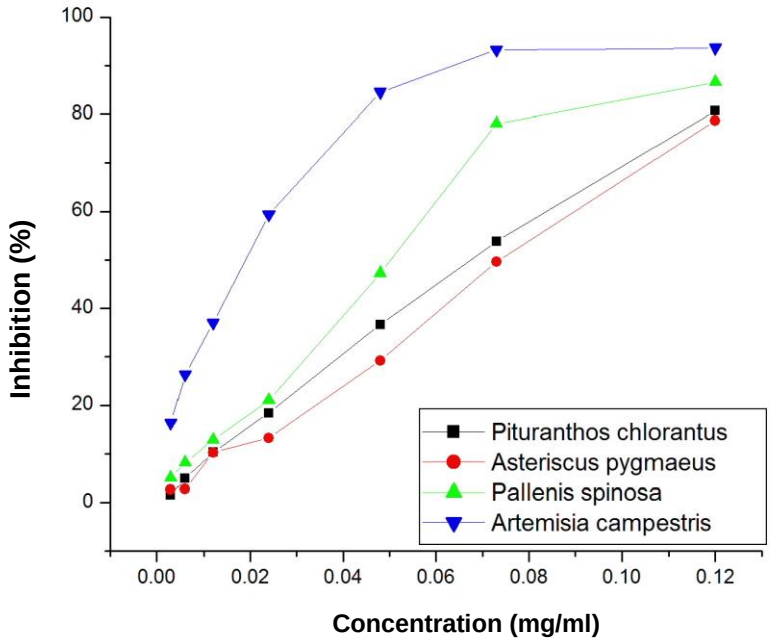

Figure 1. DPPH radical scavenging activity aqueous extract of *P. chloranthus*, *A. campestris*, *P. spinosa* and *A. pygmaeus.*

Highest phenol and flavonoid contents were noted in *A. campestris* extracts and lowest in *A. hispanicum* (Table 2).

Methanolic extracts showed the highest amount of flavonoids and aqueous extracts showed the lowest amount. This fact may be due to low solubility of these compounds in water. According to their flavonoid contents, the ranking order of the five species was as follows: *A. campestris* > *P. spinosa* > *P. chlorantus* > *A. pygmaeus* > *A. hispanicum*. Concerning phenol contents, results proved that the solvents for extraction vary individually by varying medicinal plant used, that is, total phenolic content of *A. campestris* aqueous extract was 192.28 ± 8.59 µg EAG/mg extract, while its methanol extract contained 280.4 ± 5.46 µg EAG/mg extract. Whereas, methanol extract of *P. spinosa* was 71.59± 4.44 µg EAG/mg extract of total phenolics, aqueous extract showed 125.71 ± 4.96 µg EAG/mg extract.

IC_{50} for DPPH radical-scavenging activity are shown in Table 2, and Figures 1, 2 and 3. The highest antioxidant activity was noted in the extracts of *A. campestris* plant, and the infusion of *A. hispanicum* presented a very weak or negligible activity. This result is in accordance with that reported by Akrout et al. (2011).

The general ranking of antioxidant activity decrease in the same order than phenol and flavonoid contents in each extract type. The correlation coefficient between IC_{50} data and the total phenolic compound contents is 0.81 and 0.70, for MeE and AqE respectively, confirming that these compounds are likely to contribute to the radical scavenging activity of these plant extracts. (Ivana Karabegovi et al., 2011). Lower correlation value of the

