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Agrobacterium rhizogenes vs auxinic induction for *in vitro* rhizogenesis of *Prosopis chilensis* and *Nothofagus alpina*

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Key words: *Agrobacterium rhizogenes*, algarrobo, hairy roots, indole-3-butyric acid, *Nothofagus nervosa*, *Prosopis chilensis*, raulí, rooting

ABSTRACT: The induction and improvement of *in vitro* rhizogenesis of microshoots of *Prosopis chilensis* (Mol.) Stuntz and *Nothofagus alpina* (Poep. *et* Endl. Oerst.) were compared using *Agrobacterium rhizogenes* (Ar) *versus* indole-3-butyric acid (IBA) in the culture media. Microshoots of *P. chilensis* (1-2 cm length), coming from *in vitro* grown seedlings, were cultivated in a modified Broadleaved Tree Medium (BTMm) containing half salt concentration of macronutrients and 0.05 $mg.L^{-1}$ benzilaminopurine (BAP). After 30 days, microshoots with 2-4 leaves were selected and cultured in BTMm-agar in presence or abscense of Ar and in combination with IBA. For *N. alpina*, the apical shoots with the first 2 true leaves, from 5 weeks old seedlings, were cultured in the abovementioned medium, but with 0.15 mgL^{-1} of BAP. After 2 months, microshoots with 2-3 leaves were selected and cultured in BTMm-agar, supplemented with 5 $mg.L^{-1}$ IBA or in liquid BTMm on perlite and, in the presence or absence of *A. rhizogenes* (Ar) and in combination with 3 $mg.L^{-1}$ IBA. Rooting in *P. chilensis* reached 100.0% when Ar infection was produced in the presence of IBA, increasing both, the number and dry weight of roots. In *N. alpina*, 90.0% of rooting efficiency was obtained when Ar infection was produced in liquid culture and in the absence of auxin.

Abbreviations: Ar, *Agrobacterium rhizogenes*; BAP, benzilaminopurine; BTMm, Broadleaved Tree Medium, modified (half macronutrient, concentration); IBA, indole-3-butyric acid; MS, Murashige and Skoog medium; PAR, photosynthecally active radiation.

Introduction

Prosopis chilensis (Mol.) Stuntz and *Nothofagus alpina* (Poep. *et* Endl. Oerst.) [Syn.: *Nothofagus nervosa* (Phil.) Dim. *et* Mil.] are two important autochthonous forest species in Argentina (Erize, 1997). Sexual propagation in nurseries is the most common multiplication technique for these woody species, but unfortunately many select forest traits are lost through sexual propagation (Burkart, 1976; Felker, 1992).

The micropropagation of superior genotypes of *P. chilensis* (Caro *et al.*, 2002) and *N. alpina* (Martínez Pastur and Arena, 1996) would facilitate a rapid vegetative propagation of desirable genetic traits. On the other hand, the faulty root system from *in vitro* grown plants negatively affects the establishment of microplants in the field. Research has been done to improve *in vitro* rooting of these species by modifications of the type and concentration of growth regulators (Jordan *et al.*, 1987; Walton *et al.*, 1990; Caro *et al.*, 2002), the concentration of mineral salts and the environmental conditions (Arce and Balboa, 1991; Martínez Pastur and Arena,

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1996; Martínez Pastur *et al.*, 2000) as well as the use of flavonoid compounds (Martínez Pastur, 2000).

A. rhizogenes is a Gram (-) bacterium commonly present in soils. It has the ability to infect plants through wounds and as a result, it induces abundant adventitious roots (Tepfer, 1984; Petit *et al.*, 1986). *A. rhizogenes* infection at the base of *in vitro* cultured microshoots of woody species has been shown to improve its rhizogenesis (Damiano and Monticelli, 1998). In fact, rooting of *Pinus spp., Larix spp., Eucalypus spp.* and *Sequoia sempervirens* was successfully achieved by using strains of this bacterium (McAfee *et al.*, 1993; MacRae and Van Staden, 1993; Mihaljevic *et al.*, 1999). The present work compared the effectiveness of the strain LBA 9402 of *A. rhizogenes* and indole-3-butyric acid in relation to the induction and eventual improvement of the root formation on microshoots of *P. chilensis* and of *N. alpina*.

