



## RESEARCH PAPER

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## Micropropagation of tuna (*Opuntia ficus – indica*) and effect of medium composition on proliferation and rooting

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### Abstract

The goal of this study was to determine micropropagation system for a mass production of Tuna (*Opuntia ficus – indica*). For this reason, explants dissected from strilled young cladodes successfully established on Murashige and Skoog (MS) medium supplemented with 5 mg l<sup>-1</sup> Benzyl amino purine (BAP). MS medium containing different combinations of BAP (5 mg l<sup>-1</sup>) and Indole acetic acid (IAA) (0, 0.25, 0.5, 1, 2 mg l<sup>-1</sup>), BAP (5 mg l<sup>-1</sup>) and Naphtalene acetic acid (NAA) (0, 0.25, 0.5, 1, 2 mg l<sup>-1</sup>) and BAP (0.5 and 1 mg l<sup>-1</sup>) and Kinetin (0.5 and 1 mg l<sup>-1</sup>) were tested for shoot development. The best results for shoot development and elongation were obtained in media containing 5 mg l<sup>-1</sup> and 0.25 mg l<sup>-1</sup> NAA. The highest multiplication rate (3.9) was observed in media supplemented with 5 mg l<sup>-1</sup> BAP and 2 mg l<sup>-1</sup> of NAA. Satisfactory rooting was achieved in MS Basal medium (5-6 cm length) without callus formation. The percentage of rooting was 100% and Plants were successfully established in a mix of pit and perlite (2:1) (100%) and acclimatization accomplished under greenhouse condition. In this study, total concentration of carbohydrates and proteins were measured in *in vivo* cultured (control) and *in vitro* propagated tuna after 3 months. As a result, no significant differences were observed between control and micropropagated tuna in protein concentration. Whereas carbohydrate content in micropropagated plants (3.24 mg g<sup>-1</sup>) was 2- fold that of the control plants (1.52 mg g<sup>-1</sup>).

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## Introduction

*Opuntia ficus-indica* belongs to Cactaceae family and its Authority is Mill, common names are Indian Fig, Tuna Cactus, Mission Prickly Pear, prickly pear and Spanish tuna. That is a big, tree-like cactus that can grow quickly to 15 ft tall. The species is native to Mexico and it was introduced into southern Europe, Africa and India very long ago (Bein, 1996).

The genus *Opuntia* (Cactaceae) has a specialized photosynthetic mechanism known as Crassulacean Acid Metabolism (CAM), whereby these plants open their stomates and take up CO<sub>2</sub> at night. This attribute leads to reduced water loss (Nobel, 1995, Taiz and Zeiger, 1998). Regarding to its high water-use efficiency (even in areas with low annual rainfall values, 120-150 mm), and its high drought-tolerance (Le Hou  rou, 1994), this cactus is a most widely used forage resource in arid and semiarid region during periods of drought and shortage of herbaceous plants and has been extensively developed for decades.

Plants are succulent with jointed, branching stems. These stems, or joints, are often cooked as a green table vegetable (Russell and Felker, 1987). In addition to all these applications, some prickly pear cactus species can be exploited in the horticultural industry as ornamental resources by virtue of their bizarre and particular morphological traits including small overall plant and cladode size, spine color, cladode shape and growth habits, epidermis color, shape and length of spines, etc. Several species such as *O. pheacantha* Engelman, *O. engelmannii* Salm-Dyck, *O. violaceae* Engelman, *O. aciculata* Griffiths, *O. basilaris* Engelman & Bigelow, *O. ficus-indica* (L.) miller, *O. tunicata* (Lehm.) Link & Otto, *O. microdasys* (Lehmann) Lehmann, *O. basillaris*, *O. imbricata* C.C. Haw DC, *Opuntia lanigera* Salm-Dyck among others, are commonly used landscaping plants in public, private, commercial and residential properties in Mexico, the Mediterranean area, Australia and south-western USA (Irish, 2001). Over the past century there has been a dramatic increase for culture of plants that is known as a multi-purpose plant since

it can be applied as natural wind break barrier, soil stabilizer, re-vegetation resource to control water and wind erosion in eroded soils (Nobel, 1994). It can be cultured as crop for the production of fruits, vegetables and forage for animal feed or utilized as raw-industrial material to produce several subproducts such as wine, candies, jellies, flour, etc. (Hegwood, 1990, Flores-Valde  z, 1995, Sa  enz-Herna  ndez, 1995).

