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Full Length Research Paper

Inflorescence bulbils of tiger lily *in vivo* and bulbils culture *in vitro*

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The present study includes two experiments; the physiological and the biotechnological experiments. The physiological experiment was designed to investigate the most interesting rare natural phenomenon of the development of vegetative reproductive organs (bulbils) in the inflorescence of tiger lily. In the other hand, the biotechnological experiment was carried out to evaluate the regeneration potential of bulbils culture *in vitro*. In the first experiment, the plants of tiger lily *Lilium lancifolium* var. Flore Pleno which were grown in greenhouse showed that the Pseudo viviparous phenomenon at the end of flowering when vegetative bulbils were induced in the inflorescence. These bulbils as pseudo viviparous structures were precisely formed in place of flowers and along the floral stalks. The flower head was completely surrounded by the extensive numbers of these bulbils; some of them continued in growth and produced new shoots. The tissue culture experiment of tiger lily var. Flore Pleno was carried out using bulbils culture for eight weeks of culture *in vitro*. The results indicate that the bulbils was shown to be a good choice as explants for micropropagation but the potential of these bulbils to produce bulblets, shoots and roots was greatly influenced by the concentrations of naphthalene acetic acid (NAA) and benzyl aminopurine (BA) in culture. However, the concentration of 1 mg/l BA combined with 0.1 mg/l NAA was shown to be the optimum for micropropagation of tiger lily var. Flore Pleno.

Key words: Bulbils, pseudo viviparous phenomenon, tiger lily, *in vitro*, micropropagation, growth regulators.

INTRODUCTION

The bulbils formation in the inflorescence is a rare phenomenon that occurs in certain plants in nature. This natural phenomenon is called Pseudo vivipary which is a kind of a specific asexual reproductive way that some plant can achieve under some conditions when the flowering process is aborted and developmental changes occurred to produce new plantlets or bulbils in place of flower instead of floral organ and seeds. This kind of asexual reproduction is widely recorded in some monocots plants in many families such as Liliaceae Alliaceae, Agavaceae, Pocceae, Saxifragaceae and polygonaceae particularly in some grasses (Elmqvist and

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Abbreviations: NAA, Naphthalene acetic acid; **BA,** benzyl aminopurine.

Cox, 1996; Kuzmanovic et al., 2012; Moore et al., 1976; Tooke et al., 2005; Szarek and Holmesley, 1996). While, most lilies produce many types of reproductive structures for their vegetative reproduction during growing, such as daughters and bulblets, but some lilies can produce aerial black tiny bulbils commonly along the stem at the point where leaves join the stems. However, the varieties of tiger lily (*Lilium lancifolium*), Splendens, Flore Pleno and Fortuni are well known for their tiny black bulbils production on stem plant. The asexual reproductive structures which were produced in plants during growing can be increased by application of flower buds removal practices (Dantuluri et al., 2002; Leclerc et al., 2005), and they can be used to propagate the plants in a traditional way in nursery or *in vitro* however, the aerial bulbils is considered to be a good source of explants without the contamination problem of soil borne diseases (Lian et al., 2009; Shu and Park, 1993; Kasai et al., 2000).

In lily micropropagation, various organs as explants were used such as pedicel, filament, leaf, root, bulb-scale and several studies concerned the effect of cytokinin and auxin concentrations the on regeneration ability of these explants to produce bulblets, shoots and roots (Kumar et al., 2006; Duong et al., 2001). It is well known that the cytokinin together with auxin play an essential role in plant morphogenesis, they have great influence on the formation of roots and shoots they have great the ratio of these two hormones can determine the kind of plant development (Werner and Schmülling, 2009; Bartrina et al., 2011). The objective of this paper was to study the development of bulbils in the inflorescence and to investigate the use of these bulbils as explants *in vitro* for micropropagation of tiger lily.

MATERIALS AND METHODS

The present studies included two experiments; the first was designed to investigate the development of bulbils during plants growing for two tiger lilies and to evaluate the application of bud removal practice for increasing the bulbils production of plants, second experiment was carried out to determine the potential of bulbils as explants for micro propagation *in vitro* of tiger lily var. Flore Pleno. The first experiment was carried out at the computerized greenhouses with environmental control systems in school of biomedical and biological sciences, University of Plymouth during 2014 at temperature around 25°C. Healthy Bulbs of the tiger lilies were grown by John Innes mixture no.3 as growing medium and the experiment included four treatments; two varieties of tiger lilies *L. lancifolium* (*Lilium tigrinum*) var. Splendens and var. Flore Pleno with or without flower buds removal practice; the flower buds were removed when they became 2 cm long, each treatment contained 12 plants. In tissue cultural experiment, the whole bulbils of tiger lily var. "Flore Pleno" were used as explants; these explants were carefully washed and sterilized with 10% v:v bleach solution (5.25% sodium hypochlorite) for 15 min and washed 3 to 4 times with sterilized distilled water before culturing. The explants were then cultured on Murashige and Skoog (MS) basal medium containing 30 g L^{-1} sucrose, 8 g L^{-1} agar, pH 5.7, supplemented with naphthalene acetic acid (NAA) and benzyl aminopurine (BA) in different concentrations; all cultures were incubated in a Gallenkamp growth cabinet under 16 h photoperiod, provided by cool-white flourescent lamps with an irradiance of 100 μmol m^{-2} s^{-1} at a constant temperature of 25°C for 8 weeks. All explants were placed in a vertical position on the agar, and this experiment contained six treatments with 12 explants per treatment, and the concentrations of BA and NAA were (1 mg/l BA) (1 mg/l BA+ 0.1 mg NAA), (0.5 mg/l BA), (0.5 mg/l BA+ 0.1 mg NAA), (1 mg NAA) and control.

The healthy bulbs were purchased from Hyde and Sons Nursery-UK; nutrient solution and chemicals were from Sigma-Aldrich Company Ltd. Data of greenhouse experiment, number and weight (g) of bulbils per plant were collected at flowering time while number and weight (g) of daughters, bulblets and weight (g) of bulb roots, stem roots, per plant were collected after 16 weeks from planting. Data of tissue culture experiment, number and weight (g) of bulblets, roots and number, weight (g), length (cm) of shoots per bulbils were collected after eight weeks of culture. The statistical analysis system (SAS, 2012) was used to show the effect of different factors in the study parameters. Significant difference-LSD test was used in this study to significant compare between means at the (0.05) level of significance.

RESULTS

In Plates 1 and 2, the tiger lilies *L. lancifolium* (*L. tigrinum*) var. Splendens and var. Flore Pleno grown in greenhouse produced a large numbers of common tiny black bulbils on plant stem and more black bulbils were recorded as Pseudo viviparous propagules after flowering in the inflorescence of var. Flore Pleno plants. The results in Table 1 indicates that the application of flower bud removal practice greatly increased the production of bulbils and daughters, and differential response to this practice was shown between the two varieties. Higher response was found in Flore Pleno compared to the other when the bulbils production was increased by No. (56.45%) Wt. (103.9%) and bulblets No. (21.99%) and Wt.(127.4%).

The treated plants of Flore Pleno showed the maximum values of bulbils No. (48.75) weight (7.85 g), bulblets No. (2.33) Wt. (3.32 g), daughters Wt. (44.66 g), ground roots Wt. (7.78 g) and stem roots Wt. (3.08 g) per plant, while the minimum values of bulbils No. (28.5) Wt. (2.07 g), bulblets No. (1.83) Wt. (1.41 g), daughters Wt. (14.85 g), ground roots Wt. (3.38 g) and stem roots Wt. (1.18 g) per plant were found in untreated plants of Splendens. Plate 3 shows the *in vitro* bulbil culture of tiger lily var. Flore Pleno for eight weeks of culture using whole bulbils as explants.

The results in Figure 1 indicate that the different concentrations of Naphthalene acetic acid (NAA) and Benzyl aminopurine (BA) showed different results related to potential of bulbils to produce bulblets, shoots and roots, however, the concentration of 1 mg/l BA combined with 0.1 mg/l NAA was shown to be the optimum for micropropagation of tiger lily var. Flore Pleno which achieved the maximum values of bulblets number (3.83) Wt. (0.19 g) (Figure 1A) and the shoots No. (6.17) Wt. (0.39 g) length (28.33 cm) (Figure 1B) while the maximum number of roots (4.67) Wt. (0.28 g) were observed at concentration of 1 mg/l NAA (Figure 1C).

Plate 1. The formation of pseudo viviparous bulbils after flowering in the inflorescence of double tiger lily var. Flore Pleno plants grown in greenhouse. **A, B, C)** Pseudo viviparous bulbils appeared on the heads of flowers, these flower heads were completely surrounded by the extensive numbers of bulbils, each head contains nearly 20 to 25 Pseudo viviparous bulbils instead of floral organs these kind of bulbils formed after flowers senescence. **D)** Pseudo viviparous bulbils also observed a long floral stalk at branching point where pedicel joins peduncle. **E)** Some of these bulbils continued in growth and produced new shoots while still attached to parent plant.

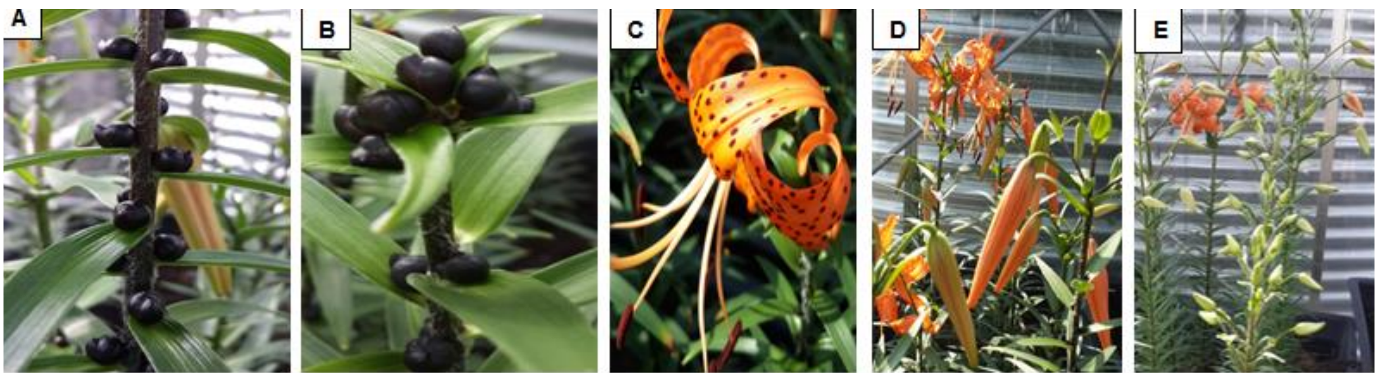

Plate 2. The tiger lilies *Lilium lancifolium* (*Lilium tigrinum*) var. Splendens and var. Flore Pleno grown in greenhouse. **A)** Both varieties produced a large numbers of common tiny black bulbils on plant stem at the point where each leave join stem before flowering time. **B)** Sometimes bulbils formed as cluster on the top of stem. **C)** The red flowers of tiger lilies *Lilium lancifolium* (*Lilium tigrinum*) var. Splendens. **D)** The plants of tiger lilies *Lilium lancifolium* (*Lilium tigrinum*) var. Splendens at flowering time. **E)** The plants of tiger lilies *Lilium lancifolium* (*Lilium tigrinum*) var. Flore Pleno. at flowering time with the red double flowers.

Table 1. The effect of the flower buds removal practice on the development of bulbils, bulblets,daughters, shoots and roots in plants of tiger liles var. splendens and var. Flore pleno.

Factor		Parameters							
		Bulbils		Bulblets		Daughters		Bulb roots	Stem roots
		WT.(g)	No.	WT.(g)	No.	WT.(g)	No.	WT.(g)	WT.(g)
Treatment	1	29.83	2.96	1.87	1.44	1.67	21.24	4.11	1.29
	2	39.33	5.19	2.13	2.41	1.75	31.01	5.09	1.89
LSD value	--	6.91*	1.40*	1.10 NS	1.40 NS	0.27 NS	4.75 *	1.05 NS	0.954 NS
Variety	SP.	29.21	2.3	1.87	1.46	1.79	16.1	2.89	0.94
	FL.	39.96	5.85	2.13	2.39	1.63	36.14	6.31	2.24
LSD value	--	6.91*	1.40*	1.103 NS	1.40 NS	0.270 NS	4.75 *	1.05*	0.954*
Treat. x Variety	1 SP.	28.5	2.07	1.83	1.41	1.75	14.85	3.38	1.18
	1 FL.	31.16	3.85	1.91	1.46	1.58	27.62	4.84	1.39
	2 SP.	29.91	2.53	1.92	1.51	1.83	17.35	2.39	0.69
	2 FL.	48.75	7.85	2.33	3.32	1.67	44.66	7.78	3.08
LSD value	--	9.77 *	1.98 *	1.55 NS	1.99 NS	0.382 NS	6.72 *	1.49 *	1.35 *

LSD test was used to significantly compare between means at the 5% level of significance. *, NS, significant and not significant at $p < 0.05$ respectively. Treatment 1, Control; Treatment 2, Flower buds removal; SP., Splendens; FL, Flore Pleno.

Plate 3. The effect of different concentrations of naphthalene acetic acid (NAA) and benzyl aminopurine (BA) on the regeneration of bulblets, shoots and roots in bulbils culture in vitro of tiger lily var. Flore Pleno after 8 weeks of culture. **A and B)** Large shoots were regenerated in bulbils culture in some treatments dependently on the concentrations of BA and NAA. **C and D)** Great growth of roots system was found in bulbils culture in some treatments and that highly dependents on the concentrations of BA and NAA. **E and F)** High bulblets regeneration was recorded in bulbils culture in some treatments dependently on the concentrations of BA and NAA.

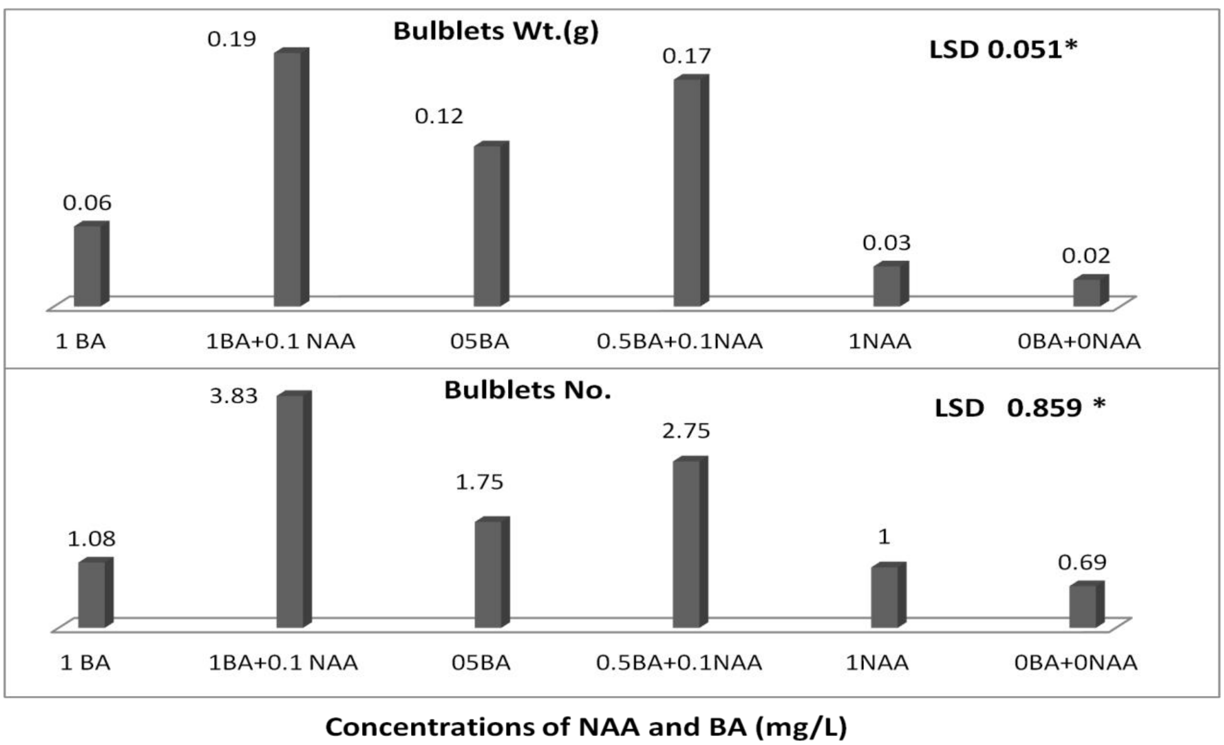

Figure 1A. The effect of different concentrations of BA and NAA on the potential of bubils to produce bulblets, after 8 weeks of culture *in vitro* of tiger lily var. Flore Pleno.

