BIOCELL
2004, 28(3): 271-277

ISSN 0327 - 9545
PRINTED IN ARGENTINA

Microcutting culture and morpho-physiological changes during acclimation in two *Lycium chilense* cytotypes

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Key words: Microcuttings, *Lycium chilense*, subcultures, endangered species.

ABSTRACT: *Lycium chilense*, a deciduous perennial shrub, is one of the endangered native species of Patagonia due to sheep overgrazing. Chances of recolonization by seeds are scarce due to the limited density of propagules in the soil and very specific requirements for germination. The objective was to develop an *in vitro* propagation protocol that would help to perform reestablishment of this species in degraded areas of the Patagonian steppe. Seeds came from two provenances with different somatic number due to differences in ploidy level. Defoliated microcuttings were planted in test tubes with different growing media and taken to a growth chamber. Rooting percentage did not differ between origins, but higher values were encountered for medium without hormones. Subcultures increased significantly rooting percentage and reduced time to rooting. The leaves from micropropagated plants were thinner, did not exhibit hairs, and had poorly developed palisade parenchyma and less epicuticular waxes. *In vitro* leaves had lower stomatal density and their stomata were less functional when compared to acclimated leaves. A repopulation program of *Lycium chilense* based on microcutting culture, specially using subcultures, is feasible.

Introduction

In the Patagonian region, Soriano *et al.* (1995) reported *Lycium chilense* Miers ex Bert., a deciduous perennial shrub from the *Solanaceae* family, endangered due to direct or indirect result of sheep overgrazing. This shrub is very important for their potential function as “engineer species” in the ecosystem (Bisigato and Bertiller, 1997), for their value as forage (Cano, 1988), and for producing substances with pharmaceutical potential (Terauchi *et al*., 1998). In this type of system, chances of recolonization by seeds are scarce due to the limited density of propagules in the soil (Bertiller, 1996; Aguiar and Sala, 1997). In addition, requirements for germination of *L. chilense* seeds are very specific and include stratification (Soriano, 1960).

Stebbins (1985) hypothesized that polyploid individuals had more invasive potential than their non-polyploid ancestors. A trait closely associated with invasive potential is a high rooting capacity. *Lycium chilense* exhibits high variability: eight botanical varieties have been described and there is a number of intermediate forms with different ploidy level (Bernardello, 1986). Regeneration of previously selected plants by *in vitro* tissue culture allows obtaining efficiently and rapidly a large number of propagules (Liu, 1991), but for each species and tissue, nutrient and environmental require-

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Received on August 11, 2003. Accepted on August 18, 2004.

ments for cell division and normal growth are different (Smeda and Weller, 1991). For the genus *Lycium* successful plant regeneration was obtained from protoplast culture (Ratushnyak and Rudas, 1989) and pollen grain culture (Zenkteler, 1972), but there is no information on rooting potential for materials with different ploidy level. In general, physical and chemical conditions of the growth medium and environmental conditions *in vitro* are related to morphological, anatomical, and physiological differences in seedlings obtained by tissue culture (Ticha *et al*., 1999; Khan *et al*., 1999). Such differences affect mainly the leaves and result in different survival rates under *ex vitro* conditions (Ziv, 1991). On the other hand, root differentiation is a developmental process that implies a set of successive physiological phases (Hausman *et al*., 1997), each of which has specific hormonal requirements (Kevers *et al*., 1997). Although root development is closely associated with level and balance of auxins and cytokinins in the medium (Noiton *et al*., 1992; Howard, 1994), this process changes according to the physiological phase (Hausman *et al*., 1997; Gaspar *et al*., 1997). Performing subcultures usually increase root development (Webster and Jones, 1989; Grant and Hammatt, 1999), but the increase in rate remains specific of each species.