In general, prickly pear cactus species can be sexual and asexually propagated. Seed propagation is only used for scientific research to study genetic variability and factors impact on the germination process (Rojas-Are  chiga and Va  squez-Yanes, 2000). Vegetative propagation, which is widely utilized, can be performed through the rooting of single or multiple cladodes (Fabbri *et al.*, 1996, Lazcano *et al.*, 1999, Mulas *et al.*, 1992), small portions of mature cladodes derived from the dissection of tissues comprising two or more areoles (Barrientos and Brauer, 1964), or by consuming fruits. Despite all these methodologies that are easy to perform and efficient, their propagation rates are low and require large spaces for propagation. Others available asexual methods include apomixis (Garc  a-Aguilar and Pimienta-Barrios, 1996, Ve  lez and Rodr  guez, 1996, Mondrago  n, 2001), grafting (Pimienta, 1974, Maldonado and Zapien, 1977), micrografting (Estrada-Luna *et al.*, 2002), and tissue culture (Escobar-Araya *et al.*, 1986, Estrada-Luna, 1988, Mohamed-Yasseen *et al.*, 1995) have been conducted by the other investigators. The last method has recently pointed out as the most potent because it provides high propagation rates, reduced requirements for space, and the production of healthy and pathogen-free plants. Recent evidences revealed that micropropagation has been extensively studied and successfully developed on cloning many cacti species including prickly pear cactus (Escobar-Araya *et al.*, Estrada-Luna, 1988).

Regarding prickly pear cactus micropropagation, recent research advances show an increased interest

for the scientific community to integrate studies in order to improve the efficiency of the propagation process and establish and introduce reliable protocols for plant transformation to engineer selected genotypes (Llamoca-Za' rate *et al.*, 1999a, b, Silos-Espino *et al.*, 2006). The first study on *Opuntia* (prickly pear cactus) micropropagation reported by Sachar and Iyer, 1959, various successful strategies have been described for different species including *O. dillenii* Haw, *O. polyacantha*, *O. basilaris*, *O. amyoclaea* Tenore, *O. echios* var. *gigantea*, *O. ficus-indica* Linne' Mill, *O. streptacantha* Lemaire, *O. robusta* Wendland, *O. cochineria* Griffiths, *O. leucotricha* De Candolle, *O. albicarpa* Scheinvar, *O. ellisiana* Griff. (Mauseth and Halperin, 1975, Mauseth, 1977, 1979, Escobar-Araya *et al.*, 1986, Estrada-Luna, 1988, Mohamed-Yasseen *et al.*, 1995, Llamoca-Za' rate *et al.*, 1999a, Estrada-Luna and Davies, 2001, Jua' rez and Passera, 2002), however, a comprehensive protocol is not available yet because most plant responses to tissue culture are highly dependent on the genotype and some important modifications and adjustments might be performed when a new species or cultivar is considered for tissue culture, especially to optimize the overall environmental culture conditions, media, plant regulators (type, concentration, and combination), etc. during the shoot proliferation stage. Rooting and plantlet acclimatization conditions might also be investigated since they may limit the success of micropropagation (Hartmann *et al.*, 1997). So far, there has been little observation about protein and carbohydrate content of micropropagated *Opuntia*. The main purpose of this study was to develop efficient systems for *in vitro* propagation of *Opuntia* and investigation of protein and carbohydrate content of micropropagated plants.