DISCUSSION

While, the tiger lilies (*L. lancifolium*) var. Splendens and var. Flore Pleno produced bulbils before flowering along the stem at the point where leaves join the stems as common bulbils, but at the end of flowering, more bulbils appeared in the inflorescence of Flore Pleno plants as Pseudo viviparous structures precisely in place of flowers and along the floral stalks. This natural Pseudo viviparous phenomenon was widely recorded in some monocots plants which belong to many families such as Liliaceae Alliaceae, Agavaceae, Pocceae, Saxifragaceae and polygonaceae (Elmqvist and Cox, 1996; Kuzmanovic et al., 2012; Moore et al., 1976; Tooke et al., 2005). It may be that the formation of these bulbils in the inflorescence of double tiger lily was, instead of seeds because this lily is commonly believed to be sterile and not capable of producing seeds as fertilization was unsuccessful. However, many studies reported that this asexual kind of reproductive way occasionally happen in

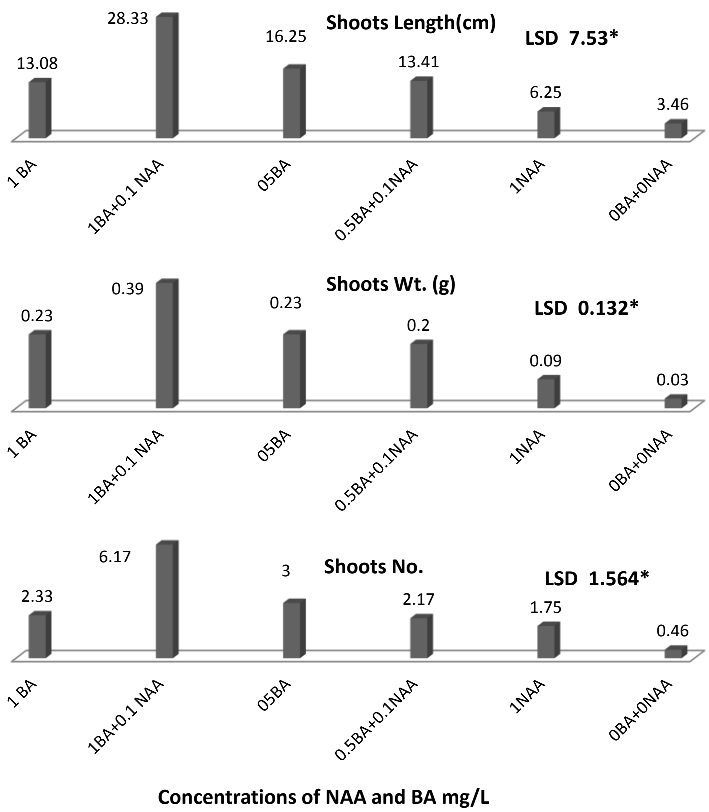

Figure 1B. The effect of different concentrations of BA and NAA on the regeneration of shoots in *in vitro* bulbils culture of tiger lily var. Flore Pleno after 8 weeks of culture.

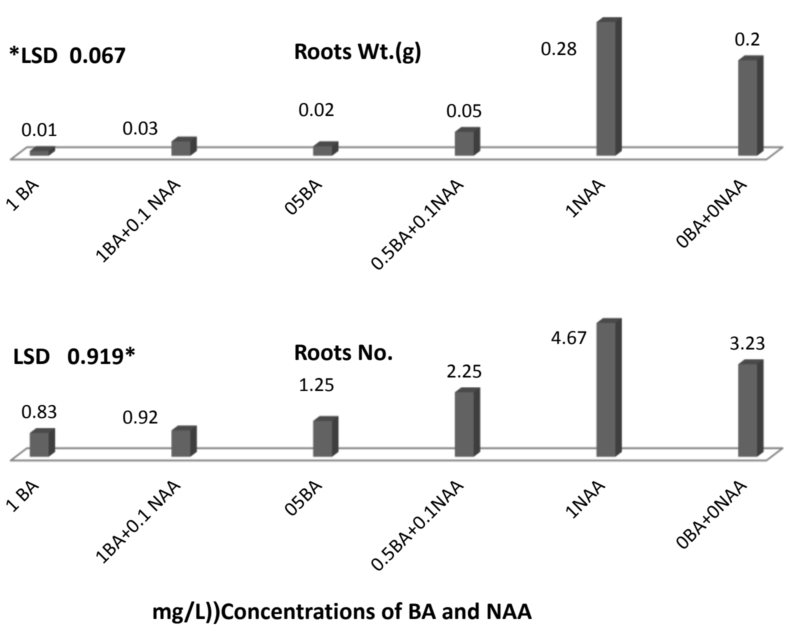

Figure 1C. The effect of different concentrations of BA and NAA on the regeneration of roots in *in vitro* bulbils culture of tiger lily var. Flore Pleno after 8 weeks of culture. LSD test was used to significantly compare between means at the 5% level of significance. *, NS, Significant and not significant at p < 0.05, respectively.

nature in specific conditions in some plants when the flowering process is aborted and some developmental changes occur to produce new plantlets or bulbils in place of flower instead of floral organ and seeds (Arizaga et al., 1995; Maria et al., 2010; Pierce et al., 2003; Tooke et al., 2005; Wang and Cronk, 2003). The results also show that the bulbils production of tiger lilies can be increased by application of flower buds removal practice and that may be due to increasing the supply of assimilates to the propagules, and this results agree with those of previous studies (Dantuluri et al., 2002; Leclerc et al., 2005).

In the study of bulbils culture *in vitro* of tiger lily var. Flore Pleno for eight weeks of culture, the results of this study show that the potential of bulbils to regenerate bulblets, shoot and roots was greatly influenced by the concentrations of NAA and BA in culture. Different concentrations of NAA and BA showed different results related to growth rate of bulblets, shoots and roots. Similar results of this effect of the growth regulators were found in several studies (Kumar et al., 2006; Duong et al., 2001), however, the concentration of 1 mg/l BA combined with 0.1 mg/l NAA was shown to be the optimum to produce bulblets and shoots while the highest growth rate of roots system as weight (g) were observed at concentration of 1 mg/l NAA. The results indicate that the bulbils is to be a good source for micropropagation of tiger liliy var. Flore Pleno particularly with no contamination problem of soil borne diseases and that agree with previous studies (Lian et al 2009; Shu and Park, 1993; Kasai et al., 2000).

Conflict of interests

The authors did not declare any conflict of interest.

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African Journal of Biotechnology

Full Length Research Paper

Effects of different culture conditions (photoautotrophic, photomixotrophic) and the auxin indole-butyric acid on the *in vitro* acclimatization of papaya (*Carica papaya* L. var. Red Maradol) plants using zeolite as support

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Plant regeneration of papaya via organogenesis and somatic embryogenesis has been successful; however, the biggest problem of *in vitro* culture of this species is the acclimatization of regenerated plants, where over 70% of the plants are lost before being planted in the field. Decreasing the relative humidity inside the culture vessel and thus increasing the ventilation, appears to have a greater effect on the adaptation of papaya plants, strengthening the function of the stomata and with this, allowing better control of water loss from the leaves. The aim of this study was to determine the effects of different concentrations of sucrose and indole-butyric acid (IBA) on rooting and *in vitro* acclimatization of plants using sterile zeolite as support and culture vessels with increased ventilation. Three concentrations of sucrose (0, 10 and 20 g L^{-1}) were studied with and without auxin and as the control treatment, the rooting culture medium with agar during 17, 27 and 37 culture days. The highest percentage of rooting was recorded at 37 culture days in the treatment without sucrose and IBA with 80.0% and zeolite as support. The best photosynthetic values were achieved when *in vitro* shoots were grown in culture medium with auxin and different concentrations of sucrose, even though they were also high in the treatment without the presence of IBA and without sucrose at 17 days of culture. The combined effect of the zeolite, auxin (IBA), without sucrose in the culture medium and increased ventilation allowed photoautotrophic culture conditions which had effect of the increasing plant survival under *ex vitro* acclimatization conditions.

Key words: *Carica papaya*, photosynthesis, roots formation.

INTRODUCTION

In Cuba, the most commercially important variety is the 'Red Maradol' and crop production exceeded 1.7 million tons in 2013 (FAOSTAT, 2014). In addition, it is sown in other countries in the Caribbean and Central America. Regeneration of papaya plants via somatic embryogenesis has been successful; however, the somatic embryos in germination have problems with root development due to the presence of a basal callus, which prevents the formation of roots or its connection to the stem, besides the low percentage of acclimatization of rooted plants (Fitch and Manchardt, 1990; Dhekney et al., 2007; Sekeli et al., 2013). Another critical aspect has been the adaptation to environmental conditions because of the high relative humidity that this species need to achieve high survival rates (Chen et al., 1991). The biggest problem that exists globally in *in vitro* papaya culture is the *ex vitro* acclimatization of regenerated plants, where more than 70% of *in vitro* plants produced are lost before being planted in the field (Malabadi et al., 2011). A plant that originated *in vitro*, differs in many aspects from those formed *in vivo* (Pierik, 1990), since its environmental conditions, substrate, light, and nutrition, are very different. It is also important to note that the growth *in vitro* is heterotrophic, while the conditions *in vivo* are autotrophic. The *in vitro* atmosphere, with a high relative humidity, low or zero gas exchange, shortage of CO_2 during most of the period, ethylene production and low photosynthetic rate, induce changes in plants grown under these conditions. After transferring the plants to *ex vitro* environment, plants have to correct all of these abnormalities in order to acclimatize to the new environment, either in greenhouse or into the field (Kadleček et al., 2001).

Furthermore, the anatomy of the leaves is influenced by light and humidity, differing anatomically from those originated from *in vivo* conditions (Brainerd et al., 1981). Because of this, the acclimatization is an important factor in the subsequent survival rates of the plants, since it is a critical stage in the process, in which the larger loss occurs. In this stage, the relative humidity should begin to decrease gradually, to allow in addition to stomata closure, better cuticle formation and reduced water loss. Moreover, for best results in *in vivo* establishment, it is necessary to have root *in vitro* development (Pierik, 1990). Decreasing the relative humidity inside the culture vessel and thus the increased ventilation, appear to have a greater effect on the adaptation of grape plants (*Vitis vinifera* L.), enhancing the stomata function and thereby enable better control of water loss from the leaves (Gribaudo et al., 2001).

In vitro photoautotrophic can be induced excluding carbohydrates from the culture medium and increasing gas exchange in the culture vessel (Kozai, 2010; Xiao et al., 2011). Photoautotrophic micropropagation is defined as micropropagation without sucrose in the culture medium, where the accumulation of carbohydrates in *in vitro* tissues cultured and their subsequent growth is completely dependent on photosynthesis and inorganic nutrients (Zobayed et al., 2004; Kozai, 2010). Therefore this may also be called photosynthetic micropropagation in culture medium devoid of sugar (Xiao et al., 2011). In photoautotrophic micropropagation, acclimatization can also be completed in the culture vessel, which is called *in vitro* acclimatization (Kozai et al., 2005).

Although the growth and physiological changes in some plant species with photoautotrophic growth have been studied (Norikane et al., 2010; Badr et al., 2011; Shin et al., 2013), there are very few reports of studies on the *in vitro* propagation of papaya and none specifically on its most critical phase - rooting. For this reason, this study aims to evaluate the effects of different concentrations of sucrose and the auxin indole-butyric acid to achieve *in vitro* acclimatization in a growth chamber with sunlight, greater ventilation of the culture vessels and using zeolite as a support for increased survival rates *ex vitro* of papaya plants obtained by somatic embryogenesis.

MATERIALS AND METHODS

Plant material and culture media

As plant material, *in vitro* shoots of papaya - variety Maradol Roja were used. These were regenerated from somatic embryos, originating from the fourth subculture in the elongation culture medium proposed by Posada-Perez et al. (2007). This culture medium contained Murashige and Skoog (MS) (1962) salt at 100% concentration supplemented with 1 mg L^{-1} of thiamine, 1.2 µM of 6-benzyl aminopurine (BAP), 1.5 µM of naphthaleneacetic acid (NAA), 100 mg L^{-1} of myo-inositol, 30 g L^{-1} of sucrose, 1 µM of riboflavin and 5 g L^{-1} of Agargel (Sigma Co.) and adjusted to a pH of 5.8. Shoots with a size between 3.0 to 5.0 cm in length, of which only the last three leaves were left, were placed in culture vessels containing three concentrations of sucrose (0, 10 and 20 g L^{-1}) combined with the presence or absence of the growth regulator, indole-butyric acid (IBA) at a concentration of 9.8 µM for *in vitro* rooting and acclimatization of shoots, using as support to the mineral zeolite (natural aluminum-silicate with excellent ionic exchange properties and a high absorption power) 1 to 3 mm granulation (Table 1). To each glass culture vessel, 97 g of zeolite

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Abbreviations: IBA, Indole-butyric acid; **BAP,** 6-benzyl aminopurine; **NAA,** naphthaleneacetic acid.

Table 1. Physical-chemical characteristics of natural zeolite (Tasajera Deposit, Villa Clara, Cuba).

Chemical composition	**(%)**
Silicon oxide (SiO_2)	70.10
Aluminium oxide III (Al_2O_3)	11.20
Iron oxide III (Fe_2O_3)	2.20
Iron oxide II (FeO)	0.30
Magnesium oxide (MgO)	0.60
Calcium oxide (CaO)	4.50
Sodium oxide (Na_2O)	1.50
Potassium oxide (K_2O)	1.30
Diphosphorus pentoxide (P_2O_5)	0.07
Water (H_2O)	4.70
Mineral composition	**%**
Clinoptilolite	40.00
Mordenite	40.00
Others (Calcite, quartz, feldspar)	20.00
Physical properties	**Value**
Size of the particle	1.0-3.0 mm
Density (δ)	0.37 g cm^{-3}
Density of the solid phase (γ)	1.77 g cm^{-3}
Total porosity (TP)	80.59% vol.

previously sterilized in an oven at 180°C for 2 h were added. Glass culture vessels with a total volume of 250 mL with 30 mL of liquid culture medium were used. They were covered with aluminum foil of 20 µm thickness. After three days of culture, the ventilation of the culture vessels was increased by opening holes on the aluminum foil covering the culture vessels in the different treatments. A second hole was made three days after the opening of the first (Figure 1A). As the control treatment, a modified version of the culture medium for rooting proposed by Posada-Perez et al. (2007) was used. This culture medium was composed of MS salts at 50% concentration, 9.8 µM of IBA, 0.4 mg L^{-1} of thiamine, 1.0 µM of riboflavin, 40 g L^{-1} of sucrose, 7.0 g L^{-1} of agar and pH of 5.8 prior to sterilization. The culture vessels and the volume of culture medium were the same as previously mentioned, but these were covered with plastic lid (polycarbonate). Forty five vessels were used with two shoots each per each variant of culture medium. Of each variant 15 culture vessels were randomly selected every 10 days, from 17 to 37 days of culture for evaluations of the morphological and physiological indicators of plants, including contamination. Survival (%) in *ex vitro* acclimatization conditions was done with plants after 17 days of *in vitro* culture conditions. This evaluation was done 20 days after being transplanted.

Culture conditions

The culture vessels with the shoots were placed in growth rooms at a temperature of 27 ± 2°C with sunlight and a photoperiod of 13 / 11 h, light / dark with a light intensity ranging between 48.0 and 62.5 µmol m^{-2} s^{-1}; measured with a light meter EXTECH 401,025 (USA). The experiments were repeated twice. The relative humidity inside the culture vessel covered with aluminum foil and two holes was between 72 to 68% and in control with culture vessel lids with plastic was 90 to 85%.

