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In vitro propagation of *Opuntia ellisiana* Griff. and acclimatization to field conditions

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ABSTRACT: The genus *Opuntia* is a valuable forage resource in arid and semiarid lands during periods of drought and shortage of herbaceous plants. However, absolute minimum temperatures in the plains of Mendoza represent a limiting factor to cultivate several species.
Opuntia ellisiana is a cold hardy species, so the goals of this study were to massively propagate it using *in vitro* culture techniques, and then to acclimatize plantlets obtained to field conditions.
Different sterilization protocols were tested. Areoles were isolated in laminar airflow cabinet, and cultured on Murashige-Skoog medium, supplemented with sucrose and different BAP and IBA combinations. Explants were grown at 27±2ºC, under a 16-h photoperiod. The shoots produced were used in the rooting assay using different auxin combinations. In the most efficient growth treatment, plantlets reached 100% shooting after 35 days of culture, and a mean length of 10.2 mm after 49 days of culture. A 100% rooted plantlets was obtained on a medium containing 5 mg L^{-1} IBA, after 12 days of culture. Acclimatization was achieved under greenhouse conditions, showing 100% plantlet survival.
This study suggests that *O. ellisiana* can be successfully micropropagated by areoles, and easily acclimatizated to field conditions.

Abbreviations: benzylaminopurine (BAP); indole-3-butyric acid (IBA); basal medium (BM).

Introduction

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_2 at night, when temperatures are lower and humidity higher than during the daytime. This invariably results in reduced water loss (Nobel, 1995; Taiz and Zeiger, 1998).

Owing to its high water-use efficiency (even in areas with annual rainfall values as low as 120-150 mm), and its high drought-tolerance (Le Houérou, 1994), this cactus is a valuable forage resource in arid and semi-arid lands during periods of drought and shortage of herbaceous plants.

Previous studies have addressed the economic feasibility of producing *Opuntia ficus-indica* Mill. cactus pear fruit (Guevara and Pizzi, 1998) and forage (Guevara *et al*., 1999) in the Mendoza plains, where soil features and rainfall values suggest that *O. ficus-indica* could be successfully grown.

However, extremely low temperatures restrict the areas where *O. ficus-indica* and other *Opuntia* species can be grown. Sensitivity to low temperatures varies greatly among species of *Opuntia*. Various commercial species, such as *O. ficus-indica* and *O. streptacantha* Lem., are killed at –5º to –8ºC (Nobel, 1995).

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Observations of several species established in the Mendoza plains have suggested that very low winter temperatures lasting 11 to 13 h are the major limiting factor affecting cultivation in this area. Some field assays showed that when night temperatures dropped to –17ºC, young cladodes from 9-month-old plants of *O. ficus-indica* were almost totally destroyed, whereas 3-year-old plants of *O. ficus-indica*, *O. spinulifera* Salm-Dyck f. *nacunniana* Le Houér., f. *nov.* and *O. robusta* Wend. had mean frost damages of 25%, 5%, and 2%, respectively (Guevara and Estevez, 2001; Guevara *et al*., 2000).

In contrast, some species show high tolerance to low temperatures, such as the spineless *Opuntia ellisiana* that experienced no damage from temperatures of -20ºC (Wang *et al*., 1997).

Despite being the slowest growing of all spineless *Opuntia* species, *O. ellisiana* exhibits high water-use efficiency (162 kg H_2O kg^{-1} dry matter). In fact its water-use efficiency is higher than that of any other plant species (including C_3 and C_4) measured under long-term field conditions (Han and Felker, 1997).

On this account, *O. ellisiana* could be a useful forage variety in locations that prove too cold for *O. robusta* Wend. or *O. ficus-indica* (Han and Felker, 1997).

O. ellisiana's cold hardiness, its economic potential for forage production, and the low availability of material for propagation justified the application of *in vitro* propagation techniques. An efficient massive multiplication in reduced space and time (Escobar *et al*., 1986; Pimienta-Barrios, 1990; Rubluo *et al*., 1996) characterizes micropropagation, while genetic stability is maintained, and plant health and vigor increase (Rice *et al*., 1992).

The main goal of this study was to achieve massive propagation of *O. ellisiana* by *in vitro* culture of areoles, and to succeed in attaining plantlet acclimatization to field conditions.