### Materials and methods

Healthy young cladodes about 4–5 cm in length were collected and excised from donor plants previously cultured under greenhouse conditions, these plant materials had been transferred from Ghasreshirin city, West of Iran, to Agricultural Biotechnology

Research Institute of Iran during September and October 2011.

To clean the cladodes, the spines-hair were carefully trimmed with regular scissors to a minimum size without damaging any other tissue and washed with running water for 4 hours. The cladodes were immersed in Mancoseb fungicide (80%) for 15 minutes and rinsed with sterile water for 3 times and then soaked in ethanol (70%, v/v) for 1 min and washed with distilled water for 2 times and immediately subjected to a surface disinfecting treatment in 50% (v/v) sodium hypochlorite plus Tween-20 (0.1%) for 15 min, then rinsed three times in sterile distilled water.

After sterilization, the explants were cultured in petri dishes with 20 ml of (Murashige and Skoog) medium (MS). This was carried out under sterile condition in laminar air flow cabinet with a photoperiod of 16 h light and 8 h of dark at  $26 \pm 2^\circ\text{C}$ .

Pairs of areoles used as initial explants were dissected from the cladodes as described by Estrada-Luna, 1988, and cultured for 4 weeks in a MS (Murashige and Skoog, 1962) medium, which was adjusted to pH 5.7, and supplemented with 3% sucrose, 0.8% plant agar and different combination of benzyl amino purine (BAP) 3, 5, 7  $\text{mg l}^{-1}$ . These were heated in a microwave oven until the media was homogenous and autoclaved at  $121^\circ\text{C}$  for 15 min. Contents of Each flasks were dispensed into sterilized Petri dishes and sealed with parafilm.

After 3 days the cladodes were cut-off and transferred into the Petri dishes, these were sealed with parafilm. The Petri dishes were transferred to a growth chamber with a photoperiod of 16 h light and 8 h of dark at  $26 \pm 2^\circ\text{C}$ . Over a period of 30 days after inoculation, data on the number of days to shoot emergence, percentage survival were recorded for explants in the establishment media.

Activated buds were transferred to proliferation medium, explants were subjected to benzyl amino

purine (BAP) 5 mg<sup>l</sup><sup>-1</sup> and different combinations of naphthalen acetic acid (NAA) 0, 0.25, 0.5, 1, 2 mg<sup>l</sup><sup>-1</sup>, and by benzyl amino purine (BAP) 5 mg<sup>l</sup><sup>-1</sup> and different treatments of indole acetic acid (IAA) 0, 0.25, 0.5, 1, 2 mg<sup>l</sup><sup>-1</sup>, and also benzyl amino purine (BAP) 0.5, 1 mg<sup>l</sup><sup>-1</sup> and concentrations of kinetin 0.5, 1 mg<sup>l</sup><sup>-1</sup>. Growth regulators and sucrose were added before autoclaving. The cultures were maintained on a 16/8 light/dark cycle under fluorescent light at 26 ± 2 ° C. The number of explants used per treatment in each independent experiment ranged from 5 to 20. This experiment was conducted three times. The number of shoots produced by each explants was recorded after 8-12 weeks of incubation. Multiplication rate was defined as the number of newly formed shoots (> 2 mm) per initial shoot. The shoot formation was recorded in 5<sup>th</sup> subculture, each experiments was repeated 3 times. The significance of differences between means values was determined by analysis of variance. Duncan's multiple range test used to compare the means of treatments at p<0.05 in order to select the best treatment for shoot proliferation and elongation in the plants.

Primary explants segments with shoots were sub cultured on fresh shoot induction medium for continued proliferation, until in some cultures the shoots (>20mm) were collected for rooting experiments. The rooting technique consisted in transferring the shoots to MS basal medium with 3% sucrose, 0.8% agar. For this rooting experiment 20 shoots were used per treatment. The numbers of roots per plant were recorded 17 days after the transfer to rooting medium. Cultivation conditions during initiation and growth of roots were the same as described in the multiplication phase. The number of initiation roots was considered for shoots with roots (>50 mm).