Materials and Methods

Plant material

Seeds were harvested from mature trees of *P. chilensis* growing in the experimental field of the Universidad Nacional del Sur (38° 15' S, 62° 11' W). They were treated with concentrated H_2SO_4 (86%) for 15 min and rinsed under tap water. Seeds were germinated in test tubes in a media containing half strength MS salts (Murashige and Skoog, 1962) 7.0 $g.L^{-1}$ agar, pH 5.7. After 30 days, 1-2 cm segments with one node from the microplantlets, were subcultured and grown on Broadleaved Tree Medium (BTMm, pH 5.8, Chalupa, 1983) with its mineral nutrients reduced to 50% of the original concentration, plus 7 $g.L^{-1}$ agar, 30 $g.L^{-1}$ sucrose and 0.05 $mg.L^{-1}$ BAP. Cultures were kept at 25 ± 2°C with a 16 h photoperiod under fluorescent white light (70 µmol m^{-2} s^{-1} PAR). After 30 days, shoots with 2 to 4 true leaves were selected to be used as explants in the experiment.

N. alpina seeds were provided by the Center of Seeds and Forest Trees of Chile (CESAF). The seeds were stratified for 30 days at 5°C in sterile humid perlite into plastic trays that were enclosed in polyethylene bags. The seeds were then shaked in an aqueous solution containing 1 $g.L^{-1}$ Captan® (PM 83%, w/v) and 0.5 $g.L^{-1}$ carbendazim (LF 50%, w/v) for 24 h and subsequently germinated in a perlite germination bed at 25 ± 2°C with a 16 h photoperiod. Beds were periodically watered with sterile distilled water and after germination, with sterile Hoagland solution (Hoagland and Arnon, 1950).

Explants were obtained from 35 day-old seedlings and consisted of the shoot tip with the first two true leaves (Martínez Pastur and Arena, 1996). This material was superficially sterilized with NaOCl (1% active chlorine) for 5 min and rinsed 3 times with sterile distilled water. Explants were then placed in glass culture tubes containing 10 ml half strength BTM, 7 $g.L^{-1}$ agar, 30 $g.L^{-1}$ sucrose and 1.78 $mg.L^{-1}$ BAP, pH 5.7 (Martínez Pastur and Arena, 1996). Cultures were kept at 25 ± 2°C with a 16 h photoperiod under fluorescent white

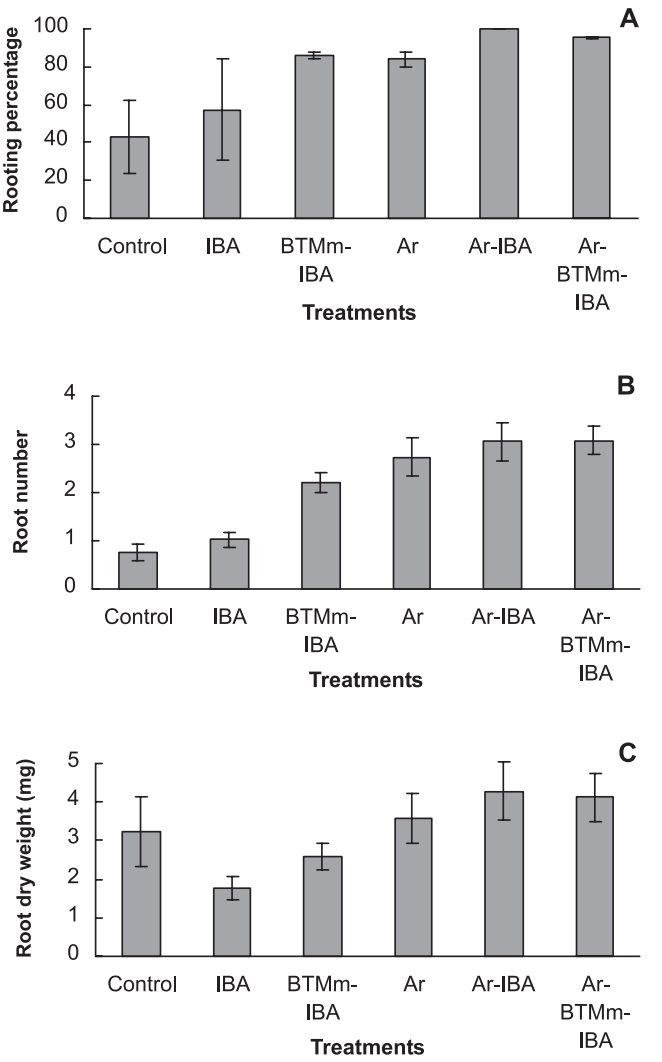


FIGURE 1. *In vitro r*ooting of *Prosopis chilensis* shoots. **A**, rooting percentage; **B**, primary root number per explant, and **C**, root dry weight. IBA: indol 3-butyric acid. BTMm: Broadleaved Tree Medium modificated. Ar: *Agrobacterium rhizogenes*.Vertical lines indicates 1 SE.