The specific goals were a) to elaborate successful protocols for cladode sterilization; b) to find the optimal growth regulators combination able to induce shooting from areoles of sterilized cladodes; c) to determine auxin concentrations able to induce rooting; and d) to acclimatize propagated material to field conditions.

Materials and Methods

Owing to low availability of *O. ellisiana* cladodes, the preliminary sterilization trials were performed with *O. ficus-indica*.

The following sterilization procedures were tested:

I. Entire cladodes washed with water and sterilized by immersion in 20% sodium hypochlorite plus 2% Tween 80 for 10 min (Mohamed-Yassen *et al*., 1995), followed by immersion in 1% benzalkonium chloride for 30 min. Subsequently, cladodes were rinsed four times with sterile distilled water.

II. Areoles were isolated using a hollow punch (1cm in diameter), then sterilized by immersion in 96º ethanol for 1 min, followed by immersion in 20% so-

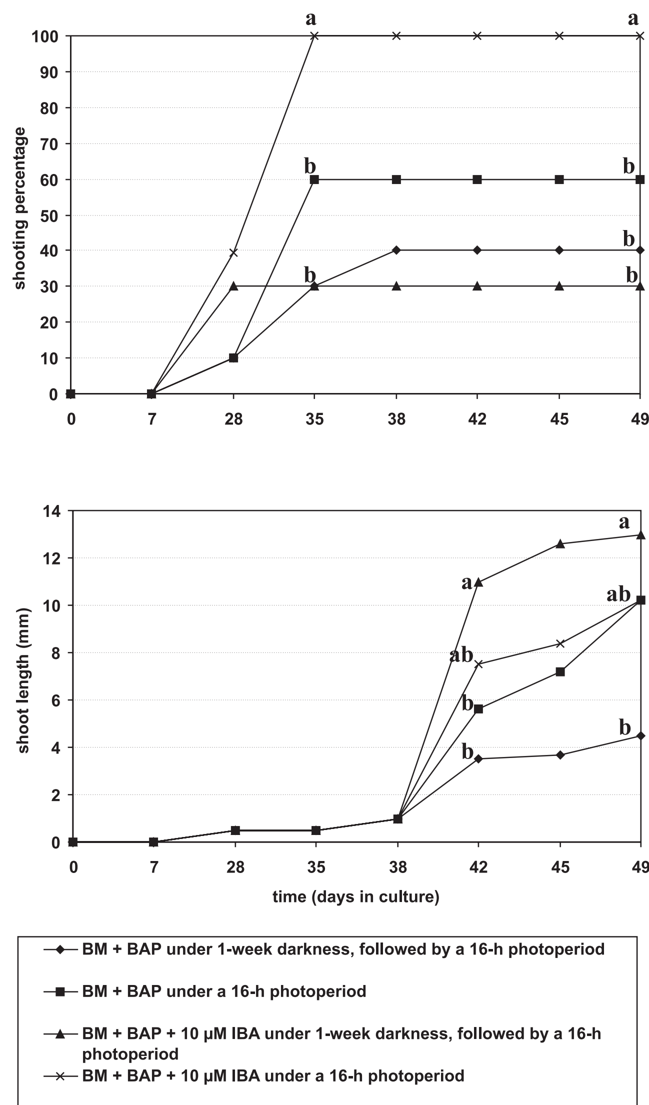

FIGURE 1. (A) Percentage of areole shooting on four different nutritive media for 49 days; (B) shoot length (mm) produced by areoles cultured on different nutritive media.
For each graph, means recorded on the same date, followed by the same letter, are not significantly different (p<0.05).

dium hypochlorite for 7 min, and finally rinsed three times with sterile distilled water (Clayton *et al*., 1990).

Results obtained (see below) led us to utilize procedure I.

A Murashige-Skoog (MS) basal medium (BM) (Murashige and Skoog, 1962) with 30 g L^{-1} sucrose and 0.8% agar was used.

Benzylaminopurine (BAP) at 10 μM, and indole-3-butyric acid (IBA) at variable concentrations were used as growth regulators.

Media were adjusted to pH 5.7 with 0.1N KOH, and autoclaved at 0.1 MPa (121ºC) for 30 min. Explants were dispensed into 20 x 150 mm glass tubes containing 10 ml of media (Mohamed-Yassen *et al*., 1995), and cultured in growth chambers at 27±2ºC, 100% relative humidity. Light had an intensity of 100 μmoles $m^{-2} s^{-1}$ at tube level.