Shoots with roots were rinsed in water to remove remains of the medium and transplanted in pots containing a mix of pit and perlite (2:1). The plants were covered with plastic bags for 1-2 weeks under greenhouse conditions. The plants were gradually acclimated by opening the cover over fourteen days.

In this study, total concentration of carbohydrates and proteins were measured *in vivo* (control) and tissue culture micropropagated tuna.

Leaf samples (0.5 g) were crushed in liquid nitrogen. Soluble sugars were extracted with 15 ml of boiling 80 % (v/v) ethanol for an hour, followed by centrifugation at 3000 rpm for 10 min. For quantitative analysis of fructose, glucose and sucrose, the alcoholic extract was evaporated to dryness and dissolved in 100 mL sterile water. Then, 5 ml of ZnSO<sub>4</sub> (5% (w/v)) and 4.7 ml of 0.3 N Ba (OH) were added and shaken thoroughly. Aliquot (45 ml) was taken into centrifuge tube and then centrifuged at 3000 rpm for 10 min. The supernatant was evaporated in other tube by freeze drier system then the pellet was solubilised in 2 ml of sterile water. In the last step the solution was filtered through Sartorius 0.45 µm filter paper and contents determined using Knauer high performance liquid chromatography (HPLC) system (Liquid Chromatography: Knauer, degasser: Biotech model 2003, injector: Knauer injector with 20 µl loop, column: Eurokat H 10 µm (300 × 8.0 mm), Detector: RI, Mobil phase: sulphuric acid 0.02N, flow rate: 1 ml min<sup>-1</sup>). Components were identified by comparison of their retention times with those of authentic standards under analysis conditions and quantified by external standard method. A 10 min equilibrium time was allowed between injections (Saeidi, 2008).

Protein concentrations were measured using a modified Bradford procedure with bovine serum albumin (BSA) as standard protein (Bradford, 1976). Protein extracts were thawed and their concentration determined by a colorimetric method. The absorbance at the wavelength of 595 nm was determined against the blank and the standard curve of absorbance versus protein concentration plotted. Reactions containing dilutions of the soluble protein extracts (unknown concentrations) were set up as mentioned earlier.

The absorbance at 595 nm determined using a spectrophotometer (Cary300, Varian, Inc.). Protein

contents of the extracts were determined from the standard curve.

Data management and analysis was performed using SPSS ver, 17 and means of comparison test by Duncan's multiple range was conducted using tests ( $P < 0.05$  and  $0.01$ ). Treatment means were compared with standard deviation (SD) of the mean.

## Result and discussion

We intended to determine the conditions that regulate shoot proliferation for massive propagation, which is the most popular method for commercial production for a number of plant species (Hartmann *et al.*, 1997) including cacti (Fay *et al.*, 1995, Malda *et al.*, 1999, Pe' rez-Molphe-Balch and Da' vila-Figueroa, 2002).

**Table 1.** Effect of IAA concentrations on *in vitro* bud proliferation of forage cacti (*Opuntia ficus-indica*).

Treatment (mg $l^{-1}$ )	New Shoots	Multiplication rate	Highest shoot (mm)
5 BAP + 0 IAA	0.55 <sup>c</sup> ± 0.07	2.3 ± 0.7	4.3 <sup>ab</sup> ± 0.2
5 BAP + 0.25 IAA	0.99 <sup>b</sup> ± 0.03	2.8 ± 0.4	4.8 <sup>a</sup> ± 0.1
5 BAP + 0.5 IAA	1.07 <sup>ab</sup> ± 0.05	3 ± 0.4	5.3 <sup>a</sup> ± 0.1
5 BAP + 1 IAA	1.24 <sup>a</sup> ± 0.05	2.8 ± 0.4	4.7 <sup>a</sup> ± 0.2
5 BAP + 2 IAA	0.44 <sup>c</sup> ± 0.05	2.43 ± 0.4	3.4 <sup>b</sup> ± 0.2

The use of Mancozeb fungicide (80%) for 15 minutes as a sterilization solution proved to be an applicable treatment method to establish plant materials of *Opuntia ficus indica*. Sterilization treatment of entire cladodes and subsequent explant excision in laminar

airflow cabinet proved to be the most successful procedure. Using *Mancozeb* in sterilizing entire cladodes resulted in reduced infection levels (Nishimura *et al.*, 2003).