Evaluation of morphological and physiological variables

After 17, 27 and 37 days of culture, *in vitro* shoots and plants were evaluated for the following morphological variables: length of the plant (cm), number of leaves, number of internodes, fresh mass of *in vitro* plant (gFM), leaf area (by the method proposed by Cardona et al. (2009) to estimate the leaf area of papaya plants), presence or absence of basal callus, number of roots, length of the roots (cm) and presence of roots (%). For the evaluations of the physiological indicators of *in vitro* shoots and plants, the contents of the pigments chlorophyll a, b and carotenoids were determined at 17, 27 and 37 days of culture using the same plants which had been used to assess the morphological variables. To determine the net photosynthetic activity and total transpiration, *in vitro* plants at 17 days of culture were used.

Chlorophyll and carotenoid pigments measurement

At 27 days, chlorophyll a, chlorophyll b and total carotenoid pigments were determined in the leaves from *in vitro* plants using the Meyer-Berthenrath´s method, modified by Stirban (1985). The absorbance of the extracts was measured at 663, 645 and 472 nm by spectrophotometry (GENESYS 6; Thermo Electronic Corporation Visionlite Vision 2.1),

Photosynthetic activity, total transpiration and stomatal conductance

For these measurements, fully extended leaves of the same position (leaves 2 and 3) of *in vitro* plants at the end of the experiment, 4 to 5 h after the start of the photoperiod were used. All measurements were performed with three leaves from different plants. The maximum photosynthetic capacity (µmol CO_2 m^{-2} s^{-1}), the total transpiration (mmol H_2O m^{-2} s^{-1}) and stomatal conductance (mmol m^{-2} s^{-1}) were measured with the equipment CIRAS-2 (Portable Photosynthesis System, UK), coupled to a universal bucket PLC6 2.5 cm^2. The area of the tray was completely covered with the leaf (1.7 cm^2). The concentration of CO_2, air temperature and relative humidity (80 to 90%) were environmental values taken into consideration. The light equipment, intensity was set at 900 µmol m^{-2} s^{-1}. Measurements were always done on all *in vitro* plants between 9:00 to 10:00 a.m.

Ex vitro acclimatization conditions

The environmental acclimatization conditions are characterized by averaged daytime temperature of 30 ± 2°C, 65 to 70% relative humidity and light intensity ranging between 224 and 457 µmol m^{-2} s^{-1} measured with a light meter EXTECH 401 025. Plastic trays with cover were used and 100% zeolite as substrate with 15 *in vitro* plants per treatment, which had been cultured for the first 17 days (Figure 1B, C). These were kept closed during the first first days after transplanting to ensure high relative humidity above 90% and thereafter it was slowly reduced by opening the lid. Irrigation was done manually with aspersion three times daily. The trays were placed in a culture house with an internal black *Saran* mesh cover with 70% shade. The survival rate (%) was determined by counting the plants that were alive at the time of evaluation after 15 days with respect to the total number of plants initially planted.

Statistical analysis

For the statistical analysis of the data, the package SPSS version 17.0 for Windows 2008 was used. For analysis of the normality of

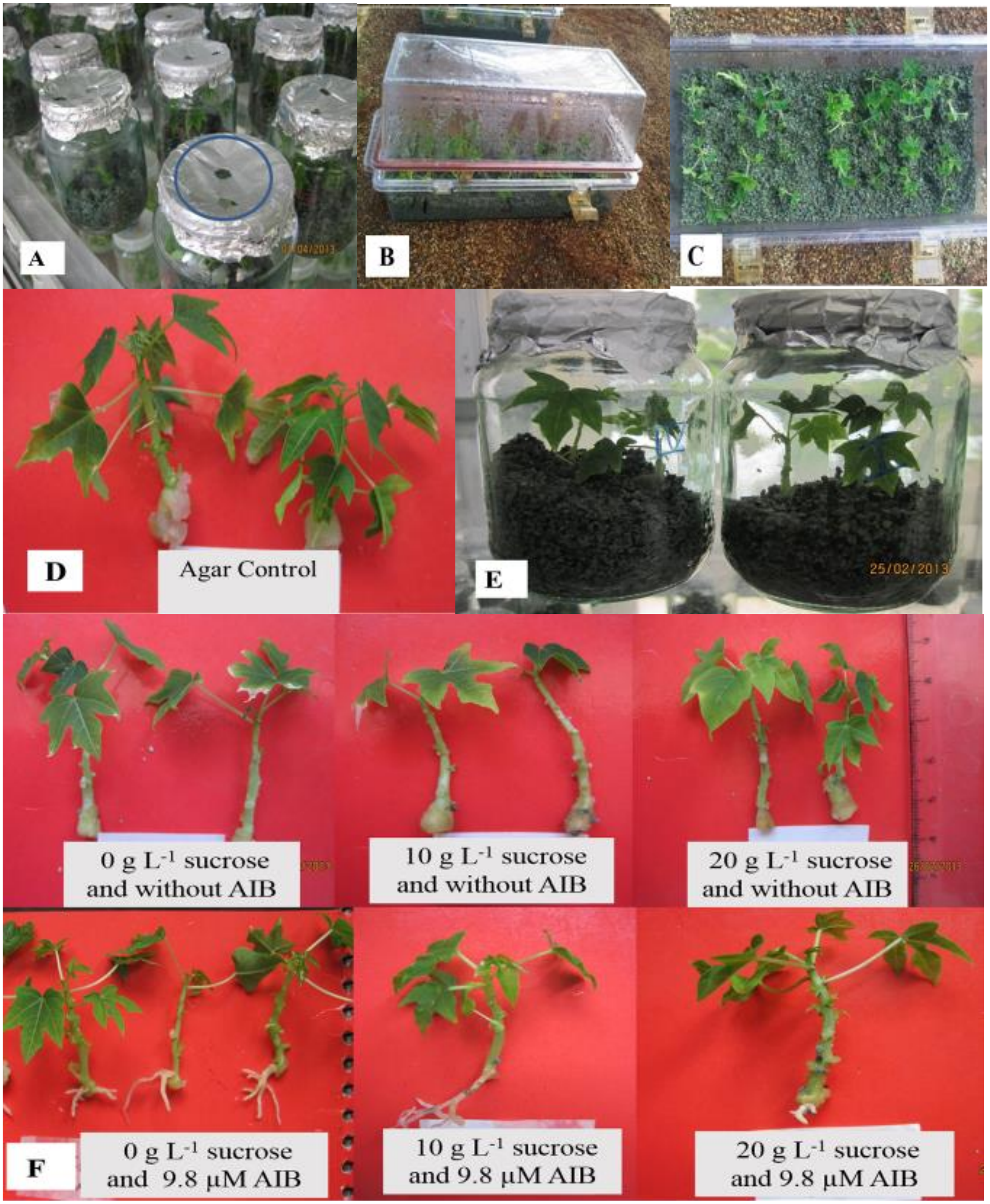

Figure 1. Rooting and *in vitro* acclimatization of papaya (*Carica papaya* L. var. Red Maradol) shoots obtained by somatic embryogenesis under different culture conditions. **(A)** Culture vessel with increased ventilation. **(B-C)** Plastic trays (polycarbonate) with zeolite as support used for *ex vitro* acclimatization plants after 17 days of *in vitro* culture. **(D)** Aspects of *in vitro* plants at the end of the experiment (37 days) in photoautotrophic culture conditions. **(E)** *In vitro* papaya plants with the formation of basal callus cultured in culture medium with agar and sucrose. **(F)** Stimulus of rhizogenesis in the presence or absence of auxin and sucrose at 37 days of culture.

Table 2. Effects of the interaction sucrose and IBA on growth and rooting of *in vitro* papaya (*Carica papaya* L. var. Red Maradol) plants growing in culture vessel with increased ventilation and zeolite as a support at 17 days of culture.

IBA (µM)	Sucrose (g L^{-1})	Height (cm)	No. of leaves	Leaf area (cm^2)	Fresh weight plant (gFW)	No. Internodes	Length of the roots (cm)	No. of roots	Rooting (%)	Survival (%)
0	0	3.63± 0.58[a]	3.10±0.99[c]	1.04±0.15[a]	0.37±0.12[b]	6.2±1.03[a]	0.0[b]	0.0[c]	0.0[c]	13.0[c]
0	10	3.31±0.37[a]	3.63±0.92[b]	1.02±0.11[a]	0.36±0.16[b]	7.8±1.45[a]	0.0[b]	0.0[c]	0.0[c]	6.6[c]
0	20	3.43±0.50[a]	4.12±0.83[ab]	1.00±0.14[a]	0.40±0.26[b]	8.5±2.44[a]	0.0[b]	0.0[c]	0.0[c]	0.0[d]
9.8	0	3.20±0.27[a]	3.70±0.82[b]	1.08±0.12[a]	0.20±0.12[c]	7.9±1.73[a]	0.14±0.18[a]	0.50±0.53[a]	40.0[a]	60.0[a]
9.8	10	3.26±0.33[a]	4.25±1.16[a]	1.05±0.14[a]	0.29±0.99[c]	9.3±2.55[a]	0.13±0.35[a]	0.13±0.35[b]	13.3[b]	33.0[b]
9.8	20	3.86±0.43[a]	3.00±0.53[c]	1.00±0.15[a]	0.48±0.28[b]	11.4±4.92[a]	0.0[b]	0.0[c]	0.0[c]	20.0[b]
9.8 (Agar control)	40	3.75±1.06[a]	4.50±0.70[a]	0.95±0.13[a]	0.85±0.77[a]	10.0±5.65[a]	0.0[b]	0.0[c]	0.0[c]	0.0[d]

Different letters within a column indicate significant differences at $p \leq 0.05$ by Kruskal-Wallis/ Mann-Whitney test (n = 15). Values ± SD.

the variables, Shapiro Wilk test was used. For the comparison between means, the nonparametric alternative analysis of variances of Kruskal Wallis test was applied. For the comparison between pairs of groups, the nonparametric Mann Whitney test was used. A bi-factorial experimental design with four repetitions was performed, analyzing the data using a multi-factorial ANOVA. Differences between groups were detected by the test of least significant difference or LSD intervals. For the statistical analysis, the statistical program Stat-graphics Centurion 2007 version 15 software was used.

RESULTS AND DISCUSSION

Effects of sucrose and auxin on rooting and *in vitro* acclimatization

Results on management leading to increased rooting and which favor photoautotrophic culture conditions are shown in Tables 2, 3 and 4, which reflect the response of the different treatments on morphological variables recorded with increased ventilation during 10, 20 and 30 days. From the earliest morphological evaluations, the positive effect of the combination that represented the treatment without sucrose and 9.8 µM IBA over other treatments was significant. For the three times that *in vitro* papaya plants were measured, the largest number of roots and increased root length were obtained with the treatment with 10 g L^{-1} of sucrose and 9.8 µM of IBA, at 17 and 27 days of culture. It is also noteworthy that in the evaluation at 27 days of culture, high values in fresh mass were achieved in the *in vitro* plants grown in zeolite without sucrose and 9.8 µM IBA, although there were no significant differences observed in the treatments without sucrose and without IBA; with 10 g L^{-1} sucrose and without AIB and the agar control. This was due to, in the case of agar control, the presence of big basal callus (Figure 1D). The callus did not form or their presence was minimal in the other treatments evaluated (photoautotrophic and photomixotrophic conditions) using zeolite as a support and with increased ventilation in culture vessels regardless of the presence or absence of auxin in the *in vitro* culture medium. In this regard Kozai et al. (2005) reported that the species Calla Lily (*Zantedeschia elliottian* L.) in photoautotrophic culture conditions prevented the formation of basal callus of *in vitro* shoots, which is the cause of the poor rooting and limited uptake of water and nutrients by the plants. Zhang et al*.* (2009) in the Chinese medicinal species (*Momordica grosvenori* Swingle) indicated that no callus was formed in those plants grown in culture medium without sucrose, free of growth regulators and photoautotrophic culture conditions. With respect to the leaf area at 27 and 37 days of culture (Figure 1E) in the treatment without sucrose (photoautotrophic culture conditions) and with the presence of IBA, the *in vitro* plants showed higher values with significant differences with the other treatments (Tables 3 and 4).

Teixeira de Silva (2014) shows that photoautotrophic culture of *in vitro* plants was possible in papaya in two varieties (Rainbow and Sunrise Solo) using plants from *in vitro* germinated seed and transferred to culture vessels Vitron® type and using as support rock wool. To the culture vessels, constant CO_2 at a concentration of 3,000 ppm was added. In the photoautotrophic conditions evaluated, plants of both varieties had a higher number of leaves and number of roots with respect to the photohete-rotrophic and photomixotrophic treatments.

Table 3. Effects of the interaction sucrose and IBA on growth and rooting of *in vitro* papaya (*Carica papaya* L. var. Red Maradol) plants growing in culture vessel with increased ventilation and zeolite as a support at 27 days of culture.

IBA (μM)	Sucrose (g L^{-1})	Height (cm)	No. of leaves	Leaf area (cm^2)	Fresh weight plant (gFW)	No. Internodes	Length of the Roots (cm)	No. of roots	Rooting (%)
0	0	4.00 ± 0.24^{a}	4.40 ± 0.96^{a}	1.23 ± 0.17^{b}	0.36 ± 0.18^{a}	10.0 ± 3.05^{a}	0.00^{b}	0.00^{b}	0.0^{c}
0	10	3.44 ± 0.38^{a}	3.88 ± 1.80^{b}	1.10 ± 0.09^{c}	0.34 ± 0.24^{a}	9.63 ± 2.87^{a}	0.00^{b}	0.00^{b}	0.0^{c}
0	20	3.30 ± 0.26^{a}	2.63 ± 1.50^{c}	1.05 ± 0.12^{d}	0.21 ± 0.99^{b}	8.25 ± 1.66^{a}	0.00^{b}	0.00^{b}	0.0^{c}
9.8	0	3.64 ± 0.39^{a}	4.60 ± 1.17^{a}	1.53 ± 0.11^{a}	0.35 ± 1.50^{a}	10.50 ± 2.27^{a}	0.31 ± 0.41^{a}	1.40 ± 1.95^{a}	53.0^{a}
9.8	10	3.54 ± 0.38^{a}	4.00 ± 1.63^{b}	1.20 ± 0.10^{b}	0.24 ± 0.10^{b}	9.90 ± 1.91^{a}	0.20 ± 0.63^{a}	0.10 ± 0.32^{b}	26.6^{b}
9.8	20	3.36 ± 0.39^{a}	2.63 ± 1.59^{c}	1.00 ± 0.08^{d}	0.18 ± 0.12^{c}	8.75 ± 2.12^{a}	0.00^{b}	0.00^{b}	0^{c}
9.8 (Agar control)	40	3.55 ± 0.21^{a}	4.50 ± 0.70^{a}	1.00 ± 0.09^{d}	0.30 ± 0.70^{a}	9.50 ± 1.41^{a}	0.00^{b}	0.00^{b}	0^{c}

Different letters within a column indicate significant differences at $p \leq 0.05$ by Kruskal-Wallis/ Mann-Whitney test (n = 15). Values ± SD.

Table 4. Effects of the interaction sucrose and IBA on growth and rooting of *in vitro* papaya (*Carica papaya* L. var. Red Maradol) plants growing in culture vessel with increased ventilation and zeolite as a support at 37 days of culture.

