BIOCELL
2002, 26(1): 25-33

ISSN 0327 - 9545
PRINTED IN ARGENTINA

Micropropagation of *Prosopis chilensis* (Mol.) Stuntz from young and mature plants

L.A. Caro[1], P.A. Polci[1], L.I. Lindström[1], C.V. Echenique[1,2], and L.F. Hernández[1,3]

[1] Departamento de Agronomía, Universidad Nacional del Sur, San Andrés 800, 8000 Bahía Blanca, Argentina.
[2] CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), 8000 Bahía Blanca, Argentina.
[3] CIC (Comisión de Investigaciones Científicas de la Provincia de Buenos Aires), 1900 La Plata, Argentina.

Key words: Algarrobo de Chile, *In vitro culture*, *Prosopis chilensis*, regeneration, rooting.

ABSTRACT: *Prosopis chilensis* (Mol.) Stuntz (Algarrobo de Chile) is an important native tree species that can be grown in arid and semiarid regions for wood and forage production and environmental protection. Developing a simple and reliable *in vitro* protocol for cloning it would enable to improve it genetically. Explants of *P.chilensis* were taken from 4 months-old plants grown in the greenhouse or from adult trees grown in a natural environment. Nodal segments 1 – 2 cm long containing an axillary bud were selected from elongating shoots. These cuttings were aseptically cultured on two agar-solid basal media, MS or BTMm, and treated with 0.05 mg L^{-1} BA and 3 mg L^{-1} of either IAA, IBA or NAA. Sucrose (3% w/v) was used as carbon source. The percentage of sprouted cuttings and whole plant regeneration as well as its shoot and root length were recorded. Number, length and dry weight of shoots and roots were also measured. Rooting was successful with cuttings taken from young or adult plants, but explants from young plants showed a better response. Culturing in BTMm resulted in significantly greater shoot and root biomass than culturing in MS. Moreover, this response was higher in young explants when IBA was used as growth regulator. This paper reports a simple and effective method to micropropagate *P. chilensis* from young and adult plants.

ABBREVIATIONS: BA: Benzyl Adenine; **BTMm:** Broadleaf Tree Medium modified; **MS:** Murashigue and Skoog Medium; **IAA:** Indole-3-Acetic Acid; **IBA:** Indole-3-Butyric Acid;
NAA: α-Naphthalene-acetic Acid.

Introduction

In arid and semiarid regions of Argentina, *Prosopis chilensis* (Mol.) Stuntz (Algarrobo de Chile) has proven valuable for production of forage and firewood, and is also characterized by its drought resistance, fast growth and significant biomass production (Jordan and Balboa, 1985). The asexual propagation from cuttings of adult trees can be used to overcome difficulties of establishing an appropriate root system in the soils predominant in these regions (Felker, 1992). An *in vitro* technique with high multiplication response would be an interesting alternative for asexual propagation of selected individual trees (Gupta *et al.,* 1981; Yang *et al*., 1995). Several attempts have been made to demonstrate applicability of tissue culture technology for *Prosopis* species (Shekhawat *et al*., 1993, Castillo de Meier and Bovo, 2000). Propagation by tissue culture has been centered on explants taken from young plants (Jordan and Balboa, 1985; Jordan, 1987; Nandwani and Ramawat, 1991).

Address correspondence to: Prof. Dr. Luis F. Hernández, Departamento de Agronomía, Universidad Nacional del Sur. San Andrés 800, (8000) Bahía Blanca, ARGENTINA.
Tel.: (+54-291) 453 4775; Fax: (+54-291) 459 5127; E-mail: lhernan@criba.edu.ar
Received on April 17, 2001. Accepted on December 12, 2001.

In vitro technology has not been used successfully for cloning adult and selected individuals of *Prosopis* species because the explants taken from mature and aged trees do not respond appropriately in culture (Jordan and Balboa, 1985; Bovo *et al*., 1991; Shekhawat *et al*., 1993). Thus, *in vitro* propagation of *Prosopis* species requires new tissue culture approaches. The aim of this study was to establish a method for micropropagation of *P. chilensis* by induction of shoot proliferation from nodal segments containing an axillary bud excised from young plants and adult trees.