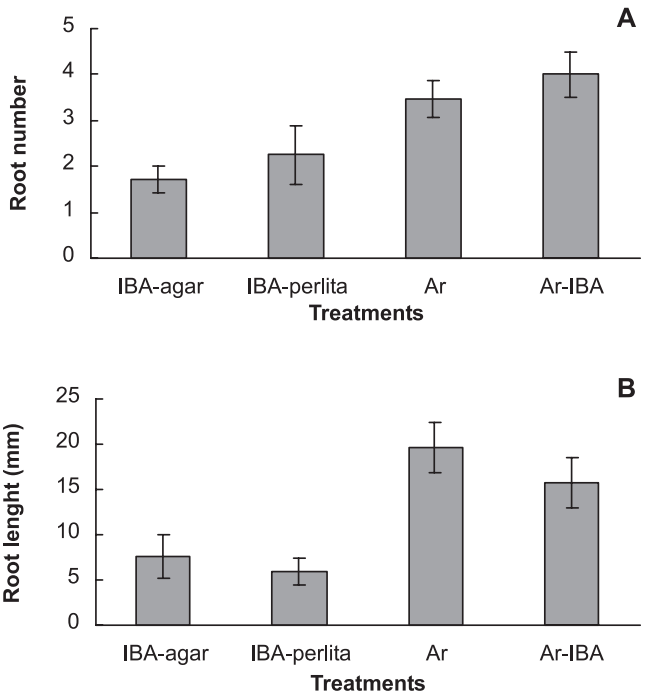


FIGURE 2. *In vitro* rooting of *Nothofagus alpina* shoots. **A,** primary root number per explant; **B,** principal root length. IBA: indol 3-butyric acid, Ar: *Agrobacterium rhizogenes*. Vertical lines indicates 1 SE.

light (57 µmol m^{-2} s^{-1} PAR). After 30 days, shoots were transferred to BTM and cultures were allowed to stabilize in initiation medium for two months.

A. rhizogenes culture

Strain 9402 of *A. rhizogenes* was cultured from an isolated colony in YEB liquid medium (Petit *et al.*, 1986) at 27°C for 12 to 16 h, i.e. until the optical density reached 0.7 - 0.8 units, measured at 600 nm in a Hitachi Model 100-60 spectrophotometer.

Rooting of P. chilensis

Shoots (Ss) containing 2-4 leaves were selected for the following treatments: 1) Ss cultivated in BTMm (control); 2) Ss immersed for 1 min in 3 $mg.L^{-1}$ IBA, prior to cultivation in BTMm (IBA); 3) Ss cultivated in BTMm containing 3 $mg.L^{-1}$ IBA (BTMm-IBA); 4) Ss inoculated with *A. rhizogenes* for 1 min prior to cultivation in BTMm (Ar); 5) Ss immersed in 3 $mg.L^{-1}$ IBA for 1 min, then inoculated for 1 min with *A. rhizogenes* prior to cultivation in BTMm (Ar-IBA), and 6) Ss inoculated 1 min with *A. rhizogenes* prior to cultivation in BTMm-IBA (Ar-BTMm-IBA). All shoots were cultivated in test tubes containing perlite saturated with the corresponding treatment medium, pH 5.8. The auxin solution was sterilized using 0.2 µm Millipore® filters. Cultures were maintained at 25 ± 2°C with a 16 h photoperiod under fluorescent white light (70 µmol m^{-2} s^{-1} PAR). After 10 days culture, the