**Table 2.** Effect of NAA concentrations on *in vitro* bud proliferation of forage cacti (*Opuntia ficus-indica*).

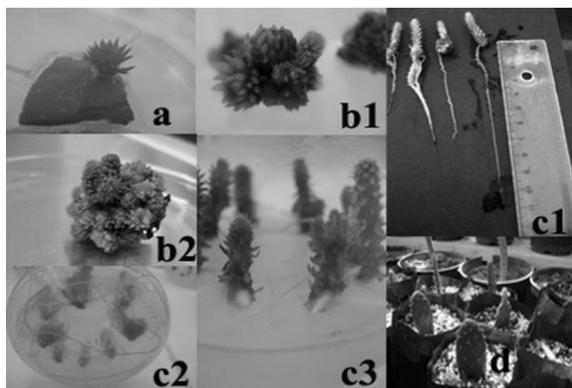
Treatment (mg $l^{-1}$ )	New Shoots	Multiplication rate	Highest shoot (mm)
5 BAP + 0 NAA	0.5 <sup>c</sup> ± 0.03	1.9 ± 0.07	3 <sup>bc</sup> ± 0.1
5 BAP + 0.25 NAA	2.3 <sup>a</sup> ± 0.09	3 ± 0.03	7.13 <sup>a</sup> ± 0.3
5 BAP + 0.5 NAA	0.17 <sup>c</sup> ± 0.04	1 ± 0.09	1.33 <sup>c</sup> ± 0.2
5 BAP + 1 NAA	2.16 <sup>a</sup> ± 0.09	1.2 ± 0.07	4.5 <sup>ab</sup> ± 0.3
5 BAP + 2 NAA	1.15 <sup>b</sup> ± 0.04	3.9 ± 0.09	4.8 <sup>ab</sup> ± 0.1

The induction medium used to culture isolated areoles was able to break dormancy in axillary buds and start new shoot development, which after growing about 2 mm in length (21 days after bud isolation) were used as secondary explants in subsequent experiments. Surviving explants showed active and uniform shoot growth and multiplication which subcultured in the different growth media containing 3, 5, 7 mg $l^{-1}$  BAP. Statistical analysis indicated that there is no significant difference among media for shoot emergence at 1 and 5% levels of significance whereas data demonstrated that the highest number of shoot emergence was in medium

supplemented with BAP 5 mg $l^{-1}$  (90.7 %) (fig.1a). The studies were conducted by breaking the bud dormancy in cacti species including *Opuntia* might be possible through the use of cytokinins alone or in combination with other plant regulators (Escobar-Araya *et al.*, 1986, Jua' rez and Passera, 2002), however previous evidence suggested that, media with cytokinins alone have proven to be more efficient for most *Opuntia* species (Johnson and Emimo, 1979, Havel and Kolar, 1983, Escobar-Araya *et al.*, 1986, Jua' rez and Passera, 2002). Previous studies have pointed out that the benzyl amino purine (BAP) is a growth promoter (Aliyu and Mustapha, 2007, Clayton

*et al.*, 1990). The method has been developed in response to the exogenous stimulus of cytokinin (BAP) in *Opuntia amytelea* buds (Escobar *et al.*, 1986).