Materials and Methods

Plant material

Single-node explants of *P. chilensis* were taken from 4 months-old plants grown from seeds in a greenhouse, and from adult trees (10 to 16 years old) growing in a natural environment in Bahía Blanca, Argentina, (38°45' LS, 62°11' LW). From the later ones, nodal segments 1 – 2 cm long containing an axillary bud were taken from elongating shoots of the current year. They were selected at the beginning of spring time (October in the southern hemisphere). Explants were surface-sterilized by immersion in ethanol (70% v/v) for 1 min and in commercial bleaching solution (0.6% active chlorine) for 30 min with one drop of Tween 80™ as surfactant. Finally all explants were washed six times with sterilized, distilled water.

In vitro culture

Explants were cultured aseptically on two basal media: MS (Murashige and Skoog, 1962) and BTMm (Chalupa, 1983). BTMm was prepared at 50% of its originally formulated concentration. Preliminary evidence (L. Caro, unpublished) demonstrated that full strength BTMm was not suitable for *Prosopis* culturing. Both media were treated with 0.05 mg L^{-1} BA and 3 mg L^{-1} of either IAA, IBA or NAA. Sucrose (3% w/v) was used as carbon source and agar (0.7% w/v) as a gelling agent. The pH of the media was adjusted to 5.8 before autoclaving at 1 atm for 30 min. Five ml of these media were dispensed in 20 ml test tubes (13.5 mm x 150 mm), and explants were positioned vertically on the surface of the culture medium.

Cultures were kept at 25 ± 2°C with a photoperiod of 16 h, and 70 μmol m^{-2} s^{-1} light intensity was provided by florescent tubes Philips™ TLD 36W/54. After 10 days, cultures were transferred to basal media without growth regulators and kept for 30 days with the same light and temperature conditions as described before.

For anatomical structure observation, transverse sections from juvenile and adult explants used for *in vitro* culture were cut on a sledge microtome before culturing. To observe the degree of tissue lignification, 5 μm thick sections were cleared with hypochlorite solution, washed in water and mounted in phloroglucinol-hydrocloric acid (O'Brien and McCully, 1981). Explant cuttings were also taken 21 days after culturing and processed by histological techniques to study their

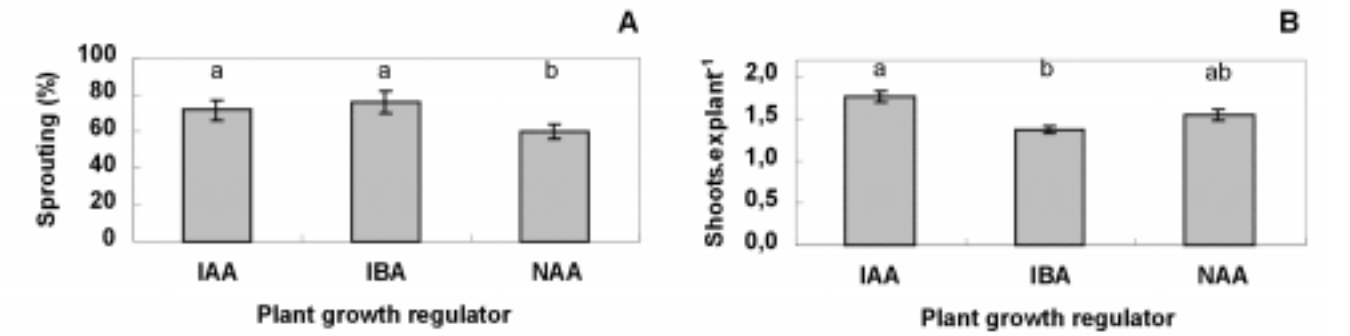

FIGURE 1: **A)** Sprouting percentages from juvenile and adult explants for each auxin. **B)** Mean number of shoots produced per explant for each auxin. Mean values with different letters are significantly different at 0.05 level of probability according to Bonferroni's test. Vertical lines = S.E.