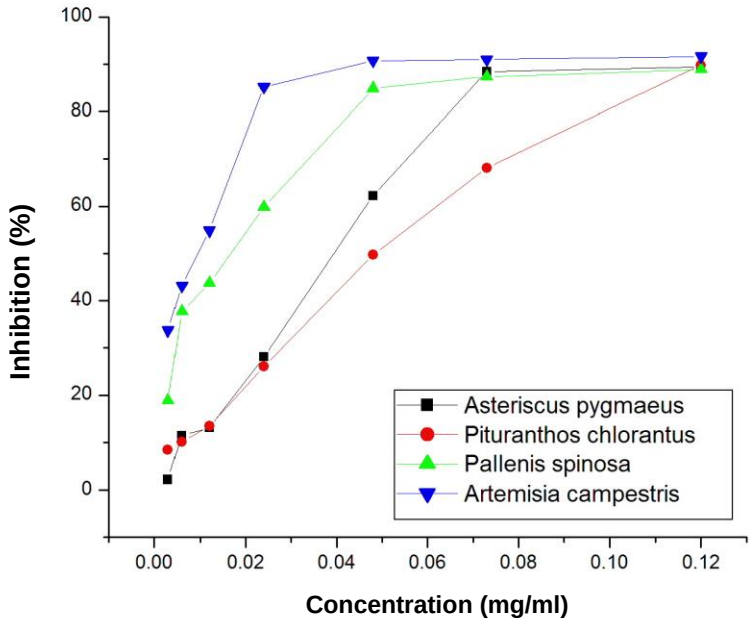

Figure 2. DPPH radical scavenging activity methanolic extract of *P. chloranthus*, *A. campestris*, *P. spinosa* and *A. pygmaeus.*

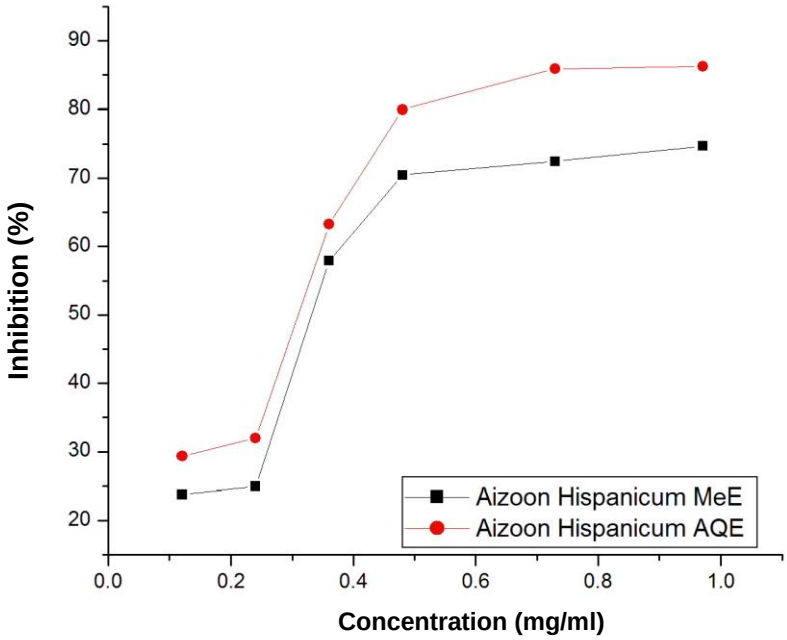

Figure 3. DPPH radical scavenging activity aqueous and methanolic extract of *A. hispanicum.*

aqueous extracts suggests that in this extracts, phenolic compounds alone are not fully responsible for the antioxidant activity of plants. A study performed by Babbar et al. (2011), showed that other water soluble constituents such as ascorbates and reducing carbohydrates as well as the synergistic effect among them could possibly contribute to the total antioxidant activity. This might indicate the relative practicability of

water as extracting solvent for the active compounds of these plants.

The implication of oxidative stress in the etiology of several chronic and degenerative diseases suggests that antioxidant therapy represents a promising avenue for treatment. In the future, a therapeutic strategy to increase the antioxidant capacity of cells may be used to fortify the long term effective treatment. The body has several mechanisms to counteract oxidative stress by producing antioxidants, either naturally generated in situ (endogenous antioxidants), or externally supplied through foods (exogenous antioxidants). The roles of antioxidants are to neutralize the excess of free radicals, to protect the cells against their toxic effects and to contribute to disease prevention (Pham-Huy et al., 2008). In the present study all the plants except *Aizoon hispanicum* showed phenol and flavonoid content and exhibit antioxidant activity. And also their use in traditional folk medicine (Table 1). Detail work by using different methods will be the aim of further investigation.

Conclusion

The purpose of this study was to evaluate, by a chemical method, the antioxidant capacity of phenolic compounds in some Algerian plants. These plants showed significant antioxidant activity, flavonoid and phenolic contents. Among the five plants studied in this work, *A. campestris* and *P. spinosa*, both belonging to the Asteraceae family, were found to be the most promising ones. These plants contain the highest amount of phenolics and have a high level of antioxidant activity. Aqueous extract exhibited higher antioxidant activity despite its lower phenolic content. This may justify the use of plant infusion in traditional medicine.

Conflict of Interests

The authors have not declared any conflict of interests.

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