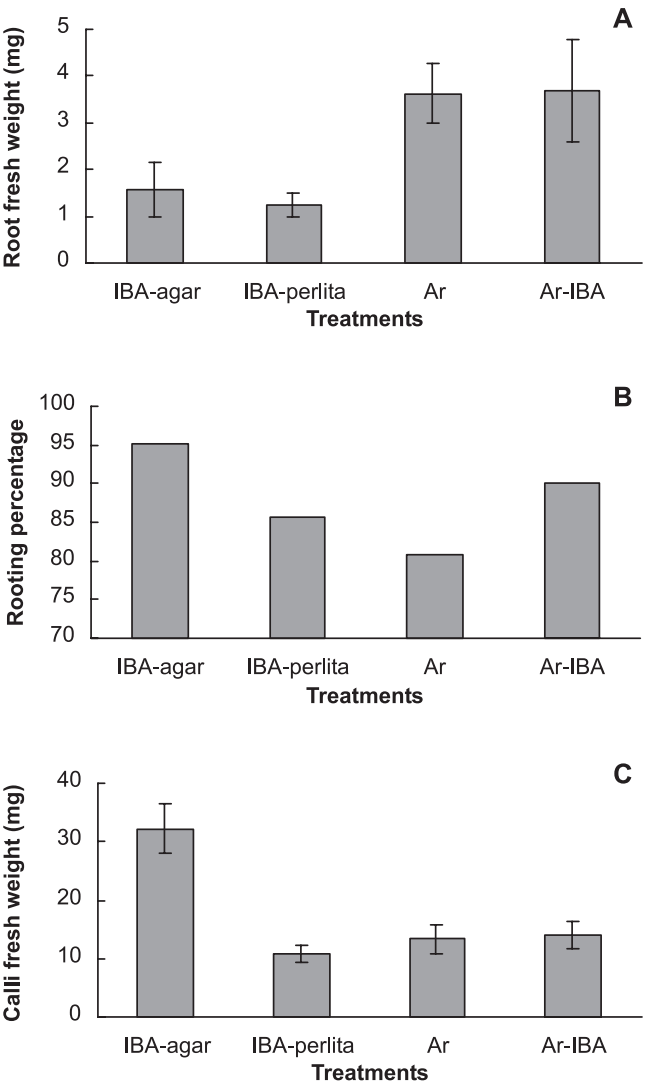


FIGURE 3. *In vitro* rooting and production of calli in *Nothofagus alpina* **A,** root fresh weight; **B,** callus percentage, and **C,** callus fresh weight. IBA: indol 3-butyric acid, Ar: *Agrobacterium rhizogenes*. Vertical lines indicates 1 SE.