For these reasons, steps considered in an acclimation process and their duration are mainly related to the morphological, anatomical and physiological features of the leaves obtained through tissue culture. The objective of this project was to develop an *in vitro* propagation protocol that would help to accelerate the obtention of saplings of *L. chilense* to perform reestablishment of this species in degraded areas of the Patagonian steppe where natural establishment is slow and scarce.

Material and Methods

For this study, material of *Lycium chilense* Miers ex Bert. was collected at sites with Mediterranean climate in the province of Chubut, Argentina: Sarmiento (long. E 69° 15’, lat. S 45° 46’) and Bahía Cracker (long. E 64° 30’, lat. S 42° 57’). Individuals from these two provenances have different somatic number as a result of different ploidy level: at Sarmiento (S)=2n:2x:24, and at Bahía Cracker (BC)=2n:4x:48.

At the above mentioned locations, seeds were harvested, stratified in the dark in Petri dishes moistened with tap water during 4 weeks at 4 °C (Soriano, 1960), and transferred to a growth chamber where they were kept in the dark under 12 h cycles of alternating temperatures (20-25 °C). After 45 days, the seedlings obtained were planted in plastic 5 L containers filled with a mixture of sieved topsoil and coarse sand (3:1). Individuals were kept in a greenhouse, at 15-20 °C, watered at field capacity. These plants (mother plants), which always remained in a vegetative stage, were used as stock to obtain propagules all year round.

We used for vegetative propagation explants of branches 1-2 years old. Segments of 2-3 cm long were washed with a mixture of detergent and water (1:9) for ten minutes and then disinfected with sodium hypochlorite 10% for five minutes. One defoliated microcutting with two nodes was planted under laminar flux on 15 ml medium in 40 ml sterilized test tube. All tubes contained 8 g L^{-1} of agar and medium B5 (Gamborg and Ojima, 1968), 0.25 strength, supplemented with sucrose (15 g L^{-1}) and the following hormone combinations: 1) Control, no hormones; 2) with indole acetic acid (IAA) 2.0 mg L^{-1} + purine amino benzyl (PAB) 0.5 mg L^{-1}, (auxins/cytokinins = 4); 3) with naphthalene acetic acid (NAA) 1.0 mg L^{-1}; and subsequently autoclaved for 30 min at 150 kPa. The test tubes were kept in a growth chamber under light supplied by fluorescent tubes (200 µmol m^{-2} s^{-1}) twelve hours per day at a constant temperature (22 °C). Explants obtained from rooted microcuttings (2-3 months) were used to perform subcultures. All the experiments were carried on using second order subcultures. Stem segments of 2 cm long, with two nodes, were planted under laminar flux in medium 1; while the mother cutting was replanted in the greenhouse. In each case, rooting percentage and time to maximum rooting were recorded. All experiments had at least 3 replicates per treatment with 30 explants per replicate. Morphological, anatomical and physiological comparisons were made on leaves coming from: a) *in vitro* seedlings, b) plants growing in greenhouse originated from seeds, and c) acclimated plants. Acclimated plants were originated by micropropagation and transferred gradually to greenhouse conditions. This was done in three steps: first, the tubes (placed in the growth chamber) were opened for two days; second, the explants were transplanted to 500 cm^3 plastic containers filled with a mixture of sieved topsoil and coarse sand (3:1), and kept for 10 days in the same growth chamber. Finally, they were transplanted again to 3 L plastic containers (same substrate), and transferred to the greenhouse under a 50% shading black net for 15 days. After this period the net was removed. During all this process individuals grew in a substrate maintained at field capacity. To measure leaf-cuticle thickness, we used a sliding microthome to obtain *in vivo* transversal cuts of fully