TABLE 1.

Three-way ANOVA analysis, including all data obtained for all factors studied.
*, ** = significant effect at $\alpha = 0.05$ and 0.01, respectively; ns = not significant.

Variables	Sprouting (%)			Plant regeneration (%)		Shoot number			Root number		Shoot length (mm)		Root length (mm)		Shoot dry weight (mg)		Root dry weight (mg)	
Source	d.f.	F	p	F	p	d.f.	F	p	F	p	F	p	F	p	F	p	F	p
1. Explant age	1	138.74	**	89.49	**	1	0.29	ns	2.05	ns	0.95	ns	4.29	*	0.21	ns	1.38	ns
2. Basal media	1	0.52	**	23.58	**	1	0.00	ns	3.49	ns	12.66	**	32.09	**	18.96	**	18.76	**
3. Auxins	2	16.81	**	42.10	**	2	4.63	**	2.72	ns	12.36	**	38.29	**	3.77	*	21.41	**
1 x 2	1	0.43	ns	0.03	ns	1	0.19	ns	0.42	ns	0.01	ns	0.02	ns	4.72	*	0.85	ns
1 x 3	2	1.62	ns	2.47	ns	2	0.41	ns	1.94	ns	0.01	ns	0.67	ns	0.54	ns	0.83	ns
2 x 3	2	0.17	**	6.98	**	2	0.25	ns	1.34	ns	3.87	*	8.22	**	1.58	ns	4.10	*
1 x 2 x 3	2	2.78	ns	1.59	ns	2	1.29	ns	1.90	ns	1.15	ns	0.83	ns	0.77	ns	0.24	ns
Error	13					486												