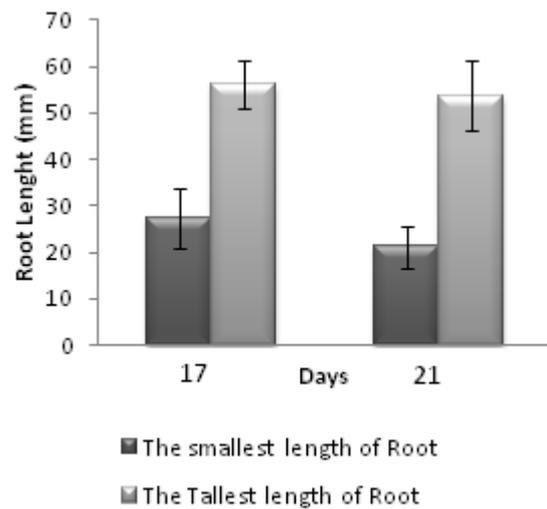
The number of newly formed shoots varied in media different growth regulators. For the proliferation stage (fig.1b1 and 2), the ANOVA detected significant effects on type of cytokinin, and concentration of the plant growth regulator. Based on establishment results media containing 5 mg $l^{-1}$  BAP significantly increased the number of shoots and thus, BAP (5 mg $l^{-1}$ ) applied in all proliferation mediums with different combination of IAA, NAA and Kinetin. The results obtained from multiplication in different combinations of IAA are stated in Table 1. The highest multiplication rates were observed on media containing BAP (5 mg $l^{-1}$ ) and IAA (0.5 mg $l^{-1}$ ). Aliyu *et al.*, 2007, reported no significant difference between the two media could be attributed to the fact that the supplements to the MS salts, that is, benzyl amino purine (BAP) and indole acetic acid (IAA) are both growth promoters and the presence of 0.25 mg $l^{-1}$  of IAA in media may have made up for the absence of 0.25 mg $l^{-1}$  of BAP.



**Fig. 1.** Plant regeneration in *Opuntia* sp. *in vitro* culture establishment (a), bud proliferation (b1, 2), root formation (c1, 2, 3) and plant acclimatization (d).

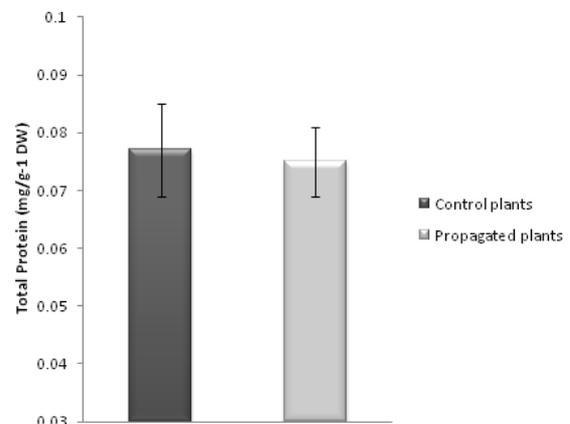
The shoots cultivated in the presence of BAP (5 mg $l^{-1}$ ) with different combinations of NAA (0, 0.25, 0.5, 1, 2 mg $l^{-1}$ ) revealed that the highest length of shoots (7.1 mm) and also the best result of new shoots number (2.33) was achieved in media with concentration 0.25 mg $l^{-1}$  of NAA (Table 2), thus being the most remarkable culture medium for the shoot proliferation stage. Shoot differentiation is the result

of the interaction between cytokinin and auxin growth regulators (BAP/NAA).



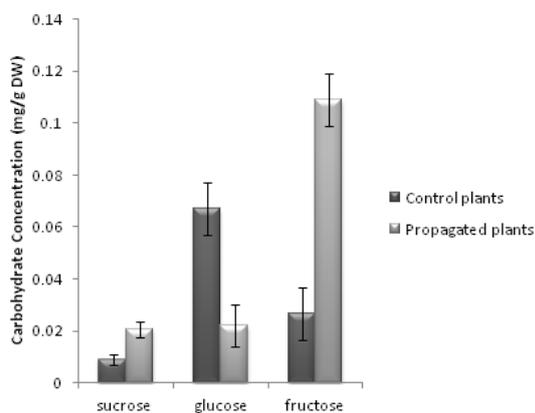
**Fig. 2.** Root length (mm) from shoots cultured on basal medium (a), Number of roots initiated from shoots cultured on basal medium (b), after 17 and 21 days .