expanded leaves. Slides were mounted in water and photographed with a Zeiss camera MC63 under a standard Zeiss microscope using 10x100 magnification. Cuticle thickness was measured on the photos with an eyepiece micrometer. To assess the stomatal aperture of *in vitro* and greenhouse plants we obtained impressions on acrylic enamel of the upper surface of apical (distal leaves, recently expanded) and basal leaves (proximal leaves in a 20 leaves branch). Stomata were considered as open when pores were apparent in the impressions under a Zeiss microscope using 40x100 magnification. Samples of the greenhouse plants were taken at 10 h:am, 2 h:pm and 5 h:pm, and those from the *in vitro* plants were taken when the test tube was open and one and two hours later, while the propagule was kept in an environment with 60% of relative humidity, at a temperature of 22 °C and with a radiation level of 200 μmol s^{-1} m^{-2}. Leaf pubescence and epicuticular waxes were observed using an environmental scanning electron microscope (ESEM). Fresh samples were placed straight on the platine, without performing any critical point and metalizing techniques. Statistical analyses were done performing one-way ANOVA for a completely randomized design. Least significant differences between means were calculated by Tuckey's test ($P < 0.05\%$). Arc-sin of square root transformation was used for proportional data.

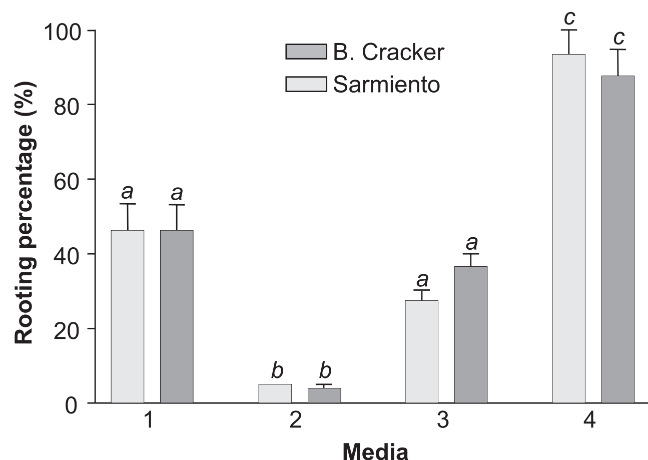

FIGURE 1. Rooting percentage of *Lycium chilense* microcuttings from Bahía Cracker and Sarmiento (mean ± s.e.): 1)medium B5 (0.25 strength); 2)medium B5 (0.25 strength) + BAP-IAA; and 3) medium B5 (0.25 strength) + NAA; 4) subcultures of rooted microcuttings in medium B5 (0.25 strength). Different letters indicated significant differences at $p<0.05\%$. Data were transformed by arc-sin of square root of y.

TABLE 1.

Days to maximum rooting (mean ± s.e.) of microcuttings from Sarmiento and Bahía Cracker: a) for the three media used (n=3); b) from stock plants or from rooted microcuttings growing in medium 1 (n=5). Different letters indicated significant differences with $p<0.05\%$. Data were transformed by square root of y.

	Origin	
	Sarmiento	**Bahía Cracker**
a)		
All media	27 ± 1.5 *a*	28 ± 1.5 *a*
b)		
Stock plants	25 ± 1.3 *a*	25 ± 1.6 *a*
Rooted microcuttings	11 ± 1.5 *b*	12 ± 1.2 *b*

Results

Rooting percentage differed significantly among growth media, but there were no differences between origins (Fig. 1). Adding BAP-IAA (medium 2) not only reduced rooting significantly, but also induced callus formation (data not shown). No differences were detected in time to maximum rooting for the different growing media or origins (Table 1). Performing subcultures increased significantly rooting percentage (Fig. 1) and reduced significantly the time to maximum rooting (Table 1). Relative changes in rooting percentage (41% increase), and in time to maximum rooting (44% decrease) were similar for both origins.