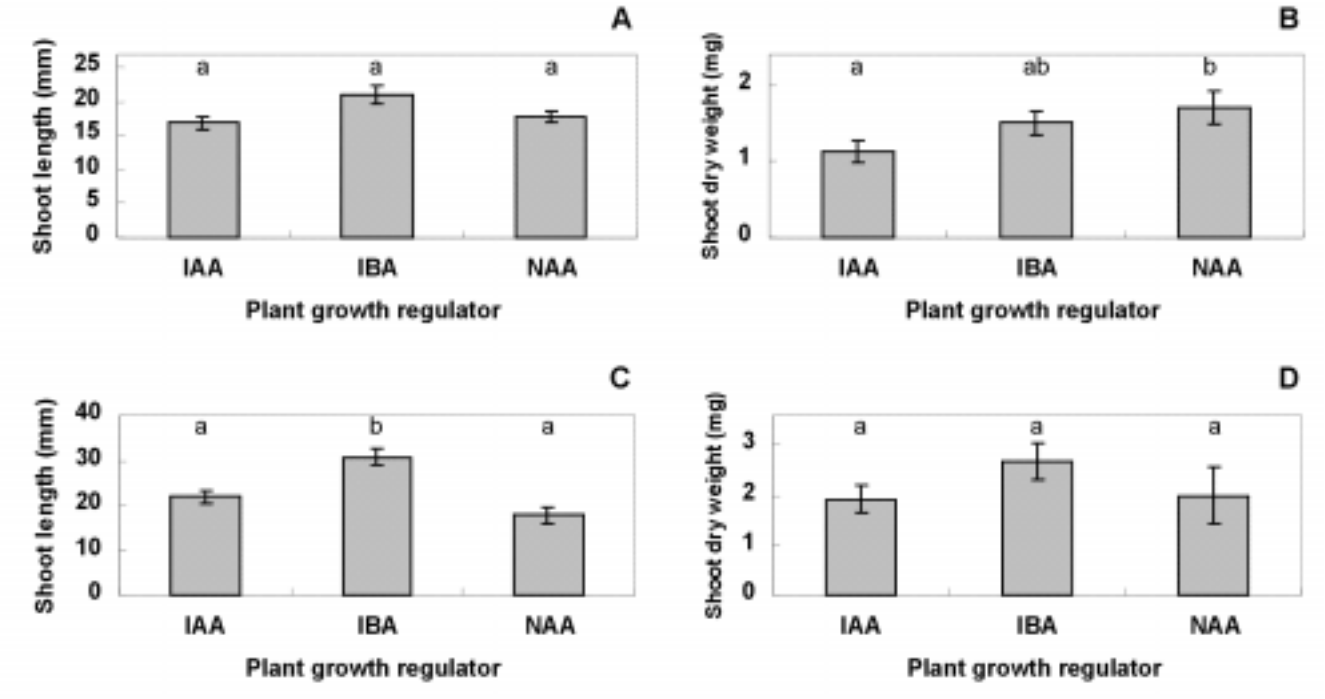

FIGURE 2: **A** and **B)** Whole plant regeneration percentages obtained in MS media for each auxin and for each type of explant, respectively. **C** and **D)** Whole plant regeneration percentages obtained in BTMm media for each auxin and for each type of explant, respectively. Mean values with different letters are significantly different at 0.05 level of probability according to Bonferroni's test. Vertical lines = S.E.

TABLE 2.

Two-way ANOVA keeping the MS basal culture medium as a reference factor
*, ** = significant effect at α = 0.05 and 0.01, respectively; ns = not significant.

Variables	Plant regeneration (%)			Shoot length (mm)			Root length (mm)		Shoot dry weight (mg)		Root dry weight (mg)	
Source	df.	F	p	d.f.	F	p	F	p	F	p	F	p
1. Explant age	1	64.77	**	1	0.39	ns	2.74	ns	2.86	ns	0.05	ns
2. Auxins	2	11.44	**	2	1.66	ns	6.55	**	3.17	*	5.02	**
1 x 2	2	1.35	ns	2	0.51	ns	0.41	ns	1.50	ns	0.83	ns
Error	6		239									

TABLE 3.

Two-way ANOVA keeping the BTMm basal culture medium as a reference factor.
*, ** = significant effect at α = 0.05 and 0.01, respectively; ns = not significant.

Variables	Plant regeneration (%)			Shoot length (mm)			Root length (mm)		Shoot dry weight (mg)		Root dry weight (mg)	
Source	df.	F	p	d.f.	F	p	F	p	F	p	F	p
1. Explant age	1	31.41	**	1	0.58	ns	1.69	ns	2.38	ns	1.72	ns
2. Auxins	2	27.70	**	2	13.93	**	36.26	**	2.55	ns	16.86	**
1 x 2	2	2.11	ns	2	0.65	ns	1.04	ns	0.39	ns	0.36	ns
Error	6		245									