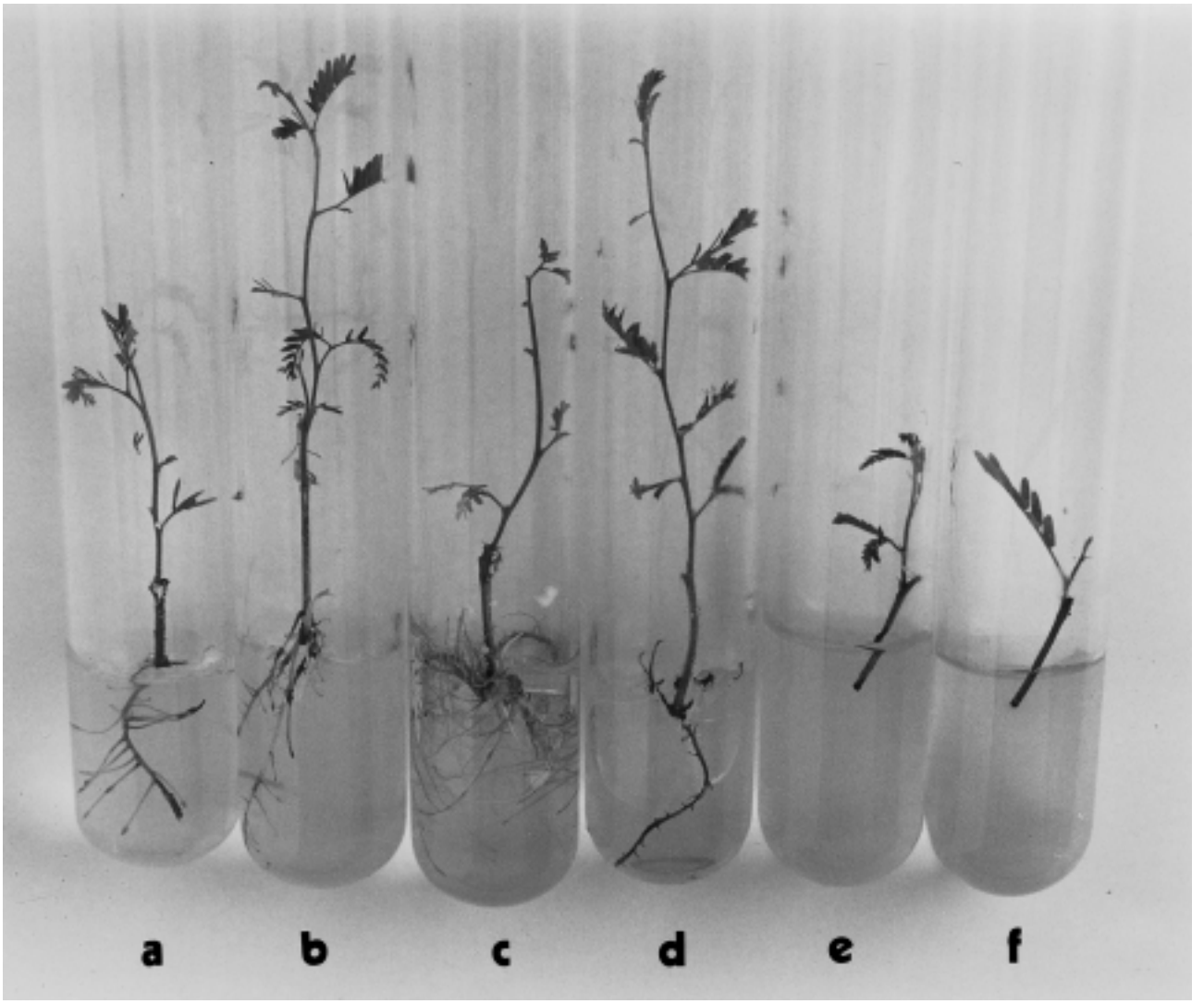
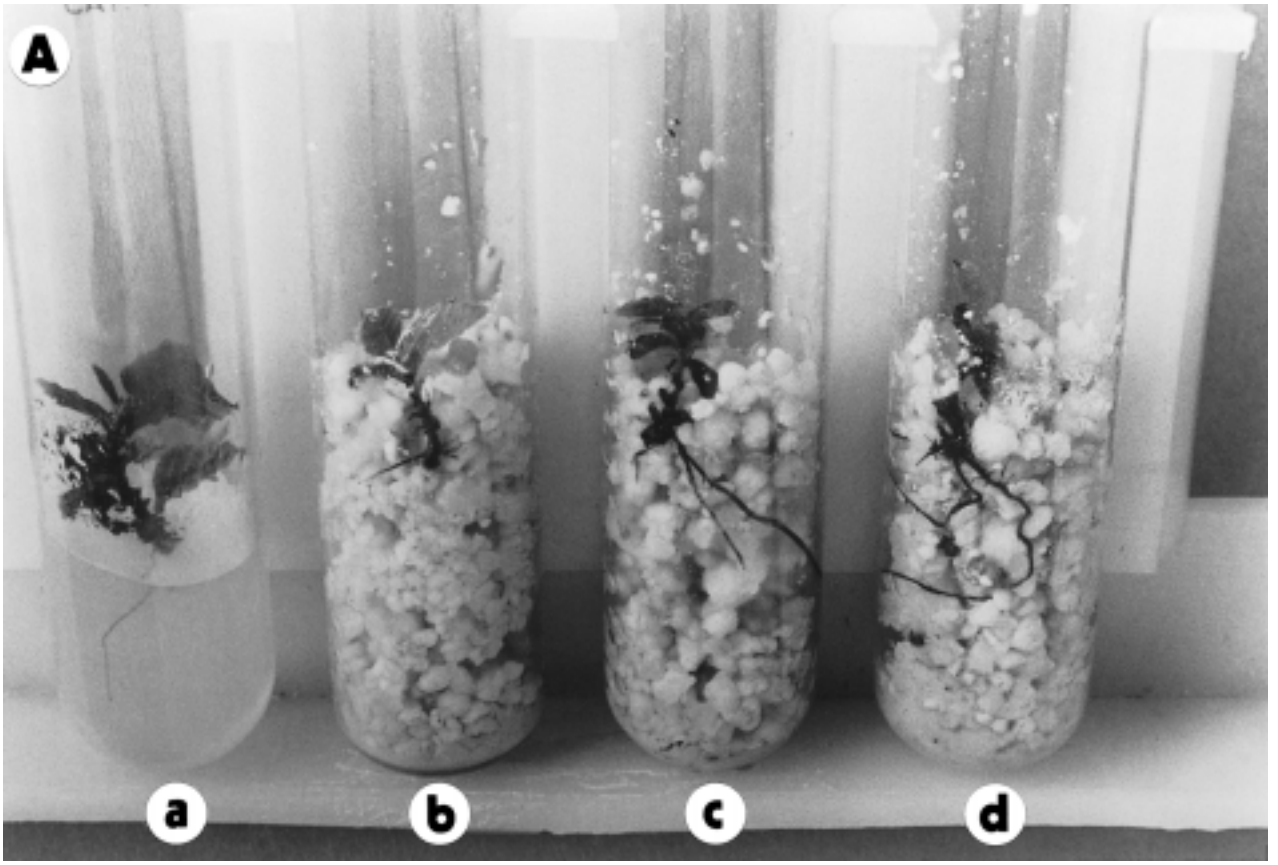