IBA (μM)	Sucrose (g L^{-1})	Height (cm)	No. of leaves	Leaf area (cm^2)	Fresh weight plant (gFW)	No. Internodes	Length of the roots (cm)	No. of roots	Rooting (%)
0	0	4.08 ± 0.57^{a}	3.80 ± 1.39^{b}	1.63 ± 0.15^{b}	0.36 ± 0.20^{ab}	11.00 ± 1.63^{a}	0.10 ± 0.25^{c}	0.60 ± 1.57^{b}	26.6^{b}
0	10	3.40 ± 0.25^{b}	3.30 ± 1.49^{b}	1.20 ± 0.04^{c}	0.29 ± 0.13^{bc}	9.90 ± 2.13^{a}	0.00^{d}	0.00^{c}	0.0^{d}
0	20	3.30 ± 0.18^{b}	3.50 ± 1.41^{b}	1.16 ± 0.11^{d}	0.19 ± 0.08^{c}	10.00 ± 1.60^{a}	0.00^{d}	0.00^{c}	0.0^{d}
9.8	0	3.70 ± 0.28^{a}	4.10 ± 0.87^{b}	1.75 ± 0.17^{a}	0.37 ± 0.15^{a}	10.30 ± 2.05^{a}	1.51 ± 0.17^{a}	1.50 ± 1.08^{a}	80.0^{a}
9.8	10	3.27 ± 0.44^{b}	3.60 ± 1.59^{b}	1.25 ± 0.05^{e}	0.21 ± 0.99^{bc}	8.80 ± 2.26^{a}	0.88 ± 1.06^{b}	0.50 ± 0.53^{b}	53.0^{b}
9.8	20	3.30 ± 0.54^{b}	2.60 ± 1.61^{b}	1.10 ± 0.08^{f}	0.33 ± 0.22^{bc}	9.90 ± 2.85^{a}	0.04 ± 0.13^{c}	0.22 ± 0.66^{b}	13.3^{c}
9.8 (Agar control)	40	3.65 ± 0.57^{ab}	5.50 ± 1.73^{a}	1.08 ± 0.06^{f}	0.65 ± 0.24^{a}	11.30 ± 2.50^{a}	0.00^{d}	0.00^{c}	0.0^{d}

Different letters within a column indicate significant differences at $p \leq 0.05$ by Kruskal-Wallis/ Mann-Whitney test (n = 15). Values ± SD.

Afreen-Zobayed et al. (2000) report that the photoautotrophic culture of sweet potato (*Ipomoea batata* L. (Lam)) significantly stimulated the growth of the leaves (leaf area) using vermiculite as substrate with respect to the control in agar. Also, Iarema et al. (2012) noted that the photoautotrophic conditions developed for the micropropagation of the Brazilian ginseg (*Pfaffia glometata* (Spreng.) Pedersen) appears to increase the leaf area of *in vitro* plants using culture medium solidified with agar. In *Limonium* spp. plants, Lian et al. (2002) report that growth in photoautotrophic conditions, the growth of the surface area of the leaf and the number had a superior effect. In *Doritaenopsis* orchid under photoautotrophic culture conditions and with increased CO_2 in the culture vessel, also achieved the best results with respect to heterotrophic culture conditions for the variables leaf area and length (Shin et al., 2013). This was also observed in the present study on papaya. However, photoautotrophic culture conditions are not also suitable for the growth of some *in vitro* plants cultured as in the case of coconut (*Cocos nucifera*

L.), where without the presence of sucrose in the culture medium, plants had a smaller leaf area with respect to those grown with sucrose (Fuentes et al., 2005).

In the treatment without sucrose and with IBA after 17 days of culture, 50% of the papaya plants rooted, and the evaluation done at 37 days it reached 80.0%. Also, the treatment with 10 g L^{-1} of sucrose and IBA, similar results were obtained, but with lower values in all the evaluations done (Table 2). The rooting percentage previously expressed, reiterates the value of this auxin as an inducer of the process with enhanced effects through the lack of or low levels of sucrose in the culture medium. In addition, the results of this study suggest that continuous exposure to the auxin (IBA) improves the frequency of root formation, which is observed in the results obtained at 17, 27 and 37 days of culture (Tables 2, 3, 4 and Figure 1F). Plants grown without sucrose and with IBA also obtained the highest values in the variables height and fresh weight at 37 days of culture (Table 4). This behaviour is good evidence of the effectiveness of the culture conditions and zeolite as a substrate, ventilation of the culture vessels, IBA as a rooting inducer, and the absence of sucrose probably favored the source-demand effect so that photoautotroph would enhance the rooting stimulus of auxin. Several authors note the superior effect that IBA has with respect to other auxins for rooting of *in vitro* shoots in papaya (Yu et al., 2000; Teixeira da Silva et al., 2007; Kumar et al., 2012; Nzilani et al., 2013; Sekeli et al., 2013). These last authors reported that the best results in the rooting of *in vitro* shoots of transgenic papaya, Eksotika variety were achieved when first placing the shoots in culture medium with 9.8 µM IBA and in darkness for 4 days and thereafter were subcultured on MS culture medium at 50% of salt concentration, 10 µM of riboflavin and vermiculite as support.

According to George (2008), IBA easily becomes IAA causing a slow release of IAA and thus providing a continuous supply of the most common active auxin in concentrations, which may be more suitable for rooting. Calamar and de Klerk (2002) report the interaction between sucrose and auxin in the rooting of apple shoots. Increasing the sucrose concentration shifted the dose-response curve of auxin. The auxin concentration at the maximum response obtained, indicates that the cells require more auxin to give the response and / or that less free auxin reaches the target cells. There are reasons to believe that sucrose enhances the sensitivity to auxin. The results obtained in this study are contrary to reported by these authors. For sucrose concentrations studied (0, 10 and 20 g L^{-1}) without auxin rooting percentages of papaya shoots were very low or zero. However in the presence of auxin (IBA) root formation was achieved, but the percentage of rooting were decreasing in the middle that sucrose concentration was increased. It seems that for these photoautotrophic culture conditions attached to the zeolite as porous support had a positive effect on the rooting of shoots than in mixotrophic culture condition with 10 and 20 g L^{-1} of sucrose. About it, Malamy and Ryan (2001) reported that when *Arabidopsis* seedlings are grown on nutrient media with a high sucrose to nitrogen ratio, lateral root initiation is dramatically repressed. Auxin localization appears to be a key factor in this nutrient-mediated repression of lateral root initiation. They isolated a mutant, *lateral root initiation 1* (*lin1*) that overcomes the repressive conditions. This mutant produces a highly branched root system on media with high sucrose to nitrogen ratios. The *lin1* phenotype is specific to these growth conditions, suggesting that the *lin1* gene is involved in coordinating lateral root initiation with nutritional cues.

Fujiwara and Kozai (1995) report that the use of porous support material improves the environment in the root zone and thereby increase rooting. A high porosity of the culture medium increases the concentration of oxygen around the rooting system, enhancing root development and improving water and nutrient absorption by the *in vitro* plants. In addition, the extensive root system produced *in vitro,* appears to contribute to the higher percentage of plant survival during acclimatization to greenhouse conditions and in the field. The results described in this study may be related to the characteristics conferred by the zeolite. According to Flores et al. (2007), zeolite drastically reduces the leaching of potassium cations (K +) and ammonium (NH_4+), also it facilitates solubilization of phosphate by the available phosphorus to plants and therefore stimulates radical development. Zeolite is a crystalline hydrated aluminum silicate with three-dimensional structures, characterized by the ability to hold and release water and exchange ions without modifying their atomic structure, exchange cations such as Ca_2+, Mg_2+, K+ and NH_4+; and various phosphate compounds, ammonium and organic matter components. It has a rigid three-dimensional structure formed by a network of interconnected tunnels creating a large surface area for the cation exchange and moisture adsorption. Similar results in terms of correlation between improved root system, improved growth and high survival rate were obtained in other plant species such as acacia (*Acacia mangium*) (Ermayanti et al., 1999); coffee (*C. arabusta*) (Nguyen et al., 1999); sweet potatoes (*I. batata* L. Lam.) (Afreen-Zobayed et al., 1999); *Eucalyptus* sp. (Zobayed et al., 2001); four Australian papaya varieties (Kaity et al., 2009) and in the variety Eksotika (Sekeli et al., 2013); Orchid (*Doritaenopsis sp.*) (Shin et al., 2013) using different types of porous materials (vermiculite, perlite and mixtures of both). This response was also reached in this work in papaya plants using the porous zeolite mineral as substrate. The presence of contaminants (bacteria and fungi) in all the treatments was quantified (Table 5). It is noteworthy that despite the increased exchange through the lid of the culture vessel, the visual presence of contamination in the treatments without sucrose was 0% for the total time of the experiment of 37

Table 5. Contamination in the culture vessel with increased ventilation at different days of culture during rooting and *in vitro* acclimatization of papaya plants.

Treatment		Percentage contamination (%)		
IBA(μM)	Sucrose (g L^{-1})	17 days	27 days	37 days
0	0	0	0	0
0	10	0	0	100
0	20	0	0	100
9.8	0	0	0	0
9.8	10	0	0	80
9.8	20	0	0	100
9.8 (Agar control)	40	0	0	0

Table 6. Effects of the interaction sucrose and IBA on the concentration of chlorophyll a and b and total carotenoids content in the leaves of *in vitro* papaya (*Carica papaya* L. var. Red Maradol) plants in culture vessels with increased ventilation and zeolite as a support at 17 days of culture.

IBA (μM)	Sucrose (g L^{-1})	Chlorophyll a (mg g^{-1}FW)	Chlorophyll b (mg g^{-1}FW)	Carotenoids (mg g^{-1}FW)
0	0	0.842 ±0.07[a]	0.519±0.04[a]	0.397±0.03[a]
0	10	0.407±0.17[a]	0.343±0.14[a]	0.296±0.05[a]
0	20	0.554±0.03[a]	0.526±0.02[a]	0.358±0.00[a]
9.8	0	0.578±0.15[a]	0.551±0.10[a]	0.311±0.07[a]
9.8	10	0.464±0.14[a]	0.449±0.13[a]	0.266±0.08[a]
9.8	20	0.765±0.08[a]	0.682±0.15[a]	0.319±0.07[a]
9.8 (Agar control)	40	0.599±0.07[a]	0.471±0.70[a]	0.286±0.14[a]

Different letters within a column indicate significant differences at p ≤ 0.05 by Tukey test (n=15). Values ± SD.

days. The treatments with sucrose showed no contamination during the assessments done at 17 and 27 days, but at 37 days, which shows the level of air cleanliness in the culture chamber and that the conditions and management developed can be used for further scaling.

Physiological parameters: Chlorophyll a, b and carotenoids

In terms of the quality of *in vitro* plants, the effects of the factors involved in the management of the contents of the active pigments in the photosynthetic process are analyzed. No significant differences were noticed for the variables chlorophyll a, chlorophyll b and carotenoids for the factors auxin and sucrose for *in vitro* shoots of papaya at 17 days of culture between the different treatments and the control (Table 6); significant differences were however observed in the assessments at 27 and 37 days as shown in Figures 2 and 3. At 27 days of culture there was a significant interaction between auxin and sucrose factors influencing the variable chlorophyll a. In Figure 2A as seen, when there was no sucrose in the culture medium without auxin, the plant produces significantly more chlorophyll a. Also, at 27 days there was a significant interaction between auxin and sucrose factors influencing the response of the variable chlorophyll b. As shown in Figure 2B when no sucrose was added to the culture medium, the plant produces significantly more chlorophyll b when there was no auxin than when the medium was supplemented with it. In the presence of sucrose, production levels of chlorophyll a and b decreased independently of the presence of auxin. With increasing levels of sucrose in the culture medium, production levels of chlorophyll a and b remain low regardless of the presence or absence of auxin. The response is quite similar for both molecules in this species. There was no interaction between the factors sucrose and auxin, only that sucrose was significant, influencing the response of the variable carotenoids content. When no sucrose was added to the culture medium, the plant produces significantly more carotenoids. As long as there is sucrose in the culture medium, this production was reduced significantly, although between the concentrations between 10 and 20 g L^{-1} of sucrose this decrease is not significant (Figure 2C).

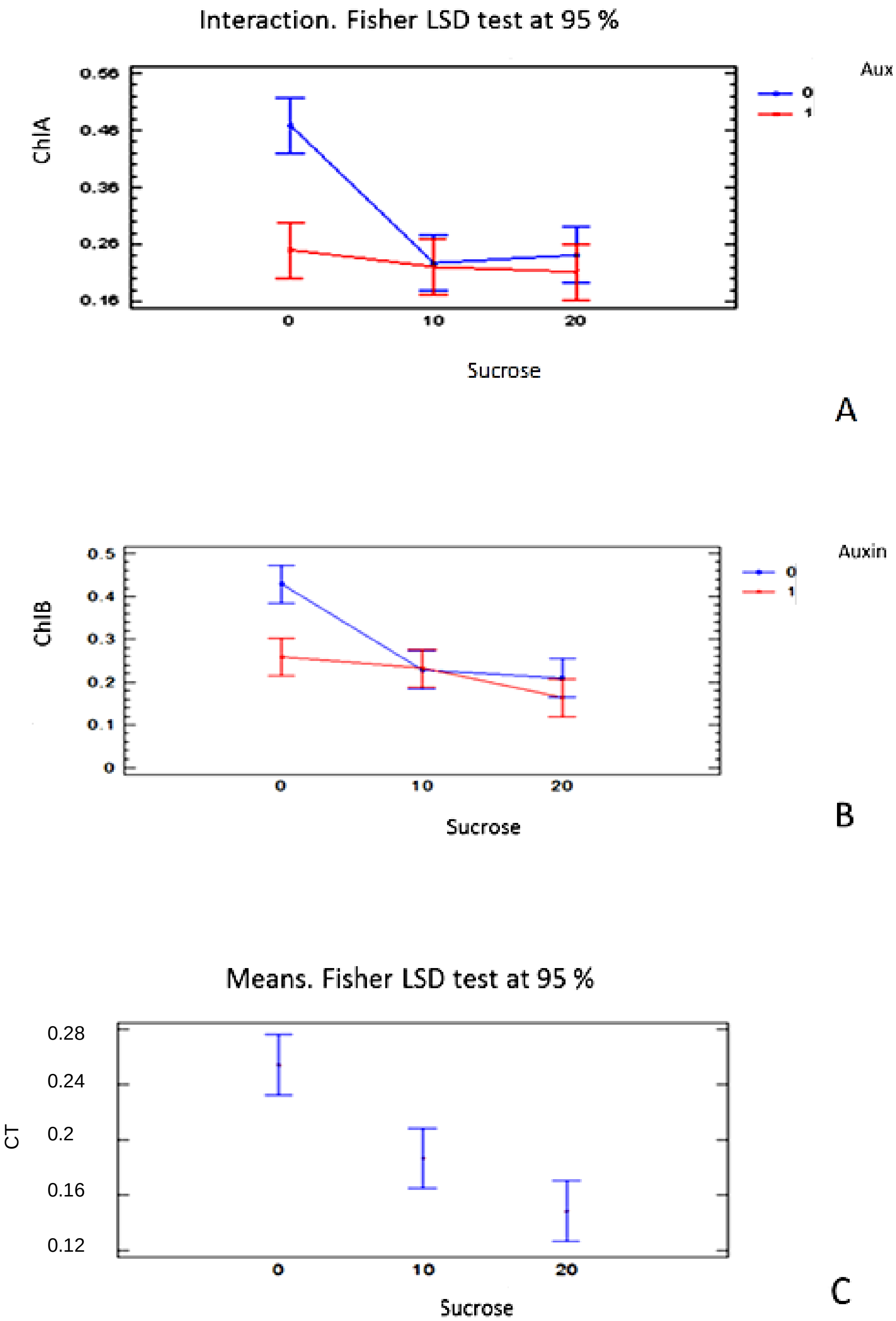

Figure 2. Effect of sucrose and IBA on the concentration of chlorophyll a, b, and total carotenoids content in the leave of *in vitro* papaya (*Carica papaya* L. var. Red Maradol) plants growing in culture vessel with increased ventilation and zeolite as support at 27 days de culture (A) Chlorophyll a (B) Chlorophyll b and (C) Carotenoids content. Reference: (0) without IBA and (1) 9.8 µM of IBA. Statistical difference between means according to the Fisher LSD test at $p \leq 0.05$.