The following experiments were designed:

1) Areole culture (n=10) for 49 days, which included the following treatments:
   a) BM + BAP, under 1-week darkness, followed by a 16-h photoperiod.
   b) BM + BAP, under a 16 h-photoperiod.
   c) BM + BAP + 10 μM IBA, under 1-week darkness, followed by a 16-h photoperiod.
   d) BM + BAP + 10 μM IBA, under a 16-h photoperiod.

   In each treatment infected and non-infected areoles were determined, as well as number and length of shooting areoles.
2) Shooting areole culture (n=10) obtained in the above assay, under a 16-h photoperiod for 48 days, which included the following treatments:
   a) Entire shoot in BM + 25 μM IBA.
   b) Transversally sectioned shoot in BM + 25 μM IBA.
   c) Entire shoot in BM + 50 μM IBA.
   d) Transversally sectioned shoot in BM + 50 μM IBA.

   Both the number of rooted shoots and the number of roots per shoots were determined.

O. ellisiana shoots developed and rooted *in vitro* were transferred to the greenhouse, where they were acclimatized so as to endure environmental conditions. Plantlets were grown in speedlings filled with sterilized substrate (organic matter: sand: soil, 6:3:1 v/v). All plantlets were watered as required and grown in the shade (80% solar radiation intercepted) for 48 h, under high relative humidity (80-90%). Subsequently some of them were taken to the field where very low relative

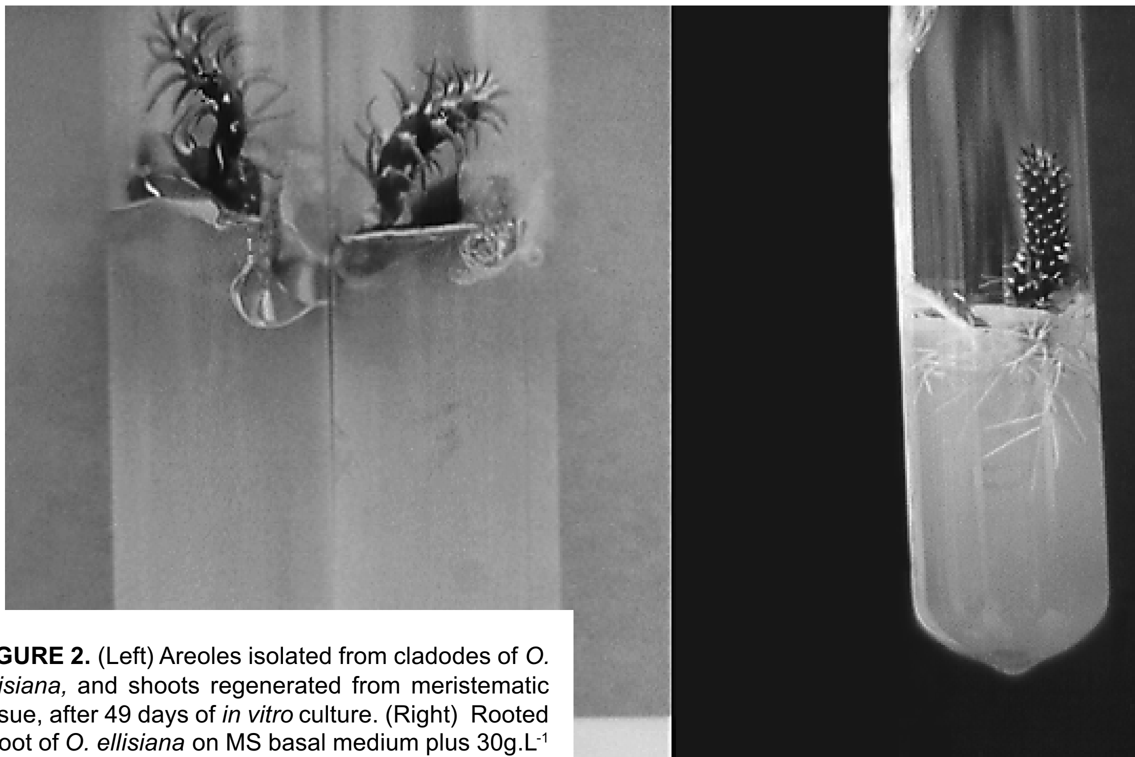

FIGURE 2. (Left) Areoles isolated from cladodes of *O. ellisiana,* and shoots regenerated from meristematic tissue, after 49 days of *in vitro* culture. (Right) Rooted shoot of *O. ellisiana* on MS basal medium plus 30g.L^{-1} sucrose, 0.8% agar and 25 μM IBA after 48 days of *in vitro* culture.

humidity prevails, and the others were subjected to decreasing relative humidity for 72 h. Plantlet survival was determined.