FIGURE 4. Rooting responses of *Prosopis chilensis* to IBA and *Agrobacterium rhizogenes* (Ar) to the following treatments: **a**, 1 min inoculation with Ar prior to cultivation in BTMm-IBA (Ar-BTMm-IBA); **b**, 1 min immersion in 3 mg.L^{-1} IBA followed by 1 min inoculation with Ar prior to cultivation in BTMm (Ar-IBA); **c**, 1 min inoculation with Ar prior to cultivation in BTMm (Ar); **d**, cultivation in BTMm containing 3 mg.L^{-1} IBA (BTMm-IBA); **e**, 1 min immersion in 3 mg.L^{-1} IBA, prior to cultivation in BTMm (IBA); **f**, cultivation in BTMm (control). After 10 days, all shoots were transferred to auxin-free BTMm (modified Broadleaved Tree Medium) for 30 days.

FIGURE 5. Rooting responses of *Nohotofagus alpina* to IBA and *Agrobacterium rhizogenes* (Ar) after 50 days culture. **A**, culture tubes containing: **a**, solid BTMm with 5 mg.L^{-1} IBA (IBA-agar); **b**, perlite satured with liquid BTMm with 5 mg.L^{-1} IBA (IBA-perlite); **c**, perlite satured with liquid BTMm and inoculation with Ar during 1 min; **d**, perlite satured with liquid BTMm with 5 mg.L^{-1} IBA and inoculation with Ar during 1 min (Ar-IBA). **B**, plants coming from treatments in tubes as in A. BTMm, modified Broadleaved Tree Medium.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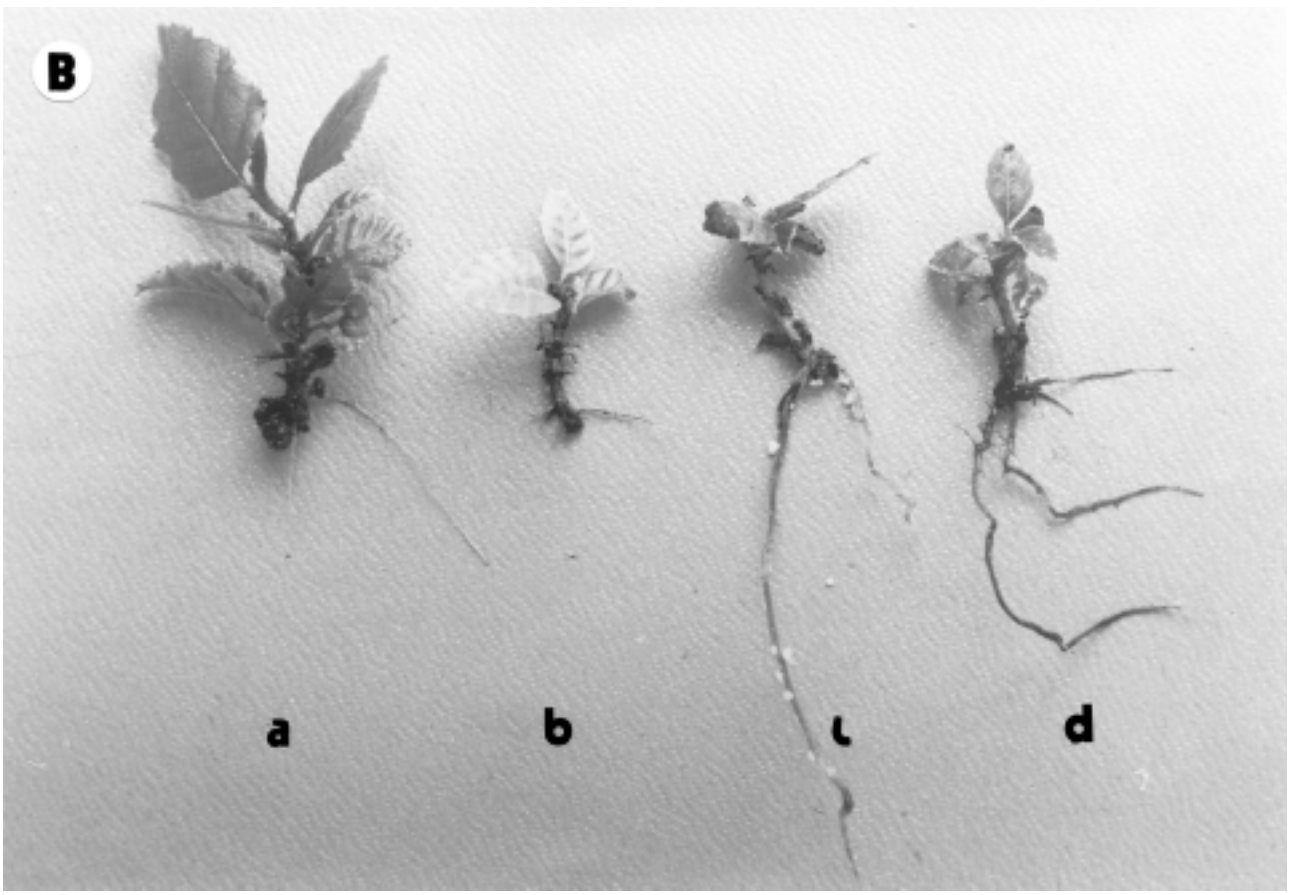
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shoots were transferred to auxin-free BTMm with 7 g.L^{-1} agar. Those inoculated with *A. rhizogenes* were subcultured in a similar medium but containing 300 mg.L^{-1} cefotaxime. After 30 days, rooting percentage, number and dry weight of roots in rooted shoots and their elongation were measured.