At 37 days of culture, there was a significant interaction between the factors sucrose and auxin influencing the response of the variable chlorophyll a (Figure 3A). When there is no sucrose in the culture medium, the plant produces significantly more chlorophyll a than when there is no auxin. In the presence of sucrose, the production

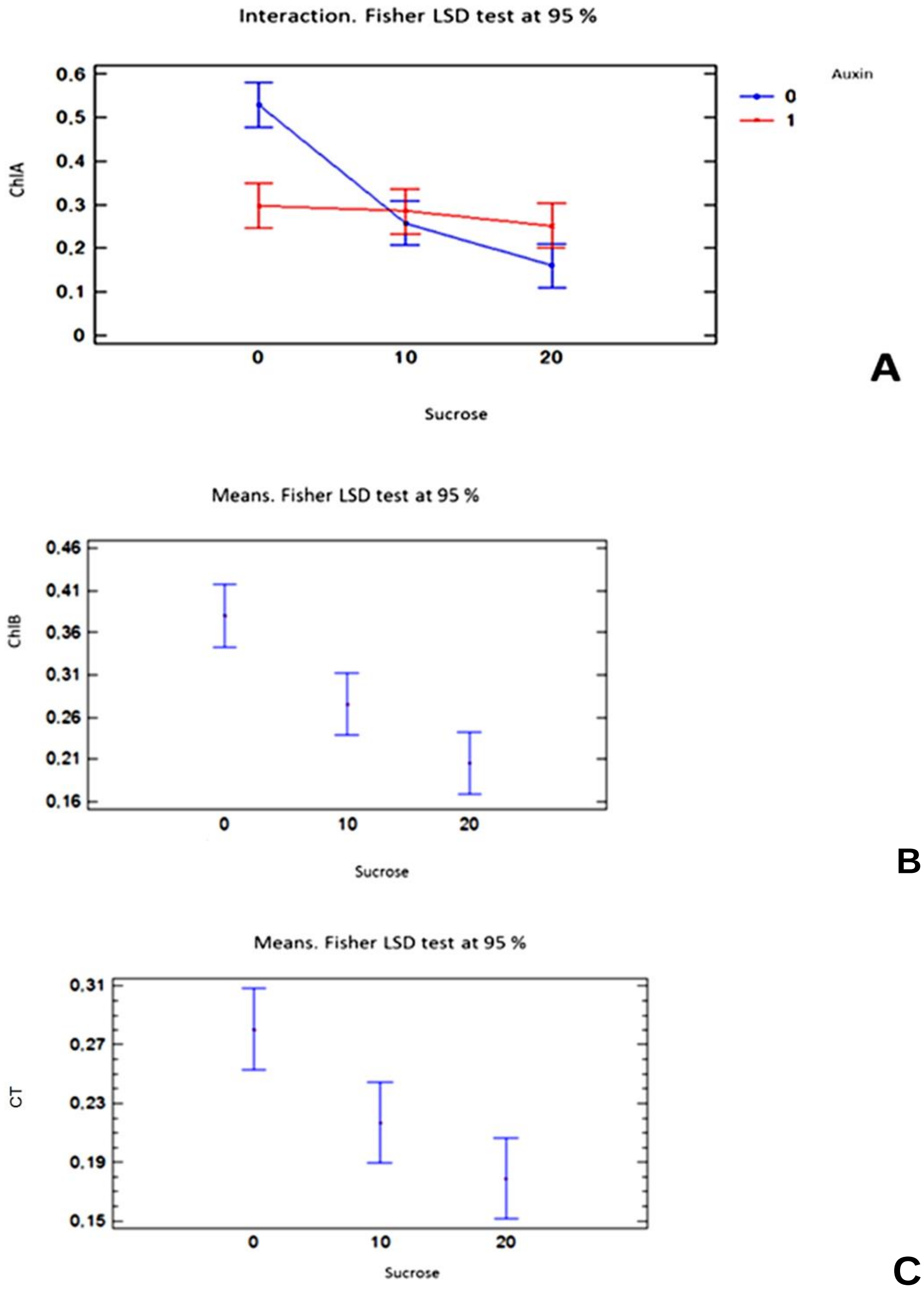

Figure 3. Effect of sucrose and IBA on the content of chlorophyll a, b, and total carotenoids content in the leave of *in vitro* papaya (*Carica papaya* L. var. Red Maradol) plants growing in culture vessel with increased ventilation and zeolite as support at 37 days of culture (A) chlorophyll a (B) chlorophyll b and (C) carotenoids content. Reference: (0) without IBA and (1) 9.8 µM of IBA. Statistical difference between means according to the Fisher LSD test at $p \leq 0.05$.

levels of chlorophyll a decreased regardless of the presence of auxin, but this decrease is significant in the case that there is no auxin for the greater concentration for sucrose, without differences in the case of 10 g L^{-1}. Sucrose alone had a significant influence on the content of chlorophyll b (Figure 3B). When there was no sucrose

Table 7. Effects of the interaction sucrose AIB on the photosynthetic activity (μmol CO_2 $m^{-2}s^{-1}$) and transpiration (mmol H_2O $m^{-2}s^{-1}$) in *in vitro* papaya plants (*Carica papaya* L. var. Red Maradol) cultured in culture vessels with increased ventilation and zeolite as a support at 17 days of culture.

IBA (μM)	Sucrose (g L^{-1})	Photosynthesis (μmol CO_2 $m^{-2}s^{-1}$)	Transpiration (mmol H_2O $m^{-2}s^{-1}$)
0	0	8.360±3.05[ab]	11.221±1.05[a]
0	10	3.766±1.56[b]	3.335±0.81[e]
0	20	3.611±1.30[b]	2.767±1.28[f]
9.8	0	8.892±1.47[a]	4.701±0.91[c]
9.8	10	8.957±1.30[a]	3.881±0.73[d]
9.8	20	8.716±1.65[a]	1.936±1.07[g]
9.8 (Agar control)	40	3.643±2.75[b]	8.194±1.56[b]

Different letters within a column indicate significant differences at p ≤ 0.05 by Tukey test (n=15). Values ± SD.

added to the culture medium, the plants produced significantly more chlorophyll b. As long as there is sucrose in the culture medium, this production decreased, without being significant between the concentrations of 10 and 20 g L^{-1} sucrose. Sucrose alone had a significant influence on chlorophyll b at 37 days of culture. It is observed that when there is no sucrose in the culture medium, the plant produces significantly more chlorophyll b. Sucrose also had a significant effect influencing the variable carotenoids. When there was no sucrose in the culture medium, the plant produces significantly more carotenoids (Figure 3C). As long as there is the presence of sucrose in the culture medium, the production of these chlorophyll pigments decreased significantly, even though between the two concentrations of sucrose this decrease is not significant.

Physiological parameters: Photosynthesis and transpiration

Best photosynthetic values were achieved when the *in vitro* shoots were grown in culture medium with auxin and different concentrations of sucrose, even though they were also high in the treatment without the presence of IBA and without sucrose at 17 days of culture. In photomixotrophic culture conditions, transpiration levels were low with respect to the heterotrophic and photo-autotrophic conditions. This is due to the presence of sucrose in the culture medium which the plant used as an energy source and it was not required for an increase in the photosynthetic activity and thus the opening and closing of the stomata, which made the transpiration levels so low. Nevertheless, the lowest levels of transpiration was obtained in plants grown in 20 g L^{-1} sucrose since the osmotic potential of the culture medium was higher, therefore for *in vitro* plants it is more difficult to take up water and hence transpiration rate was lower. For photoautotrophic conditions in the absence of the auxin, the plants did not have any roots at 17 days of culture, which resulted in a high rate of photosynthesis, but also high transpiration and having no roots to take up water for photosynthesis they had to have a greater stomata activity for the intake of CO_2, causing a greater transpiration (Table 7).

Plants grown in photomixotrophic conditions and without auxin, presented the lowest values of photosynthesis. In this regard, Rolland et al. (2002), Amiard et al. (2005); Jo et al. (2009) refer to plants that were grown in culture medium with sucrose, exhibited reduced photosynthesis, probably due to the presence of a sufficient energy source (sugar) and other metabolic activities. Franck et al. (2006) reported that sucrose plays a central role in the mechanism mediating control of the regulation by decreasing photosynthesis. The low rate of substrate regeneration for the carboxylation of ribulose bisphosphate (RuBP) due to the accumulation of soluble sugars in the leaves is the possible result in the inhibition of photosynthesis (Azcon-Bieto, 1983).

However, results obtained by these authors indicate that a greater amount of starch granules found in the chloroplasts of leaves of plants grown in the greenhouse probably were part of the storage product. On the contrary, in *in vitro* seedlings they did not show any starch granules, probably because the rate of photosynthesis is low or exogenous sucrose caused a negative feedback on the enzyme level of the plastid for starch biosynthesis (Krapp and Stitt, 1994). However, plants grown in photomixotrophic conditions and auxin, had high photosynthetic rate equal to those grown in photoautotrophic conditions, this might be because these plants began to develop their rooting system, which offset the loss of water for photosynthesis, making efficient use of water (Table 7).

Photosynthesis in plants grown on agar (heterotrophic control) was very low compared with their high transpiration rate, a reason that adds to the justification for the zero survival assessed at 17 days after planting in the acclimatization phase. This demonstrates the low ability to control water loss of these plants in heterotrophic culture conditions. The levels of photosynthetic pigments also corresponded with this result given their involvement in the photosynthetic process. When sucrose was zero, the contents of carotenoids and chlorophylls were high and also appear to achieve good performances of the collecting antennas and the light producing complexes which integrate the photosystems involved and which constitute the pigments analyzed and other components. Although, the content of chlorophyll is not a direct indicator related to the photosynthetic capacity (Fujiwara et al., 1992); this is a good indicator of the state of the photosynthetic apparatus (Seon et al., 2000). This happened when the *in vitro* papaya plants were evaluated at 17 days of culture in the different treatments with and without photoautotrophic culture conditions where there were no significant differences observed among them; however, when determining the photosynthetic rate, there were significant differences between the different treatments as shown in Table 7. In this regard, Iarema et al. (2012) obtained the same response on analyzing the content of chlorophyll pigments and carotenoids of *in vitro* plants of Brazilian ginseg [*P. glometata* (Spreng.) Pedersen] cultured in the absence of sucrose and in the culture vessel with greatest level of exchange or ventilation and hence had an increase in the photosynthetic activity.

However, other authors reported the increase of photosynthetic pigments and increased photosynthetic activity in *in vitro* shoot cultured of *Limonium* spp. (Lian et al., 2002) and in *Dendrobium candidum* Wall. ex Lind (Xiao et al., 2007). The results obtained in this study support those reported by Kozai and Kubota (2005) on the benefits of photoautotrophic micropropagation over conventional micropropagation. The benefits from a biological point of view include: (1) promoting growth and photosynthesis; (2) high rates of survival and a smooth transition to environmental conditions *ex vitro*; (3) elimination of morphological and physiological disorders; (4) no callus formation at the base of the explant and (5) less plant lost due to contamination by microorganisms.

Ex vitro acclimatization

The treatment without sucrose and 9.8 µM IBA reached the highest percentage of survival which are suitable for rooting percentage that had *in vitro* papaya plants at 17 days of culture (Table 2 and Figure 1F). The treatments with the presence of auxin had the highest percentages of rooting, which corresponded to those of the greatest survival. The problem of very low survival is confirmed if appropriate management strategies are not performed that guarantee better quality of *in vitro* plants, with emphasis on their rooting pattern, and treatments without IBA at 17 days of culture (Table 2). Afreen-Zobayed et al. (2000) report that, in sweet potato 90% achieved survival of plants cultured *in vitro* in photoautotrophic conditions compared to 73% of those grown on agar. Kozai et al. (2005) reported in the species Calla Lily (*Zantedeschia elliottian* L.), 95% survival (photoautotrophic conditions) at 12 days after transplanting to acclimatization phase relative to 60% of plants grown in heterotrophic conditions. Also, in the species China fir (*Cunninghamia lanceolata* (Lambert) Hooker) only 16% survival in *in vitro* plants cultured in heterotrophic conditions was obtained and 95% in photoautotrophic. However, Jo et al. (2009) report that the best results in *ex vitro* acclimatization was reached for *Alocasia amazonica* plants cultured with 3.0% sucrose and not those that were cultured in autotrophic conditions.

Conclusion

The management of papaya plants var. Red Maradol obtained through somatic embryogenesis during the transition *in vitro-ex vitro* integrated by using zeolite as a support, the combination of zero or low levels of sucrose, increased ventilation and use of auxin IBA (9.8 µM) as an inducer of rooting, improve the quality of the plants and thus their survival.

Conflict of interests

The authors did not declare any conflict of interest.

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The effects of exotic weed *Flaveria bidentis* with different invasion stages on soil bacterial community structures

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A new exotic weed, *Flaveria bidentis*, is spreading in central China where it forms dense monospecific patches modifying the structure of different native ecosystems and threatening native aboveground biodiversity. However, little is known about the consequences of such an invasion for soil bacterial community, especially its effect pattern at different invasion stages. In this study, soil samples were taken in native ecosystems that were uninvaded, partially invaded (transition), and severely invaded by *F. bidentis*. The bacterial richness and diversity in *F. bidentis* in rhizospheres soil was evaluated using denaturing gradient gel electrophoresis (DGGE) analysis. Different stages of *F. bidentis* invasion can trigger changes in soil physicochemical properties in particularly in available N and P *F. bidentis* invasion significantly decreased the richness of soil bacterial community, and the decline contents were positively correlated with invasion progress. In the invaded soils, bacterial species in *Proteobacteria*, *Chloroflexi* and *Actinomycetes* decreased with invasion, with the greatest reduction in relative abundance occurring for *Proteobacteria*, which was the dominant species in the native soils. Invasion of *F. bidentis* corresponded with an alteration in the structure of soil bacterial community, and soil microbial biomass as well, thus soil environment modification was expected to promote spreading of this exotic weeds in turn.

Key words: Biological invasion, *Flaveria bidentis*, soil nutrients, soil bacteria, polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE).

INTRODUCTION

Biological invasion has become a global ecological and economic problem. Understanding the impact of invasive species on the local ecological systems has gained increased attention in invasive ecology (Ehrenfeld et al., 2001). Invasive species employs very complex and multiple mechanisms and strategies (Mack et al., 2000). Once establishing in a new environment, exotic weeds can replace the native species, causing above vegetable

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Abbreviations: MBC, Microbial biomass carbon; **MBN,** microbial biomass nitrogen.

structural changes in the native ecological system. Soil microorganisms play an important role in the successful spread of exotic weeds (Hierro et al., 2005). Altered soil microbial communities and resulting effects on ecosystem processes may be an invisible legacy of exotic weed invasions by rhizosphere microbe and the host plants have an inter-dependent and mutual constraint relationship, which is the reason that why certain rhizosphere microbial communities only co-exist with specific plant species (Schloter, 2003). It has also been suggested that exotic weeds could disrupt mutualistic associations within native microbial communities (Richardson et al., 2000; Callaway and Ridenour, 2004; Stinson et al., 2006). Alteration in the structure and function of soil microbial communities could eventually lead to changes in the vegetation structures (Callaway et al., 2013). Among soil microbial communities, bacteria associated with plant roots are fundamentally important in plant nutrition, growth promotion, and disease interactions (Marschner et al., 2001). For this reason, there has been considerable interest in characterizing the structure and function of rhizosphere communities. The bacterial community composition in the rhizosphere is important for the performance of the plant, as bacterial species can have beneficial, neutral or harmful relationships with the roots (Atkinson and Watson, 2000; Sylvia and Chellemi, 2001). It is well accepted that different plant species can be selected for specific rhizosphere communities (Burke et al., 2002; Costa et al., 2006). The differences in root-derived substrates are claimed to explain the plant specific rhizosphere bacterial communities that have been observed for different plant species grown under similar conditions (Marschner et al., 2001; 2002; Smalla et al., 2001).

A new exotic weed, *Flaveria bidentis* (L.) Kuntze, commonly called “yellowtop”, is increasing spreading in central China. This species originated from South Africa and was first found in 2001 in suburbs of Tianjin and a few cities of Hebei province (Liu, 2005). It invades roadsides, abandoned field or even arable fields, out-competes natural vegetation, and forms a dense population (Huangfu et al., 2011). This weed tolerates environmental stresses of salinity and cold temperature, and could become troublesome for the development of sustainable agriculture (Gao, 2004). The function and population of rhizosphere microbes also undergo various alterations to allow the establishment of the invasive species. However, there is little information on the impact on soil bacterial diversity and mechanism upon invasion by the exotic weeds (Lorenzo et al., 2010).