Leaf thickness was significantly lower for seedlings obtained by micropropagation and growing *in vitro* (221 ± 12 μm) than for seedlings obtained from seeds and growing in the greenhouse (544 ± 47 μm). However, leaves of micropropagated plants generated during acclimation in greenhouse had similar thickness to those originated from seeds (514 ± 27 μm). Unlike leaves of plants cultivated in the greenhouse (Fig. 2C), leaves from micropropagated plants did not have hairs (Fig. 2A). In addition, the palisade parenchyma of these leaves was

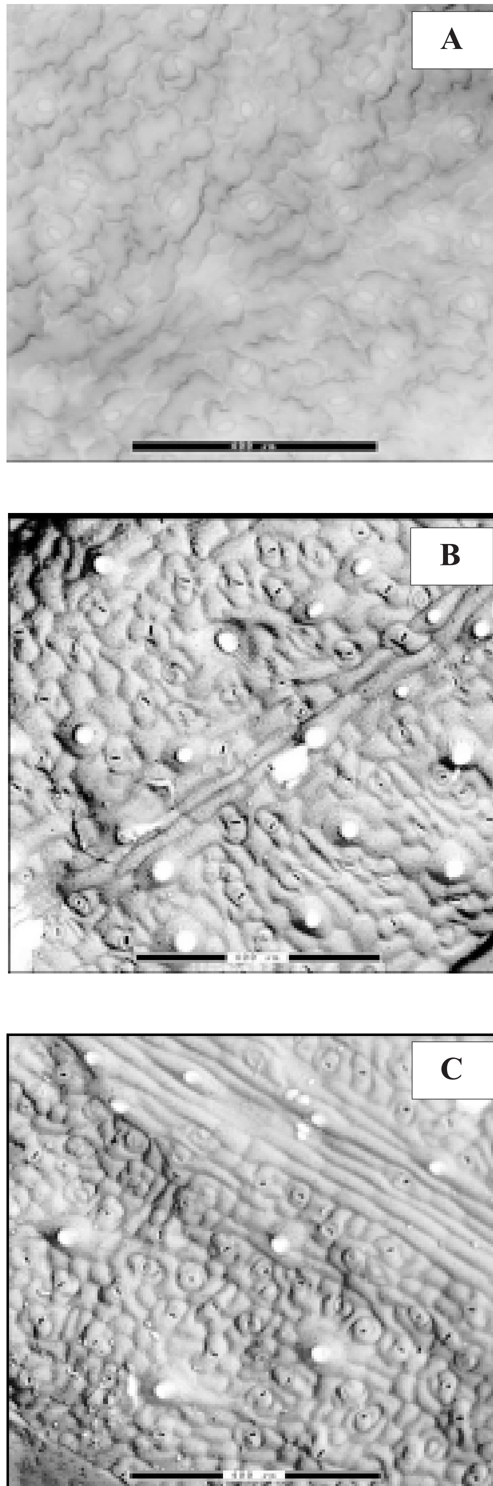

FIGURE 2. Leaf surface of *Lycium chilense* from: A) *in vitro* plant; B) acclimated plant; and C) mother plant (X 115).

poorly developed, and their epicuticular waxes had no sculptures (data not shown). Once they were transferred to the greenhouse, these individuals obtained by micropropagation developed new leaves with single or multicell hairs (Fig. 2B), well-developed palisade parenchyma, and epicuticular sculptures (data not shown).

Stomatal density differed significantly between *in vitro* and greenhouse leaves (1.37 ± 0.07 and 3.80 ± 0.21 stomata mm^{-2} respectively). Leaves from *in vitro* plants located at proximal and distal branch positions had lower stomatal density than leaves of equivalent positions in greenhouse plants, although this parameter did not change along the stem, neither in *in vitro* nor in greenhouse leaves (Fig. 3). Observations made by ESEM revealed that the stomata of *in vitro* leaves did not have epicuticular waxes regularly displayed (Fig. 4A) compared to those of acclimated plants (Fig. 4B).