Data were subjected to an analysis of variance (ANOVA). Mean comparisons were tested using Duncan´s test. Angular transformations were made for the analysis of percentage data.

Results

Sterilization procedure I, in which entire cladodes were immersed in Tween 80, sodium hypochlorite and benzalkonium chloride solution, proved to be the most efficient, with only 12% areoles infected. This infection percentage was remarkably lower than that of sterilization procedure II, wherein 80% areoles were infected.

A 100% areole shooting was obtained on BM + BAP + 10 µM IBA, under a 16-h photoperiod on the 35th day of culture. The areoles cultured in BM + BAP, under a 16-h photoperiod presented 60% shooting after 35 days in culture. Areoles which were subjected to BM + BAP, under 1-week darkness, followed by a 16-h photoperiod; and BM + BAP + 10 µM IBA, under 1-week darkness, followed by a 16-h photoperiod, showed at the end of the assay a shooting percentage close to 40% (Figs. 1-A and 2). It is worth noticing that only one shoot was obtained for each areole.

The greater shoot length (13 mm) was obtained by culturing areoles on BM + BAP + 10 µM IBA, under 1-week darkness, followed by a 16-h photoperiod, on the 49th day. This shoot length was significantly higher than that registered in BM + BAP under conditions of 1-week darkness, followed by a 16-h photoperiod (Fig. 1-B).

The highest rooting percentage of shoots was 100% for the following treatments: entire shoots in 25 µM IBA; sectioned shoots in 25 µM IBA; and entire shoots in 50 µM IBA, after 19 days in culture (Figs. 2 and 3-A).

A high number of roots per rooted plantlet was obtained by culturing entire shoots on BM+25 µM IBA, after 19 days in culture. However, the greatest number of roots per rooted plantlet was obtained with entire shoots cultured on BM+25 µM IBA and BM+50 µM IBA, and was achieved after 33 days of culture. Growing entire shoots on a BM supplemented with any of the IBA concentrations used produced 16 roots per rooted plantlet on the 33rd day. This was significantly higher than the number reached using the treatment including sectioned shoots in 25 µM IBA; and the latter was in turn higher than that obtained from the treatment using sectioned shoots in 50 µM IBA (Fig. 3-B). The same results were observed after 48 days in culture, at the end of the assay.

Both acclimatization procedures applied to *in vitro* regenerated plantlets were successful, plantlets showing a 100% survival when transferred to soil.

Discussion

Sterilization treatment of entire cladodes and subsequent explant excision in laminar airflow cabinet