This study aimed (1) to examine the effects of different stages of *F. bidentis* invasion on soil physicochemical properties and (2) detect the effects of different degrees of *F. bidentis* invasion on the community structure of bacteria in soils. We hypothesize that (1) increasing stages of *F. bidentis* invasion enhance soil nutrient element concentrations (especially soil N) because invasive plants have high nutrient cycling rates, especially for N (van Kleunen et al., 2010; Laungani and Knops, 2009; Jones and Chapman, 2011), and that (2) *F. bidentis* invasion significantly increases the richness and diversity of the soil bacterial community along the invasion gradients. Also, the changes in soil bacterial communities were associated with soil physicochemical properties. Towards these aims, we used the PCR-DGGE approach together with cloning and sequence analysis of 16S rRNA fragments of soil bacteria upon the invasion process by *F. bidentis*. In our findings will provide fundamental knowledge for soil bacteria diversity upon invasion by alien plant species.

MATERIALS AND METHODS

Site description and sampling

The sampling sites were collected in wasteland ecosystems, a typical system *F. bidentis* infestation (Zhang et al., 2010), located in the Xian County in north China (38° 15′ 30″N, 115° 57′ 50″E) with temperate continental monsoon climate, mean annual precipitation of 560 mm, mean annual temperature of 12.3°C, and its average frostless period lasts 189 days. Geographically, the experimental site had flat land, a uniform landscape, and a similar terrain and soil origin with very minor disturbance by human and animals, and very minimal habitat variation. The soil is alluvial type where *F. bidentis* plants grown as monocultures had formed alternate successions. The following three sites (soil types) with three different levels of invasion by *F. bidentis* were sampled: (a) native soils (the control) mainly dominated by native herbaceous plants, including *Setaria viridis* (L.) Beauv, *Digitaria ciliaris*, *Phragmites australis* and *Echinochloa crusgalli* with coverage of over 60%; (b) transition soils where *F. bidentis* plants covered 10 to 30% of the plot, and (c) invaded soils where *F. bidentis* covered over 60% of the plot. Soil samples were collected on August 10 in 2009. In each of the sampling sites, six plots (repetitions) were randomly chosen each covering 3 × 3 m area with about 10 to 20 m apart from each other. The five points Quincunx sampling scheme was used to collect soil samples in each plots at the 0 to 20 cm depths, and soils within the same plot were pooled and mixed together equally as one replication, thus 18 soil samples collected in total were placed in plastic bags for transport to laboratory. Prior to sampling, all plants and organic matter debris on the ground were removed. Samples were stored at -20°C until analysis. From 1000 g of each soil sample, 20 g were homogenized and subsamples of 5 g were taken for further analysis. To verify the impact pattern found with this invasive plant, sampling was done in following year. Twice sampling data was pooled for soil physicochemical parameters analyses and only once PCR-DGGE fingerprinting was presented given the fact that there were no inter-year differences between treatments detected.

Determination of soil nutrients

Soil NH_4^+ and NO_3^- were extracted by shaking 20 g of fresh soil in 100 ml of 2 M KCl solution for 1 h. Soil extracts were analyzed with the FIAstar 5000 Auto Analyzer system. Total N and P in soil samples were analyzed with oven-dried samples, 48 h at 70°C. The Kjeldahl method was used for analyzing the total nitrogen (N) content of the soil. Soil mineral N was extracted using 2 mol L^{-1} KCl, then the concentrations of NO_3^--N in the KCl extracts were determined by hydrazine sulfate colorimetry and the concentrations of NH_4^+-N by indophenol blue colorimetry (Mulvaney, 1996). Total phosphorus (P) was extracted using the $HClO_4$-H_2SO_4 method, and available P was determined using the sodium bicarbonate method.

DNA extraction from soil samples

Total DNA was isolated from soil samples using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., CA), following the instruction of the maximum yield method. After a final purification, the soil DNA was visualized on 1% (w/v) agarose gels to assess its purity and molecular size. The final DNA extracts obtained from the soils were color-free, indicating that they did not contain high amounts of humic compounds.

Specific PCR of 16s rRNA gene fragments

For amplification of 16s rRNA fragments, a pair of universal primers consisting of the 357f- GC and 518r (Muyzer et al., 1993) were used to amplify the V3 region of bacterial 16S rRNA. Primer sequences were 357f- GC (5'-GCclamp-CCTACGGGAGGCAGCAG-3'), and 518r (5'-ATTACCGCG GCTGCTGG-3'). The PCR reaction were carried out in a final volume of 50 μl containing 2 μM of $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, 50 ng of isolated DNA, 5 μl 10 × PCR buffer, and 2.5U of Ex Taq™ polymerase (TaKaRa Inc., Dalian, China). Touch-down PCR procedure was performed for increasing both the specificity and sensitivity of PCR assays in a thermal cycler (Bio-Rad) (Labbe et al., 2007). After preincubation at 95°C for 5 min, samples were amplified with denaturation for 1 min at 94°C, annealing for 1 min (temperature decreasing 0.5°C per cycle from 65 to 55°C, and then 15 cycles at 55°C), primer extension for 3 min at 72°C, followed by one final extension at 72°C for 15 min. Aliquots (5 μL) of PCR mixture were examined by electrophoresis in an agarose gel (1%, w/v) stained with ethidium bromide to check fragment size and integrity.

DGGE Patterns

DGGE was performed with 8%(w/v) acrylamide gels containing a linear chemical gradient ranging from 40 to 60%.The gels were allowed to polymerize overnight. DNA samples containing 20 μl of the PCR products were electrophoresed in 1× TAE buffer at 60°C at a constant voltage of 120 V for 8 h, and all DGGE analysis was done in the Dcode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gels were stained for 30 min with SYBR gold nucleic acid gel stain (Invitrogen Molecular Probes, Eugene, USA) (10,000-fold diluted in 1× TAE) and photographed under UV light with a video imaging system. Band detection and quantification of band intensity was performed using Quantity One 4.62 software (Bio-Rad, USA). DNA band intensity was normalized by dividing the band intensity of each band by the mean band intensity of the gel. Therefore, both band position and intensity are expressed as relative values. Each peak represents individual groups of species having 16S rRNA sequences with similar melting behavior. The band intensity indicates the relative abundance of the group under these PCR conditions.

Sequence analysis of DGGE bands and phylogenetic analysis

Selected DGGE bands that occurred in majority of samples were excised from the gels and eluted (Kowalchuk et al., 1997). The criteria for selection of bands were that (i) they appeared as a single band in the pool of lanes, (ii) they represented bands in high abundance in the community or (iii) they were of relatively low abundance in the DGGE pattern. It is noteworthy that, in some cases where multiple clones were generated from a given excised band, more than one phylotype was detected from that band. In total, we sequenced 20 different clones, corresponding to 17 excised DNA fragments. These sequences of 16S rRNA genes obtained were submitted to the GenBank to determine the closest known relatives of the partial 16S rRNA sequences and the phylogenetic affiliations are shown in Table 2. Eluted DNA was then amplified using the 518r and 357f primer pair without GC clamp, and PCR products were ligated onto pMD19-T vector (Takara) and transferred into *Escherichia coli* JM_{109} competent cells. After positive cloning selection, the white colonies were further screened with vector primer pMD19-T to confirm the positive clones. The positive colonies were cultured in LB broth overnight at 37°C with constant shaking. Aliquots of 500 μL bacterial stocks were mixed with sterile glycerine (50%) and stored at -70°C. The clones of each of excised bands were chosen for sequencing. Sequencing was carried out at Shanghai Biotech Company. Searches in GenBank with the BLAST program (Altschul et al., 1997) were performed to determine the closest known relatives of the partial 16S rRNA sequences obtained. Multiple alignments of the sequences were performed using Clustal X (Thompson et al., 1997). A phylogenetic tree was constructed by the neighbor-joining method in MEGA 4.1 (Tamura et al., 2007). The confidence values for the branches of the phylogenetic tree were determined using bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. The similarity between sequences was calculated using the GENETYX computer program (Yumoto et al., 1999).

Measurement of soil microbial biomass carbon and nitrogen

Microbial biomass carbon (MBC) and nitrogen (MBN) were measured by a chloroform-fumigation extraction method modified from Vance et al. (1987). Six aliquots of wet soil from each sampling replications equal to 20 g dry weight were placed into 100 ml beakers. Three samples were fumigated whereas the other three were not. Soil was placed on top of the internal shelf in a vacuum desiccators drier that had internal diameter of 29 cm. Below the shelf, 60 ml HPLC grade chloroform and glass beads (to prevent explosion) were put into a 100 ml beaker. After addition of 50 ml 1 mol/L NaOH, the soil was covered with a few layers of wet filter paper to maintain moisture content of the steamed soil. After sealing the drier with Vaseline, vacuum was turned on and chloroform started to boil. Degas was topped after 5 min, and the samples were stored in darkness at 25°C for 24 h. After removal of the beaker containing chloroform, the soil was degassed again to remove chloroform residuals. The non-steaming treated soil was placed into a separate drier, and the chloroform was replaced with distilled water. After putting the fumigated soil into a 150 ml flask and addition of 60 ml 0.5 mol/l K_2SO_4 (soil: water = 1:4), the mixture was shaken at 25°C and 200 rpm for 30 min. The extracts were filtered through mid-speed filter paper, and the filtrates were measured immediately or stored at -15°C for later analysis. Soil microbial biomass carbon and nitrogen contents were measured using a multi NC3100 TOC/TN instrument (Analytik Jena AG, Germany). MBC and MBN of soil microbes were calculated using the differences in organic carbon and nitrogen between fumigated and non-fumigated soil, divided by the conversion factor of 0.45 (Joergensen, 1996). Data from the same soil type were pooled for analysis.

Statistical analysis

One-way ANOVA and Duncan's Test as post hoc test were used to check the differences in soil nutrients and microbial biomass C and N, bacterial richness and diversity between different soil types. Richness, defined as number of species, was calculated as the total

Table 1. Changes in soil nutrient contents of different type of soil in *F. bidentis* invaded area.

Type of soils	NS[a]	TS	IS
Organic matter ($g{\cdot}kg^{-1}$)	76.02±0.83[b]	77.56±0.06[b]	80.36±0.51[a]
Total N ($g{\cdot}kg^{-1}$)	0.81±0.02[b]	0.86±0.01[b]	1.00±0.01[a]
Total P ($g{\cdot}kg^{-1}$)	0.75±0.05[a]	0.73±0.41[a]	0.64±0.13[a]
NH_4-N ($mg{\cdot}kg^{-1}$)	7.40±0.49[c]	12.92±1.53[b]	19.33±0.81[a]
NO_3-N ($mg{\cdot}kg^{-1}$)	2.30±0.03[c]	4.72±0.06[b]	6.28±0.06[a]
Available P ($mg{\cdot}kg^{-1}$)	9.20±0.25[a]	6.50±0.10[b]	4.63±0.03[c]

[a]IS, invaded soils; TS, transition soils; NS, native soils; [b]different lowercase letter within same row means difference at *P*=0.05 level.

Table 2. Phylogenetic affiliation of sequences retrieved from DGGE bands.

Band	Closest relative	Similarity %	Accession number
1	*Kaistobacter* sp.	99	FJ889336.1
2	*Bacteroidetes* bacterium clone	100	GU552199.1
3-7	*Actinobacterium*	98	EU328001.1
3-9	*Legionella* sp.	98	GU598197.1
4-1	Uncultured bacterium clone	99	EU133920.1
4-2	*Nitrosospira* sp. clone	100	GU472862.1
5	*Acinetobacter* sp.	97	GU827519.1
6	*Mycobacterium houstonense*	94	EU977810.1
7	*Chloroflexus* sp.	100	AB257633.1
8	gamma proteobacterium	94	FJ568411.1
9	*Devosia* sp.	100	FN594685.1
10	*Streptomyces rochei* strain	100	HM153701.1
11	*Rhodocyclaceae* bacterium	99	EF606817.1
12	*Bacillus* sp.	98	EU043767.1
13	*Chloroflexaceae* bacterium enrichment	94	EU918581.1
14	*Acidobacteriaceae*	99	AM936873.1
15-17	*Chelatococcus* sp	98	AM412118.1
15-19	Uncultured bacterium clone	97	EU546566.1
16	*Nocardioides* sp.	100	GU202324.1
17	Uncultured alpha proteobacterium clone	98	GU552196.1

number of bands per sample, and diversity, defined as number of different species and their relative frequency (Lorenzo et al., 2013). Based on DGGE results, cluster analysis of soil bacteria was performed using the Ward's method by the Quantity-One software (Bio-Rad). The Shannon–Wiener index of general diversity, *H* was calculated using the following function:

$$H=-\Sigma P_i \log_2 P_i \quad (1)$$

Where, *Pi* is the importance probability of the bands in a track. *H* was calculated on the basis of the bands on the gel tracks using the intensity of the bands as judged by peak heights in the densitometric curves (Ampe and Miambi, 2000). The importance probability *Pi* was calculated as:

$$Pi=ni/\Sigma n\ i \quad (2)$$

ANOVA was performed with the SPSS 17.0 for Windows software package, and mean comparison was done using the least significant difference (LSD) test at $P < 0.05$.

RESULTS

Changes in soil nutrient contents status

Compared with native soils, the *F. bidentis* invaded soils had obviously higher contents of soil organic carbon, total nitrogen, NO_3^-, and NH_4^+, but lower contents of soil available phosphorus (P<0.05). For example, it was increased by 5.7 and 23.4% in soil organic carbon and total nitrogen, respectively, while available phosphorous was reduced by 49.6% in invaded soil ($P < 0.05$, Table 1).

Impact of invasion by *F. bidentis* on bacteria diversity

16S rRNA fragments amplified from DNA extracted directly from soil samples were compared and only three

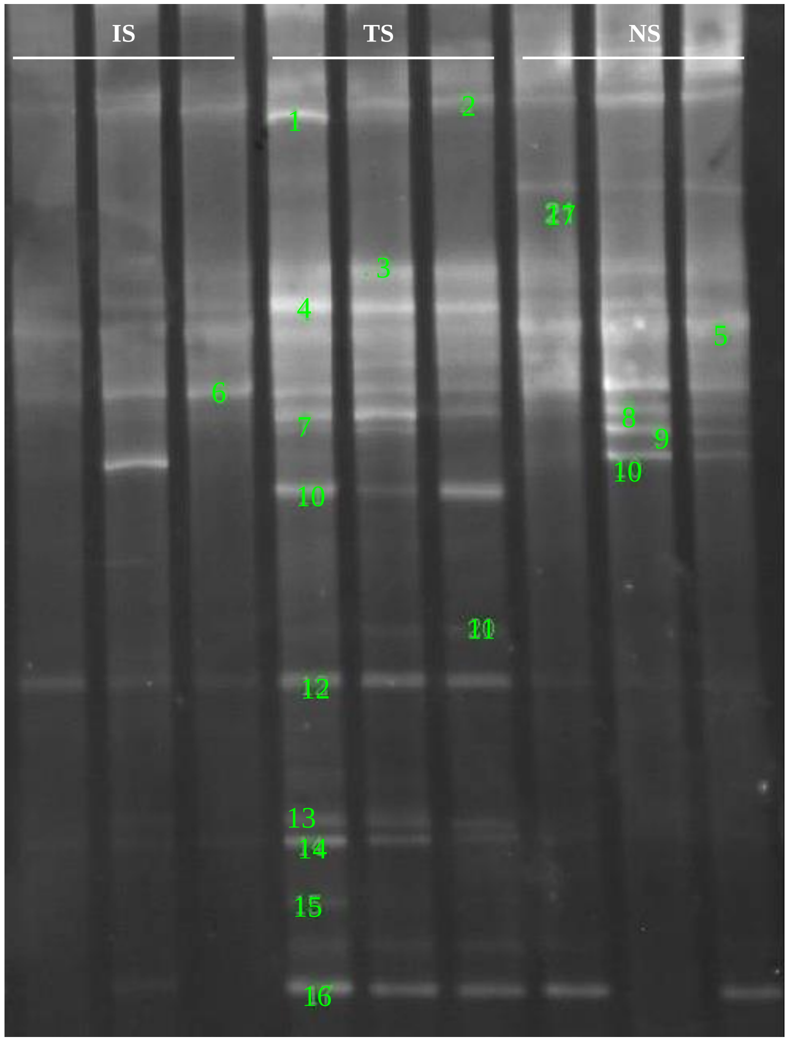

Figure 1. DGGE analytical result of bacterial 16S rRNA V3 fragments in different type of soil in *Flaveria bidentis* invaded area. IS, invaded soils; TS, transition soils; NS, native soils.

replications of each site were presented due to a relatively high similarity of the DGGE patterns obtained for each of the replicates, which also suggested a low degree of variability caused by sampling (Figure 1). DGGE profiles of amplified 16S rRNA fragments from DNA extracted from the rhizosphere bacterial fractions revealed significant differences of the bacterial fingerprints from different *F. bidentis* invasion stages. Both the strains and number of bacterial reduced in well-invaded soils (Figure 1). Bands that were shared among all the soil samples included bands 2, 4, 6, 12 and 16, indicating that bacteria carrying these genes were common to all types of soil and were not affected by the invasion. The *H'* were ranked in descending order as native soils (2.96) > transition soils (2.58) > invaded soils (2.33) ($P < 0.05$). Consequently, compared with the control (native soils), the invasion of *F. bidentis* reduced bacterial diversity with invasion progress.