In the *in vitro* plants all stomata were open right after the opening of the test tube, and remained open (100% of stomatal aperture) for one hour not reacting to a 30 ± 2% drop in air relative humidity. After two hours leaves were wilted and a significant but small stomata closure occurred in apical leaves only (94.3 ± 1.2% of stomatal aperture), probably as a consequence of the

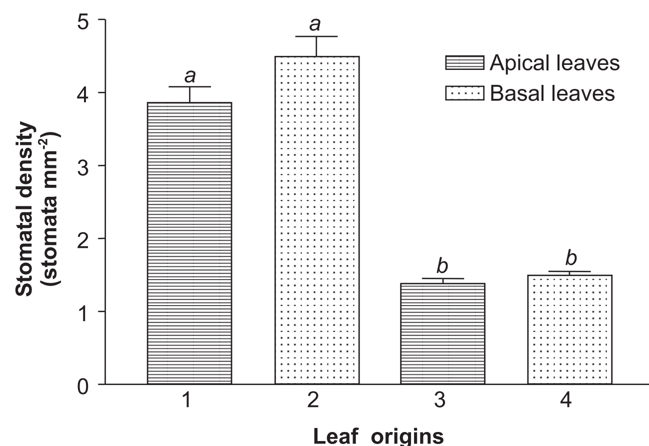

FIGURE 3. Stomatal density of apical and basal leaves of *Lycium chilense* (mean ± s.e.): 1) & 2) greenhouse; 3) & 4) *in vitro*. Different letters indicated significant differences at $p<0.05\%$. Data were transformed by log of y.

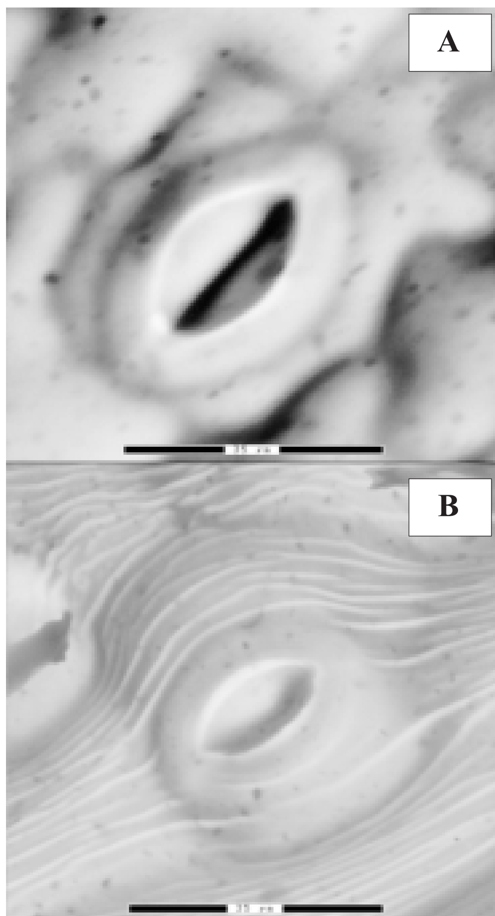

FIGURE 4. Stomata and epicuticular waxes of A) *in vitro*; B) acclimated leaf of *Lycium chilense* (X 1350).

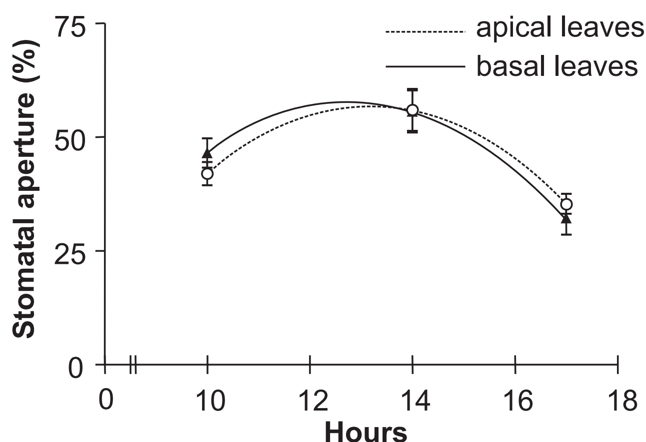

FIGURE 5. Daily stomatal aperture of apical and basal acclimated leaves of *Lycium chilense* plants (mean ± s.e.). Data were transformed by arc-sin of square root of y.

leaf water status. Leaves of micropropagated plants acclimated in greenhouse, grown under good soil moisture, had a normal stomata aperture behavior (Fig. 5).