Rooting of N. alpina

After 2 months culture, 2-3 cm long shoots (Ss) with 2 or 3 leaves were chosen to be used as explants in rooting assays. The treatments were: 1) solid BTMm with 5 mg.L^{-1} IBA (IBA-agar); 2) perlite satured with liquid BTMm with 5 mg.L^{-1} IBA (IBA-perlite); 3) perlite satured with liquid BTMm and inoculation of Ss with *A. rhizogenes* during 1 min (Ar); and 4) perlite satured with liquid BTMm with 5 mg.L^{-1} IBA and inoculation with *A. rhizogenes* during 1 min (Ar-IBA). BTMm was the same medium used in the culture initiation stage but without BAP. The pH was adjusted to 5.7. To improve rooting, the cultures were incubated at 25 ± 2°C in darkness for one week (Martínez Pastur and Arena, 1996) and then kept with a 16 h photoperiod under fluorescent white light (56 μmol m^{-2} s^{-1} PAR) until the end of the experiment. For Ar and Ar-IBA treatments, shoots were wounded at the base with a sterile scalpel prior submerging them in the *A. rhizogenes* culture. Then, they were gently blotted with sterile tissue paper, transferred to the corresponding rooting medium and incubated at 25 ± 2°C in darkness for one week. At day 7, inoculated Ss were inmersed for 15 min in 100 ml rooting medium containing 1.0 g.L^{-1} cefotaxime and then transferred to the corresponding rooting medium containing 300 mg.L^{-1} cefotaxime. Cultures were maintained at 25 ± 2°C with 16 h photoperiod (white-cold fluorescent light, 56 μmol m^{-2} s^{-1} PAR) until the experiment was concluded at day 50.

For all treatments, number of rooted explants, number of roots per explant, length of the main root, number of secondary roots, fresh weight of roots, number of explants producing callus and callus fresh weight were measured.

Experiment design and statistical analysis

For both species a randomized design was used with 25 explants per treatment and two replicates for each experiment. Data were evaluated using either one or two way analysis of variance and the mean values of treatments were separated using the multiple comparison media test of Tukey-Kramer (Ott, 1984).

Results and Discussion

Rooting of P. Chilensis

The average rooting percentage was significantly higher ($P = 0.03$) in the treatments including *A. rhizogenes* inoculation to those which were not inoculated, reaching 100% in Ar-IBA treatment (Fig. 1 A). These results agree with those reported by Benavides and Radice (1998) for *Simmondsia chinensis*.

Explants grown in the culture medium containing IBA (BTMm-IBA) showed an increase in the number of primary roots per explant compared with those grown in the medium auxin-free (2.2 and 0.8, respectively). With *A. rhizogenes* (Ar) the number of primary roots observed per explant was significantly higher ($P < 0.001$) in comparison with the control (no bacterium inoculation) with mean values nearly to 3.0 roots per explant. However, the root average number per explant increased up to 3.0 when IBA was supplied together with *A. rhizogenes* in both treatments Ar-IBA and Ar-BTMm-IBA (Fig. 1 B).