Analysis of the DGGE profiles found were different in soil bacterial community between different soils. The transition soils and the native soils were clustered firstly at similarity index of 0.89 as one group, while the invaded soils distinctly separated from them with similarity of 0.68 ($P < 0.05$). The impact of this exotic weed on soil bacteria was a continuous process; as the invasion intensified some bacterial strains diminished in the soil. By comparing sequencing results, it was found that 20 sequences belonged to six different bacterial phyla with the majority in the division of *Proteobacteria* (Table 2). The similarity of the closest relatives of the partial 16S rRNA fragments of all sequenced bands ranged between 94 and 100%. Bands 2, 4-2, 7, 9, 10 and 16 showed the highest sequence similarity of 100%, respectively. On the other hand, other bands have various similarities with the

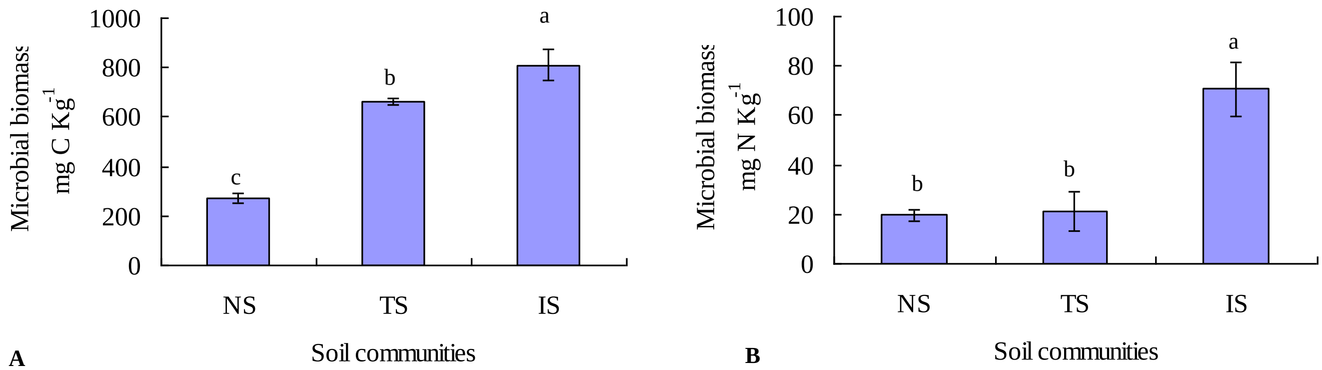

Figure 2. Mean microbial biomass (±SE) carbon (a), and nitrogen (b) for the different soil communities. NS, native soils; TS, transition soils; IS, invaded soils.

similar sequences in the NCBI database. The bands 1, 11 and 14 showed the higher sequence similarity (99%) with those of genus *Kaistobacter*, *Rhodocyclaceae* and *Acidobacteriaceae*, respectively, while bands 6, 8 and 13 only got 94% similarities with assigned sequences, respectively. According to the intensity of the band, a bacterial species that exhibited the higher sequence similarity to genus *Kaistobacter* (99% similarity) (band 1) was one of the most predominant during invasion of *F. bidentis*.

Impact of *F. bidentis* invasion on soil MBC and MBN

This result indicates that *F. bidentis* invasion led to alteration of microbial carbon metabolism in the soil (Figure 2). Upon the invasion of *F. bidentis*, soil MBC increased accordingly. The MBC content was ranked in descending order as following: invaded soils>transition soils> native soils. MBC was almostly 200% higher in the invaded soils than that of native soils (Figure 2a, $P < 0.05$). The same pattern was found for MBN which increased significantly after invasion by the exotic weed (Figure 2b). However, non-significant effect was detected between the transition soils and the native ones in MBN. Based on MBC and MBN, invasion of *F. bidentis* increased soil nutrient level as suggested in Table 1.

DISCUSSION

The results obtained partially support our original hypothesis. Firstly, invasion of *F. bidentis* lead to significant increases in soil N, organic matter, but decrease in available P. Because the genus of *Flaveria* is extensively cloned by arbuscular mycorrhizal fungi, the fungi known to help plant uptaking phosphorus from soils that are P-deficient for plant growth (Aziz et al., 1995; Bagayoko et al., 2000), further research should focus on possible competitive relationship in uptake of phosphorus between *F. bidentis* and native plant species. As a result, the decrease of phosphorus was attributed to the high uptake by this exotic weeds and the competition of exotic weeds with the soil community. Secondly, we found that invasion of *F. bidentis* was associated with significant increases in total soil N, C, organic matter and exchangeable P, but reduced soil bacterial diversity index, contrary to findings of Sanon et al. (2009). According to the positive feedback hypothesis, exotic weeds may cause soil-based ecosystem processes change following invasion, and such changes could establish positive feedbacks that enhance the spread of the exotic (Ehrenfeld et al., 2001). Our study suggests that number and diversity of soil bacteria changed, that the invasion of *F. bidentis* propagated certain groups of bacteria while suppressing others. Even some bacterial were common to all type soils, but plant species will eventually alter structure of soil bacterial community (Briones et al., 2002).

Many phytopathogenic organisms, bacteria as well as fungi, have coevolved with plants and show a high degree of host specificity (Raaijmakers et al., 2009). As the invasion progressed, the soil bacterial community structures also underwent significant changes as suggested by similarity analysis of the DGGE fingerprints where invaded soils clustered separately from native soils in the cluster analysis.

A decrease of bacterial diversity and increase of microbial biomass could be also caused by an increase of fungal biomass (Schimel et al., 1999). Alteration of native microbial community composition may further decrease competition from native plants and therefore support *F. bidentis* dominance as suggested by Rudgers and Orr (2009). *F. bidentis* can release allelopathic compounds (kaempferol, quercetin) (Xie et al., 2010; Iwashina, 2003), which may inhibit the growth of many microorganisms. It was found that extracts of *F. bidentis* from both leaves and roots reduced seed germination and seedling growth of native plant species (Huangfu et al., 2011). Therefore, allelopathic compounds produced by *F. bidentis* may be responsible for alterations in microbial biomass pools but further study is needed.

Our previous study has shown that *F. bidentis* invasion significantly decreases soil pH values (Zhang et al., 2010). This result may be mainly attributed to the fact that this invasive plant has high ammonium uptake rates as suggested by our study (unpublished data). The metabolic activities and community structure of soil microorganisms were highly correlated with soil pH values (Hackl et al., 2005; Högberg et al., 2007). Thus, we believe that changes in soil pH values mediated by *F. bidentis* invasion can enhance the succession of soil microbial communities in the rhizosphere and facilitate further invasion. With the widespread introduction and invasion of exotic weeds there are many studies that investigate alteration of basic ecosystem structure and function.

However, studies concerning invasive processes, information about changes in the impact over time is rarely available (Souza-Alonso et al., 2015). Some studies found that changes in soil properties as C or N contents and microbial properties soil ecosystem parameters are more pronounced after a long period of invasion (Marchante et al., 2008). Nevertheless, recent findings suggest that both ecological and adaptation processes may increase or attenuate the impact of invaders on the resident community, and that the impact of an invasive species on soil characteristics and on the structure and function of microorganisms does not necessarily remain constant or accumulate over the course of invasion (Strayer, 2012; Dostál et al., 2013). Our study sought to determine the effects of different stages of plant invasion on soil bacterial communities to better understand the mechanism of plant invasion. Different stages of *F. bidentis* invasion can trigger changes in soil physicochemical properties, in particularly in available N and P. *F. bidentis* invasion significantly decreased the richness of soil bacterial community, and the decline contents were positively correlated with invasion progress. Changes in the soil physicochemical properties and community structure of soil bacterial communities mediated by *F. bidentis* invasion may play an important role in facilitating further invasion.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLGEDMENTS

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Extraction and characterization of *Retama monosperma* fibers

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The aims of this study were to determine the good conditions for fibers extraction from *Retama monosperma* leaves and their mechanical, physical and chemical characteristics. The fibers were extracted using a range of NaOH concentration from 1 to 16% in a period of treatment of 1 to 24 h, coupled with a physical treatment. For the evaluation of physico-mechanical characteristics, 200 samples were performed in the tensile test. The biochemical composition of the fibers was determined after separation of the parietal compounds. The results show that the best fiber yield was 11.51% obtained by a treatment of 14% NaOH for 8 h, followed by a physical treatment. The fibers biocomposition was 87.3% of cellulose, 7.5% of hemicelluloses and 1% of lignin. The Young's modulus was 13.3 GPa, tensile strength was 110 MPa and density was 1.3 g/cm^3. The average fiber length was 155.7 mm. The fibers yield and characteristics showed that *R. monosperma* plant may in future be suitable source for natural fibers.

Key words: *Retama monosperma* young stems, fibers, extraction, characterization.

INTRODUCTION

The composites industry began since the 20th century, and in the same time, fibers industry saw an exponential growth. In that period, the most fibers used frequently in composite industry were synthetic fibers such as carbon, graphite, and glass fibers. The impact of this industry on the environment was very heavy. Today, natural fibers seem to be an effective solution for the production of fully biodegradable materials for replacing of some synthetic fibers (Belaadi et al., 2013; Mylsamy and Rajendran, 2010). Among the natural fibers, vegetable fibers have many advantages; availability, recyclability, low-cost, eco-friendly, no toxicity, biodegradability, mechanical performance and easy extractability (Bledzki and Gassan, 1999; Reddy and Young, 2005; Béakou et al., 2008). Without cotton and wood fibers, the annual world production of vegetable fibers is 6200 kt (Rajendran, 2011). The Jute fibers are half of the global production, followed with coco 16 and flax 13% (FAO, 2010). At present, the most used plant in the extraction of fibers are sisal, hemp, flax and bamboo by using different plants parts such as: bast, leaf, seed, fruit, wood, stalk, and grass fibers (Mawaikambo, 2006). Currently, researchers do many studies on the characterization of new lignocellulosic fibers as Okra (De Rosa,

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2010; De Rosa, 2011), *Posidoniaoceanica* (Khiari et al., 2011), *Artichoke* (Fiore et al., 2011) and *Grewia tilifolia* (Jayaramudu et al., 2010). In Algeria, there are many plants which can be used in the extraction of fibers (Kaid-Harche, 1985; Kaid-Harche et al., 1990; Benahmed et al., 2006) but until now there are any studies on these plants. In this study we focused on *Retama monosperma*; this plant is natively from North Africa and some parts of southern Europe. In Algeria, this plant occupies a considerable area (Thoma, 1968). To the best of our knowledge, actually, the only use of this plant is for dune fixation and for fighting against desertification.

The aims of this study were the establishment of a fiber extraction protocol from *R. monosperma* leaves and characterization of extracted fibers. In general, for fibers extraction there are three different ways; chemical, mechanical and biological. In this study we used coupling techniques, chemical and physical. After extraction, the fibers were characterized with the different tests.

MATERIALS AND METHODS

Plant materials

In this study, we used a freshly harvested young leaves.

Fibers extraction

Physicochemical procedure

For the first time, an extraction protocol of *R. monosperma* fibers is established. The determination of the extraction parameters appears mandatory to define the appropriate protocol for the best fibers yield. The procedure used to obtain fibers is based on the principle of treatment combination, (chemical and physical) unfolding in four steps: pretreatment, chemical dissociation, physical dissociation and post-treatment.

Pretreatment

Pretreatment aims to eliminate the protoplasmic content. To this end three samples of 25 g were studied: i. the first sample (T1), was treated for 24 h with a mixture of chloroform / methanol (v/v); ii. the second sample (T2), was placed in an acetone bath for 10 min followed by another bath of isopropanol for 10 min as well. The sample was then transferred into an ethanol bath for 20 min at 90°C and last, it was washed with water; iii. The third sample (T), non-treated was used as a control.

Chemical dissociation

Alkaline dissociation was carried out by using sodium hydroxide (NaOH) at 14% for 24 h at 70°C. Then, the three samples were washed with water to neutralize them, and to finally separate the fibers.

Physical dissociation

It consists in proceeding to an autoclaving. Pressure and temperature are important factors in fibers dissociation. The three samples underwent the same treatment for 30 min at a pressure of 1.0 bar and a temperature of 121°C.

Post-treatment

After drying in ambient air, the fibers were separated by a manual carding. This step consists in removing the impurities and obtaining fine fibers.

Optimization of extraction conditions

Effect of NaOH concentration

Different concentrations of NaOH were tested (4, 6, 8, 10, 12, 14 and 16%) to determine the optimal concentration for the best dissociation of fibers. The treatment for each one has been 24 h.

Effect of processing time

The experience to determine the reaction time of the alkaline solution was carried out at different periods: 1, 2, 3, 4, 6, 12 and 24 h.

The effect of pressure

The experiment was conducted on two samples; one underwent a pressure of 2.2 bar at a temperature of 121°C, while the second (control) underwent no pressure.

Fiber characterization

Density

The fibers were dried at 100°C for 24 h, cut at the same length and put in pycnometer for density.

Fibers tenacity

The strength of fibers was determined with a Zwick tensile testing machine. The fibers were placed with clips between two rods separated with 2 cm. A tensile force was applied to the fiber breakage. The test was repeated 200 times.

Fineness

According to Fiore et al. (2011), fiber fineness was defined by the separation degree expressing the number of fiber bundles contained in 1 mg of raw material. The fibers were manually parallelized and cut to a length of 1 cm each. The fiber bundles were placed one by one on a balance with a clamp, until the weight of all fibers reached 1 mg. The number of fibers counted represents the separation degree.

Swelling test

Swelling of fibers due to water absorption was observed with a microscope provided with graduate objective. Three fibers removed from fiber bundles were placed in parallel direction on a glass slide. The fiber diameter was measured after 2 h distilled water immersion. Fibers diameter measurements were taken before and after immersion. The percentage in fiber diameter due to swelling was determined on 30 different fibers.

Table 1. The result of the extraction of fibers.

Sample	Weight (g)	Yield (%)
T	1.41	10.6
T1	1.27	9.6
T2	1.07	6.72

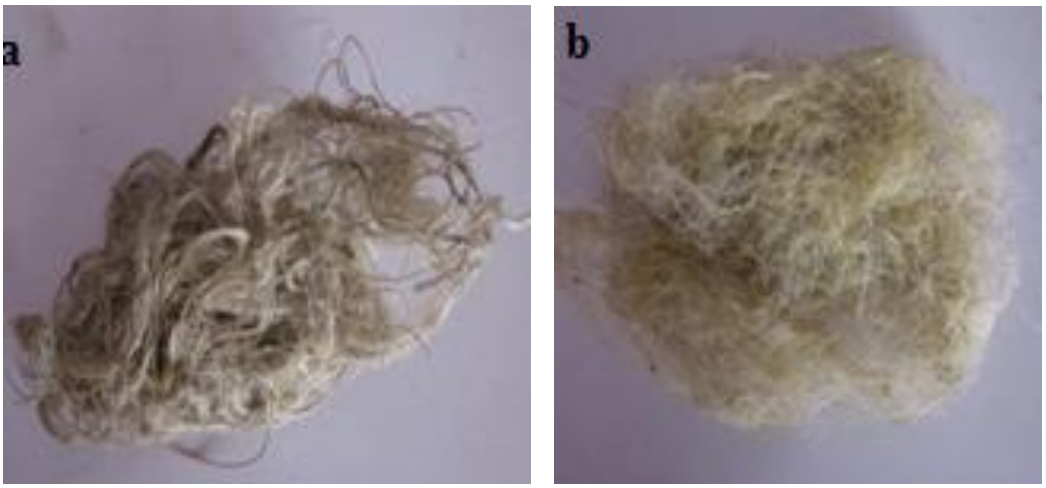

Figure 1. Fibers aspect. **A.** Before combing. **B.** After combing.