Discussion

The differences in ploidy level did not affect the rooting behavior of *Lycium chilense* as we expected. On the other hand, the rooting medium employed clearly modified rooting percentage. Although variation to external auxin supply response was observed before (King and Stimart, 1998), in our experiments auxins did not improve rooting. Since the segments we used for micropropagation came from woody adult branches, this lack of response was probably caused by the presence of cells that were determined for root formation in the segments with nodes (Altamura, 1996). Marks and Simpson (2000) reported a similar case in which segments with nodes of woody species do not respond to external supply of auxins. This ability to develop cells capable of generating new roots during ontogeny may explain *L. chilense* capacity to propagate vegetatively in nature.

Our results with the auxins/cytokinins ratio used in medium 2, confirm that *Lycium chilense* respond as predicted by the classical model of morphogenesis in cultured tissues proposed by Skoog and Miller (1965). Similarly, when *Vigna unguiculata* was in a medium with auxins/cytokinins = 2, Soh *et al*. (1998) observed absence of root development. However, our medium 3, which was supposed to increase endogenous IAA level (Liu *et al*., 1998) and was free of cytokinins, did not promote rooting above the basic medium also. Even with their rather limited range of treatments, our experiments indicate the need to further explore the effects of consecutive subcultures (e.g. third or fourth order ones) on rooting capacity.

The leaves of *L. chilense* grown *in vitro* were characterized by their low thickness, lack of pubescence and epicuticular waxes, and poorly developed palisade layer. All these features were similar to those observed in other species under equivalent growing conditions (Preece and Sutter, 1991). As the morphological and anatomical shade responses in leaves were practically the same (Smith and Whitelam, 1997), there is a general agreement around the idea that these responses could be the consequence of the low light environment. Assuming this as an irreversible consequence of the propagation method, it is important for each species to know whether the *in vitro* leaves or the *ex vitro* ones maintain the

growth of the plant during the acclimation phase. This could alter the water balance of the plant, as the water loss from each type of leaf is different. Opposed to Estrada-Luna *et al*. (2001) findings in *Capsicum annum*, leaf stomata of *L. chilense* that developed *in vitro* were not functional ex vitro; nevertheless the water loss could in part be mitigated by the reduced number of stomata per area unit. While Volenikova and Ticha (2001) found that the stomatal density is higher in the apical leaves of the *in vitro* stem of *Nicotiana tabacum*, in *L. chilense* this parameter remains low in all leaves. Although the new *Lycium's* leaves had a higher stomatal density, since they had a normal stomatal functioning, no dehydration was observed in our conditions. Moreover, the acclimation procedure allowed the generation of new leaves before a negative water balance in the plant occurred.

A repopulation program of *Lycium chilense* based on tissue culture techniques seems possible, specially using second order subcultures. However, as *Lycium*'s explants had poor morphological, anatomical and physiological leaf features in relation to water economy and this could not be reverted, the acclimation ex vitro has to be specially considered in order to establish normal leaf development and thus, a successful survival.

Acknowledgements

The Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and the Universidad de Buenos Aires (UBA) supported this work. We thank Dr. M. Bertiller and Dr. A. Bisigato for the supply of the plant material; and A. Reynoso and P. Ratto for assistance with electron microscope. We are grateful to Dr. Roberto Fernández and Dr. W. Batista for helpful comments; and to Dr. R. Golluscio and Prof. L. Mroginski for critically reading the manuscript.

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