The proliferation rates reported in our work are in the range of those found by other authors. Balch, 1998, reported a proliferation rate of some species, the highest proliferation rates occurred in medium with cytokinin (BA) only, whereas all the species of the genera *Echinocereus* and *Ferocactus* required cytokinin (BA) and auxin (NAA) in addition. These results agree with observations of several authors, who also reported that a single culture medium may not be suitable for optimal shoot proliferation in different genera and species of cacti (Martínez-Vázquez and Rubluo, 1989, Infante, 1992).



**Fig. 3.** Total protein content in control and micropropagated tuna after 3 months.

Areoles which were subjected to 0.5 mg<sup>-1</sup> BAP and 0.5 mg<sup>-1</sup> Kinetin and 1 mg<sup>-1</sup> BAP and 1 mg<sup>-1</sup> Kinetin, showed no significant differences in shoot length and number of new shoots. Further, our investigation pointed out shoots cultured on MS medium supplemented with different combination of BAP and kinetin showed no significant differences in shoot proliferation. Mackay *et al.*, 1995, reported that shoots poorly grew on medium supplemented with 2iP or kinetin with excessive yellowing of the leaves and necrosis.



**Fig. 4.** Carbohydrate contents (sucrose, glucose and fructose) in control and micropropagated tuna after 3 months.

Rooting initiation started within 7 days and 100% areole rooting was obtained on MS basal medium, under a 16-h photoperiod on the 17th day of culture. Result demonstrated that there was a significant difference between 17<sup>th</sup> and 21<sup>th</sup> days of culture. The highest root length (56mm) and root number (5.2) were achieved after 17 days (fig. 1c1, 2 and 3 - 1a and b).

Several studies revealed that rooting is favored in most plant species when auxins are available in the culture media (Hartmann *et al.*, 1997), however, several *Opuntia* species have successfully been rooted and normally grown without auxins (Escobar-Araya *et al.*, 1986, Estrada-Luna, 1988, Mohamed-Yasseen *et al.*, 1995, Garcí'a- Saucedo *et al.*, 2005, Estrada *et al.*, 2008).

The acclimatization procedure applied to *in vitro* regenerated plantlets was successful, plantlets showing a 100% survival when transferred to pots containing pit and perlite (2:1) (fig.1d). After transplantation, plantlet survival was 100%, which is similar to the reports for other prickly pear cactus species (Escobar-Araya *et al.*, 1986, Estrada-Luna, 1988, Mohamed-Yasseen *et al.*, 1995, Jua' rez and Passera, 2002, Estrada *et al.*, 2008) and most micropropagated cacti (Ault and Blackmon, 1987, Clayton *et al.*, 1990, Johnson and Emino, 1979).

In our experiments on all media, any physiological disorders or morphological abnormalities such as callus formation were not observed.

As a physiological result, protein concentration presented no significant differences in *in vivo* (control) and micropropagated tuna (fig.3). Whereas, carbohydrates concentrations showed significant differences (2-fold) between control and micropropagated seedlings. The distribution of sucrose and fructose contents was different between control and micropropagated tuna. Fructose and sucrose contents were highest in micropropagated tuna whereas glucose content did not change (fig.4). It has been demonstrated that cactus is rich in carbohydrates and calcium; it is attractive as an animal feed because of its efficiency in converting water to dry matter (Nobel, 1995).

However, at higher concentrations sucrose molecules could enter cells intact and bring about differentiation. Thus the formation of phloem (Wetmore *et al.*, 1964), the tissue which carries the sucrose from the leaves, is stimulated in the growing intact plant by the presence of sucrose (Jacobs & Morrow, 1958, Torrey, 1963).

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