Root dry weight was also significantly higher in material coming from Ar-IBA and Ar-BTMm-IBA treatments ($P = 0.007$) (Fig. 1 C). However, differences in root length were not found among treatments ($P = 0.63$).

Irrespective of the treatment, length of rooted shoots (22.8 mm) was significantly greater ($P < 0.001$) than that of the shoots without roots (9.4 mm). This effect may be atributted to a major exploration of culture medium by the roots and a better assimilation of nutrients by the explants.

It is important to emphasize that although treatments with inoculation of *A. rhizogenes* produced better rooting, treatment with IBA alone in the culture medium (BTMm-IBA) produced a good percentage of rooted shoots (86.0%), with more than 2 (up to 6) roots per shoot and having a good root biomass (2.6 mg average dry weight). Tipical response for each treatment is shown in Fig. 4.

In a preliminary hardening trial (unpublished results) eighteen plants, 9 inoculated with Ar and 9 treated with IBA, were grown *ex vitro* and maintained under nursery conditions for five months. The survival was 88.9% and 77.8%, respectively. No differences were detected after transplanting between both rooting treatments.

Hence, if micropropagation in *P. chilensis* is looked for, then the IBA treatment, being easier to perform, would be the method of choice. However, rhizogenesis mediated by *A. rhizogenes* would otherwise be presum-

ably advantageous if the *in vitro* differences in its favor in both volume and radical weight were to be translated in a superior survival and growth in field conditions.

Rooting of N. alpina

Rooting percentages were 33.3 %, 19.0 %, 90.4 % and 70.0 % for IBA-agar, IBA-perlite, Ar y Ar-IBA treatments, respectively. Combination of IBA and *A. rhizogenes* in the rooting medium did not improve the rooting performance to the level achieved by the shoots incubated with *A. rhizogenes alone.*

The treatments including *A. rhizogenes* infection significantly increased the number of main roots with regard to the IBA-agar treatment ($P < 0.005$) (Fig. 2 A). The main root length was significantly higher in the Ar treatment in comparison with both the IBA-perlite ($P < 0.001$) and IBA-agar ($P < 0.005$) treatments. The length of the main root was significantly higher with Ar-IBA than with IBA-perlite (Fig. 2 B). There were no secondary roots in the explants coming from either IBA-agar or IBA-perlite, while *A. rhizogenes* produced approximately four secondary roots per explant.

There were no differences among treatments in the fresh weight of the roots, but when *A. rhizogenes* infection was made, a tendency to increase fresh weight was observed (Fig. 3 A). All treatments produced a high ratio of calli formation, i.e. 95.2%, 85.7%, 80.9% and 90.0% for IBA-agar, IBA-perlite, Ar and Ar-IBA treatments, respectively (Fig. 3 B). The average fresh weight of calli from IBA-agar treatment was significantly higher ($P < 0.001$) than in the IBA-perlite, Ar and Ar-IBA treatments (Fig. 3 C). Tipical rooting response for each treatment is shown in Fig. 5 A and B.

The above mentioned results demonstrate a positive influence of the 9042 strain of *A. rhizogenes* on *in vitro* rooting in *N. alpina*; infection with *A. rhizogenes* also improved the rooting in *Pinus* and *Larix* spp. which are recalcitrant woody species (McAffe *et al.*, 1993). It is well known that both quantity and quality of the *in vitro* roots will affect the microplant behavior during the acclimatization phase. It is expected that a better root system, as the one obtained using *in vitro* infection with *A. rhizogenes*, would improve the *ex vitro* performance (survival rate and quality of the plants) of the micropropagated *N. alpina* plantlets. Our previous results show as a whole, that inoculation with the strain 9042 of *A. rhizogenes* markedly improves the quality of the root system in *N. alpina* in comparison with the rooting protocol reported by Martínez Pastur and Arena (1996).

Despite the fact that explants rooted in perlite (IBA-perlite) exhibited lower main root lengths and fresh weights than those cultured in IBA-agar medium, the higher root number and the lower callus fresh weight obtained in the former treatment, suggest that perlite is to be preferred over solid agar as solid support.

The choice of either rhizogenic route, will ultimately depend on the behaviour of microplants under field conditions with respect to survival and growth. Also, for both species, the use of hairy roots would have a potential to facilitate a different and possibly faster route for the improvement of these tree species through genetic engineering.

Acknowledgments

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