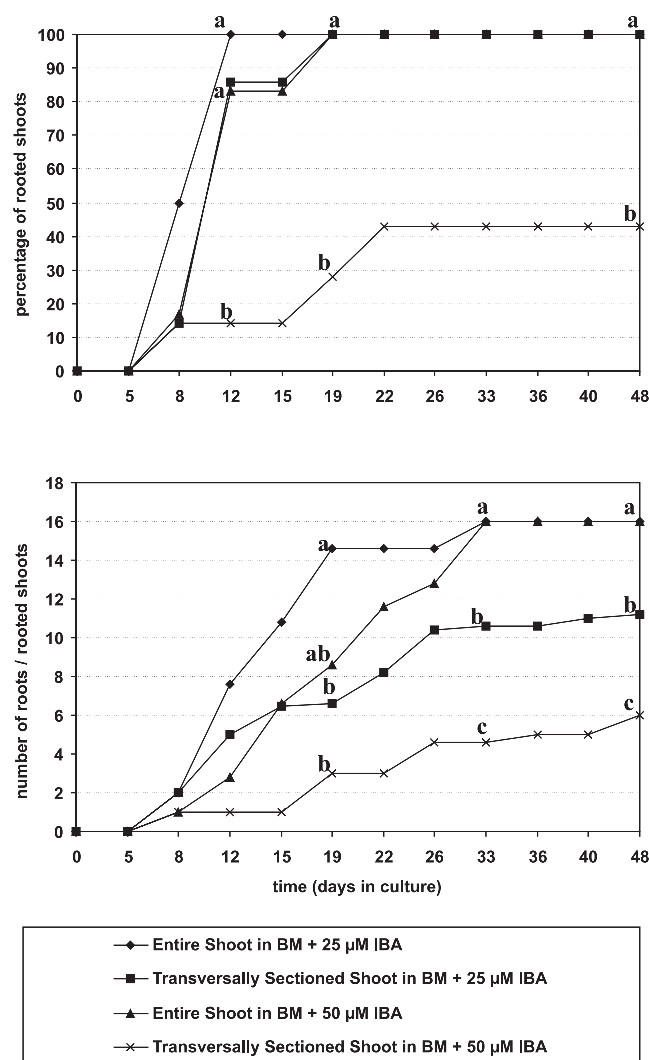

FIGURE 3. (A) Percentage of rooted entire and sectioned shoots, on a culture medium with different concentrations of IBA, for 48 days; (B) number of roots from entire and sectioned shoots cultured on nutritive media.
For each graph, means recorded on the same date, followed by the same letter, are not significantly different (p<0.05).

proved to be the most successful procedure. Using benzalkonium chloride in sterilizing entire cladodes resulted in reduced infection levels, as was also found for other plant materials (Flachsland *et al*., 1997).

The culture medium with BAP and IBA, under a 16-h photoperiod, showed the highest percentage of areole shooting (100%) along with a high shoot growth rate, thus being the most efficient culture medium for the optimal multiplication of this plant material. Previous studies have pointed out that the optimal hormone combination may be unique for each cactus species (Johnson and Emino, 1977, 1979). However, recent articles emphasize that low levels of auxin with cytokinin increased axillary shoot production in some cactus species (Clayton *et al*., 1990). Cytokinin is considered to be essential for the development of cactus axillary shoots (Mauseth, 1977). *Opuntia amyclea* buds developed in response to the exogenous stimulus of cytokinin (BAP) (Escobar *et al*., 1986). The lateral buds of tobacco plants can also be stimulated to grow when exposed to a nutritive medium high in cytokinin (Murashige and Skoog, 1962).

Shoot and root differentiation is the result of the interaction between cytokinin and auxin hormones (BAP/IBA). This interaction cannot always be controlled because endogenous hormones (synthesized by the tissues in culture) are influenced by exogenous growth regulators (Rubluo *et al*., 1996). These endogenous hormones are also affected by light availability (Taiz and Zeiger, 1998). The lengthening of shoots recorded in the treatment using 10 μM IBA under 1-week darkness was probably the result of the absence of light. Rooting is favored by auxin availability. Apical meristems produce auxins, so treatments with entire shoots yielded the greater number of roots and the higher rooting percentage.

After transplant, plantlet survival was 100%, similar to that reported by other authors (Ault and Blackmon, 1987; Clayton *et al*., 1990; Johnson and Emino, 1979; Mohamed-Yassen *et al*., 1995; Vyskot and Jára, 1984). Moisture requirements of plantlets were minimal during acclimatization. They can be directly placed in the open, under environmental moisture conditions, to avoid fungal infection (Smith *et al*., 1991). Plantlets obtained showed the typical cladode shape (Fig. 4), which is a major trait of *in vitro* regenerated plants. It is to be noted that plants grown from seeds in an irrigated field maintained the juvenile cylindrical form for a longer time and grew very slowly, even after four years (Escobar *et al*., 1986).

After 7 months of culture a total 1,200 plantlets were obtained. By early autumn the propagated and acclimatized plantlets of *O. ellisiana* were sent to the Jardín Botánico in Puerto Madryn (Chubut); to the Dirección de Recursos Naturales Renovables, Malargüe (Mendoza); and to the Estación Experimental de Ganado y

FIGURE 4. *O. ellisiana* acclimatized under greenhouse conditions after 15 months.

Pasturas Naturales El Divisadero, Santa Rosa (Mendoza), where the plants were grown in the field. Plants survived the freezing winter temperatures in all three places (-9ºC, -14ºC, and -14.8ºC respectively, for several hours); however *O. ficus-indica* plants, forming the control group, were killed.

The results of this study suggest that *O. ellisiana* can be successfully micropropagated by areoles, and easily acclimatized to field conditions.

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