Floatability

Thirty single fibers were subjected to the following treatment: with a metal hook, one fiber was placed horizontally to the surface of deionized water till the fiber touched the water surface. The hook was removed gently, and the behavior of the fiber was observed (folating or sinking) for 5 min.

Chemical composition (Chaa et al., 2008)

A method based on the compounds selective dissolving was applied to determine the amount of each ligno-saccharide components (lignin, pectin, cellulose and hemicelluloses) presents in *R. monosperma* fibers.

Fibers biometrics

Fiber’s length and width of 30 fibers and fibrils were determined under microscopes provided with graduated objective.

RESULTS AND DISCUSSION

Extraction of fibers

Chemical dissociation

In Table 1, the obtained results show that the first batch of samples T (non-treated) presented a yield of 10.6% (w/w) which is higher than the batches T_1 (9.6%, w/w) and T_2 (6.72%, w/w). The batch T_2 gave the lowest yield of fibers. Treatment with acetone and isopropanol caused a considerable decrease in the yield which was estimated at 3.88% (w/w) compared with the untreated samples T (Table 1). T_1 also presented a low fiber yield compared to untreated samples. These results show that the preprocessing step for the removal of cellular contents leads to lower fiber yield. That proves that in the process of extracting fibers, sodium hydroxide was sufficient to eliminate the cell content and also to partially separate the fibers.

Physical dissociation

Autoclaving combines pressure and temperature. These two factors are important in the separation of fibers. Such treatment followed by a carding allows to separate fibers from impurities to obtain fine fibers ready to be used in the industry (Figure 1). Table 2 shows that most of the plants used in the production of fibers have yields which do not exceed 9% except for the case of banana leaves, while the *R. monosperma* gave a higher yield 10.6% (Table 1). This is explained by its high fiber content and their facility to extract.

Table 2. Extraction rate of fibers in several plant species (Arsene et al., 2007).

Fiber	Extraction (%)
Bagasse	4.00
Feuille de Bananier	9.84
Tronc de bananier	4.46
Coco	8.77
Tissu de coco	1.74
Eucalyptus	8.20
Sisal	1.33

Table 3. Yield fibers according to the concentration of NaOH.

NaOH (%)	Weight (g)	Output (%)
16	1.30	5.2
14	2.40	10.6
12	2.29	9.16
10	1.72	6.88
08	1.54	6.16
04	1.25	5.00
03	1.25	5.00
02	1.04	4.16
01	0.0	0.00

Optimization of the extraction of the fibers

Effect of the concentration of NaOH

The results obtained (Table 3) show that the yield of fiber was positively proportional to the concentration of sodium hydroxide. The best yield obtained was 10.60% with a concentration of 14% of Soda. Beyond this concentration, the yield decreases. It is also important to note that at a concentration less than 4% of sodium hydroxide, the separation of the fibers is achieved only if a mechanical pressure is applied, and at a concentration of 1%, the separation of fibers is impossible. These results show that the concentration of sodium hydroxide has an effect on the separation of fibers. This phenomenon is due to the swelling of the cellulose fiber after the relaxation of the natural crystalline structure of the cellulose. Fengel and Wegener (1983) have reported that the different alkali solution (KOH, $Ca(OH)_2$, NaOH) and its concentration have an effect on the degree of swelling and in the transformation into cellulose-II which affects the quality of the fibers. Also, author researchers reported that treatment with $Ca(OH)_2$ decreased the tenacity of fibers more than treatment with NaOH (Arsene et al., 2007). The treatment with sodium hydroxide changes the topography of the surface of the fibers, removing the components of the cuticle, the pectin, and partially the lignin and the hemicelluloses (Mwaikambo et al., 1999).

Table 4. Yield fibers according to treatment time.

Treatment time (h)	Output (%)
05	0.0
08	5.6
12	4.4
21	4.4

The recent studies have shown that Na+ has a favorable diameter for penetrate between crystalline structures and with presence of water molecules to create spaces. In this structure, the -OH groups of the cellulose are converted to -ONa groups, expanding the dimensions of the molecules as it showed on the following reaction:

$$\text{Cell-OH + NaOH} \longrightarrow \text{Cell - O}^{-}\text{Na}^{+}\ \text{H}_2\text{O+ [surface impurities]}$$

Subsequent washes with water will remove the Na-ion bonds. NaOH allows a complete transformation of cellulose I network to cellulose II, unlike other alkaline solutions that only lead to a partial transformation of the network (Johnson, 1979; Shenouda, 1979).

Effect of treatment time

Table 4 shows that treatment for 8 h in sodium hydroxide gave the best results than the others treatment time. During the extraction, the reaction of sodium hydroxide disassociates the fibers by breaking the bonds between lignin and polysaccharides of the cell walls (Wang and Sain, 2007). The extraction rate changes in relation to the concentration of NaOH and processing time. Table 4 shows that the ideal time for *R. monosperma* fibers extraction was 8 h. More or less than 8 h processing time, the yield was low, because less than 8 h was insufficient for the reaction of fiber extraction and over than 8 h, the NaOH has degraded the cellulosic fibers, which was undesirable. Sandy and Bacon (2001) reported that alkaline extraction can cause degradation of the cellulose leading to the extraction of nanofibers.

Effect of pressure

The pressure is also an essential element for a good separation of fibers; the pressure facilitates the separation of fibers. The Application of pressure of 2.2 bars gave a yield of 11.51%, whereas without autoclaving the yield was 9.48%.

Fiber's characterization

Mechanical characteristics

The results of tensile test shows that Young's modulus of

Table 5. Mechanical properties of *Retama monosperma* and some principal fibers (Bledzki and Gassan, 1999; Sandy and Bacon, 2001; Bismarck et al., 2005; Elenga et al., 2009; Elenga, 2009; Agu, 2014).

Fiber	Strength (MPa)	Elongation to failure (%)	Young's modulus (GPa)
Retama monosperma	110	4.6-4.7	13.3
Flax	345-1035	1.3-3.3	27.6
Sisal	600	3	12
Jute	396-773	1.5-1.8	26.5
Hemp	690	1.6	30-60
cotton	287-597	7-8	5.5-12.6
Raffia textilis	148-660	2	28-36
Raffia farinifera	500	4	12.3
Kenaf	700	3	55

Table 6. Density of some vegetable fiber (Sandy and Bacon, 2000; Béakou et al., 2008; Elenga et al., 2009).

Fiber	Density (g/cm^3)
Retama monosperma	1.3
Flax	1.5
Sisal	1.5
Jute	1.3
Hemp	1.15
Cotton	1.5-1.6
Rhectohyllum camerunese	0.947
Raffia texillia	0.75

Table 7. Water absorption of some vegetable fibers (Savastano et al., 1999; Ramakrishma and Sundararajan, 2005; Toledo Filho et al., 2005).

Fiber	Water absorption (%)
Retama monosperma	53
Sisal	190-250
Bamboo	145
Jute	281

13.3 GPa was found for *R. monosperma* fibers (Table 5). Compared to the *Raffia farinifera* (28-36 GPa), Young's modulus value of *R. monosperma* fiber was lower and it was about half that of Jute fiber (26.5 GPa) and Flax fiber (27.6 GPa). But it was higher than cotton which its Young's modulus ranged between 5.5 and 12.6 GPa according to the literature (Agu, 2014). Thus, the *R. monosperma* fibers appears to be more flexible than *R. farinifera,* flax and jute but more rigid than cotton. *R. monosperma* fibers tensile strength was 110 MPa (Table 5). Mechanical properties have a direct relationship with cellulose crystallinity (Sanadi, 2004; Sena Neto et al., 2013), length (Morlier and Khenfer, 1991), microfibrillar angle, cellulose content, molecular structure (Mukherjee and Satyanarayana, 1986), and fibers orientation (Djoudi et al., 2009). The elongation to failure was about 4.6 to 4.7%. It was higher than Flax (1.3 to 3.3%), Sisal (2 to 2.5%) and Hemp 1.6% but it was lower than cotton (7 to 8%).

Density

R. monosperma fibers density was 1.3 g/cm^3. Table 6 shows that *R. monosperma* fibers density was same like that of jute and sisal fibers but lower than Flax and Sisal fibers density. *Raffia texillia* fibers density is lower than all vegetable fibers (Elenga et al., 2009). There are a negative correlation between the density and Young's modulus. When the density is lower, the young's modulus and strength are higher. In general, vegetable fibers present densities lower than synthetic's fiber like glass fibers (2.5 g/cm^3) (Bledzki and Gassan, 1999).

Swelling test using optical microscope

The absorption capacity of *R. monosperma* fibers was lower than that of all vegetable fibers represented in Table 7. It was three times lower than Bamboo fibers.

Floatability

The test shows that *R. monosperma* fibers had a hydrophobic character, which was due to its hydrophobic surface. The hydrophobic surface and the limited absorptive character may be due to treatment with NaOH.

Chemical composition

Polysaccharides composition of *R. monosperma* fibers was: 87.3% cellulose, 7.5% hemicelluloses, 4.2% pectin

Table 8. Fiber wall chemical composition (Bledzki and Gassan, 1999).

Fiber	Lignine	Hemicellulose	Cellulose
Flax	2	12	64.1
Sisal	9.9	12	65.8
jute	11.8	12	64.4
Ramie	0.6	13.1	68.6
Retama monosperma	1	7.5	87.3

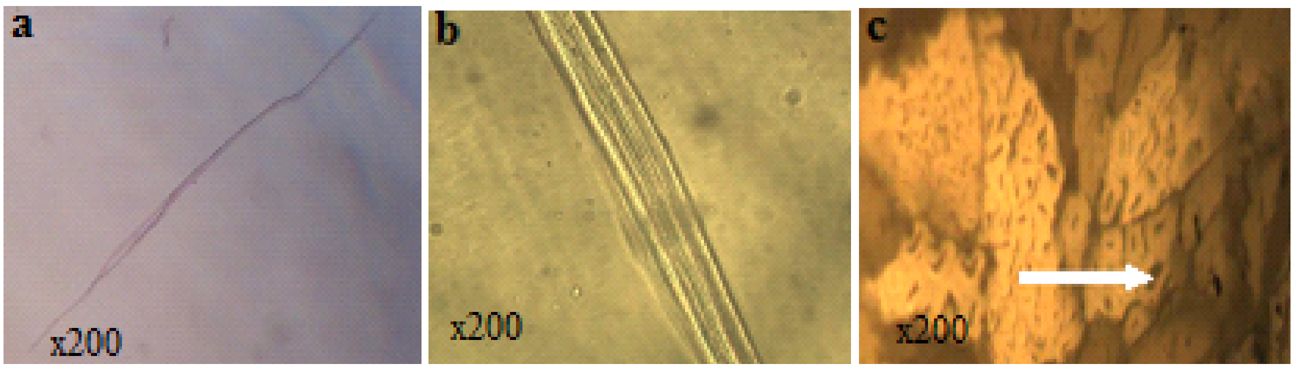

Figure 2. Microscopic observation of *Retama monosperma* stems and fibers. **a)** Microscopic observation of fiber cell of *Retama monosperma* (x200). **b)** Cross section of fibrous bundles *Retama monosperma* (x200). **c)** Micoscopic observation of *Retama monosperma* fibers cross-section (x200) .

and 1% lignin. The results show that *R. monosperma* fibers were rich in cellulose 87.3%; richer in cellulose than Flax 64.1% and Ramie 68.6%. Fibers with a higher cellulose fraction are more suitable for fibrous applications (Sena Neto et al., 2013). Cellulose is the main structural component of the lignocellulosic fibers, as it provides strength and stability to the cell walls and to all the fiber structure (Paster et al., 2005). Therefore, the cellulose content in a fiber or fiber bundle affects its properties and consequently, its applications. On the contrary, the percentage of the fraction of the hemicelluloses of *R. monosperma* fibers was lower compared to other plants (Table 8).

Morphometric characterization

R. monosperma fibers had an average length of 155.7 mm (Table 1). Compared to kenaf fibers (3 to 7 mm) (James et al., 1999) and to cotton fibers (0.83 mm) (Ververis et al., 2004), *Retama monosperma* fibers were longer. This characteristic interested the textile and biocomposites industry. The morphological charac-teristics of fibers, length and width are important factors in mechanical characteristics of fibers (rigidity or flexibility). In general, the length of plant fibers is between 100 and 150 mm and width from 10 to 50 µm (Fogtdal, 1990) (Table 9).

Morphology and ultrastructure of R. monosperma fibers

Figures 2a and 2b shows the morphological difference between fiber and fiber cell. A fiber (Figure 2b) is composed of many fibers cell called elementary fibers (Figure 2a). The microscopic observation (Figure 2c) shows that elementary fibers have a lumen (indicated with arrow). This characteristic is very interesting for thermal and acoustic insulation. Although, no study has been performed on the insulation performance for each plant fiber. Kymäläïnen and Sjöberg (2008) and Hepworth and Brus (2000) reported that there are a link between fiber porosity and thermal property. The SEM observations show clearly the morphology, shape and microstructure of *R. monosperma* fibers (Figure 3a, b and c). One of these fibers was separated from the bundle (Figure 3, a, arrow).

Conclusion

This is the first published paper on the extraction of *R. monosperma* fibers from leaves. Our study shows that, this species is very rich in fiber and it is easy to extract them with an interesting yield compared to several plants already exploited, which makes possible its valuation for industrial purposes, especially, if it is a wild plant

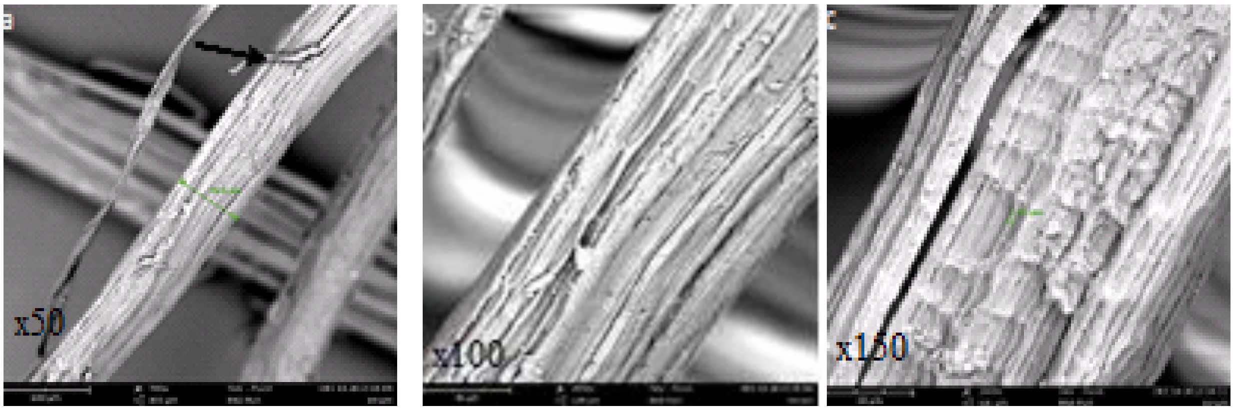

Figure 3. Scanning electron microscopy observation of *Retama monosperma* fibers. **a)** X50. **b)** X100. **c)** X150.

Table 9. Morphological characteristics of *Retama monosperma* fibers.

Average length of elementary fibers (µm)	Average length of fibers (mm)	Average width of elementary fibers (mm)	Average width of fibers (mm)
700	155.7	0.002	0.116

widespread in Algeria and which requires little water. Also, their fibers exhibit interesting properties such as higher cellulose content (86%), high elasticity (4.6 to 4.7%) and low density (1.3mg/cm^3). These characteristics enable *R. monosperma* fibers to be the preferable plant fibers in textile and composite industry. Finally, we recommend further studies for better understanding of the chemical, physical and mechanical characteristics. Furthermore, structural studies such as cellulose crystallinity as well as plant age and seasonal variation are needed for efficient exploitation of this species in Algeria.

Conflict of interests

The authors did not declare any conflict of interest.

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