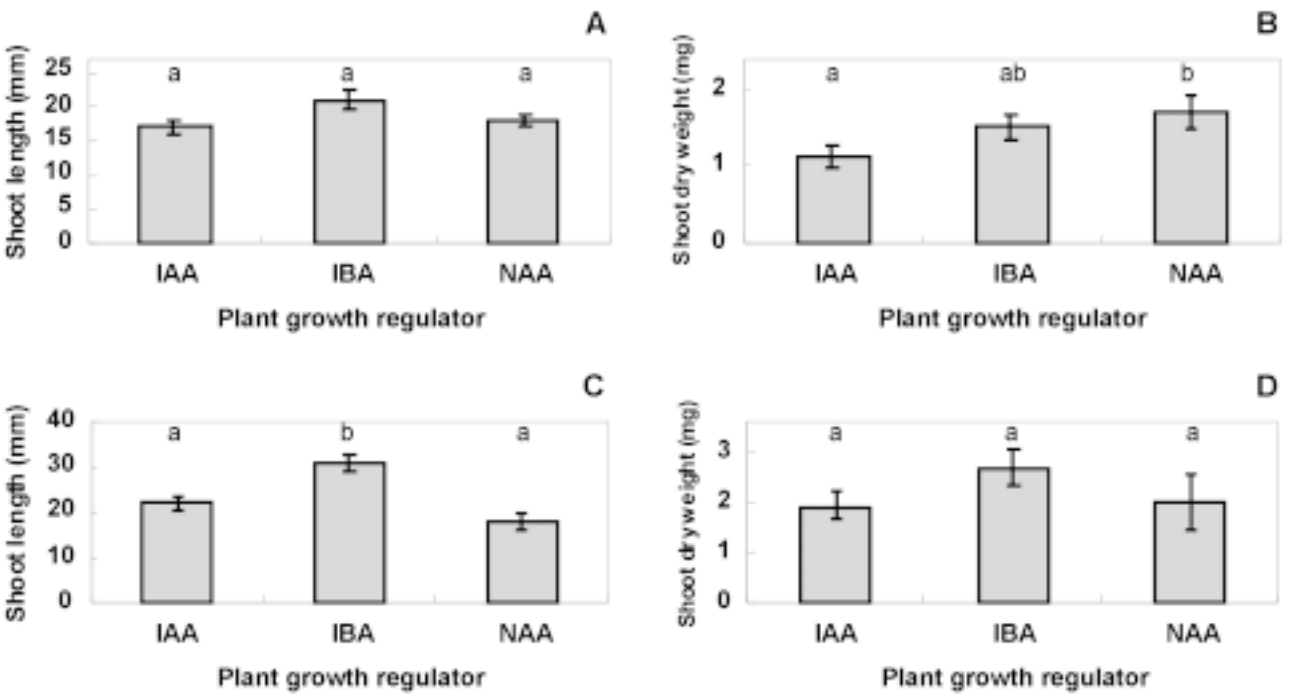

FIGURE 3: **A)** Shoot length (mm) from juvenile and adult explants cultivated in MS media **B)** Shoot dry weight (mg) from juvenile and adult explants cultivated in MS media. **C)** Shoot length (mm) from juvenile and adult explants cultivated in BTMm media. **D)** Shoot dry weight (mg) from juvenile and adult explants cultivated in BTMm media. Mean values with different letters are significantly different at 0.05 level of probability according to Bonferroni's test. Vertical lines = S.E.

anatomical structure, the adventitious roots origin and to confirm if vascular connections between the adventitious roots and the parent axis were stablished (Esau, 1977). Cuttings were obtained with a rotary microtome, and stained and mounted following conventional techniques (O'Brien and McCully, 1981).

Experimental design and statistical analysis

A completely randomized design was used with three factors: explant age (juvenile, adult), basal culture medium (MS, BTMm), and auxins used (IAA, IBA, NAA), resulting a total of twelve treatments. Each treatment was applied to 60 replicates.

After 30 days of subculturing, the percentage of sprouted cuttings (S%) and whole plant regeneration (SR%), shoot and root number (SN, RN), shoot and root length (SL, RL), and shoot and root dry weight (SDW, RDW) were recorded. SDW was obtained weighing only shoots larger than 0.5 mm newly differentiated from the explants.

For statistical analysis, ANOVA and Bonferroni tests were performed (Steel *et al*., 1996) over the responsive explants with the statistical software SYSTAT v.7.0 (SPSS, Inc. 1997).

Results and Discussion

Independently of the basal medium, treatments using the auxins IBA and IAA showed higher sprouting response (S%) than the treatment with NAA (Fig. 1 A). The highest mean value (95%) in S% was obtained when IBA as grow regulator, juvenile material as explant and BTMm as basal medium were used. Sprouting in juvenile explants was always significantly higher that in adult ones ($P < 0.01$). For this variable significant differences were not presented ($P = 0.484$) between basal media (Table 1).

Most of the explants produced only one shoot regardless of culture media or explant origin ($P > 0.50$). In a few cases up to 5 shoots per explant were observed. IAA treatment promoted more shoot growth per explant than IBA (1.75 and 1.37 shoots per explant, respectively; $P = 0.008$) (Fig. 1 B).

As for root number per explant (RN) differences were not observed due to explant age ($P = 0.153$), basal medium ($P = 0.062$) or growth regulator ($P = 0.067$) (Table 1).

When the variables whole plant regeneration (%SR), shoot and root length (SL, RL) and shoot and root dry weight (SDW, RDW) were analyzed using a

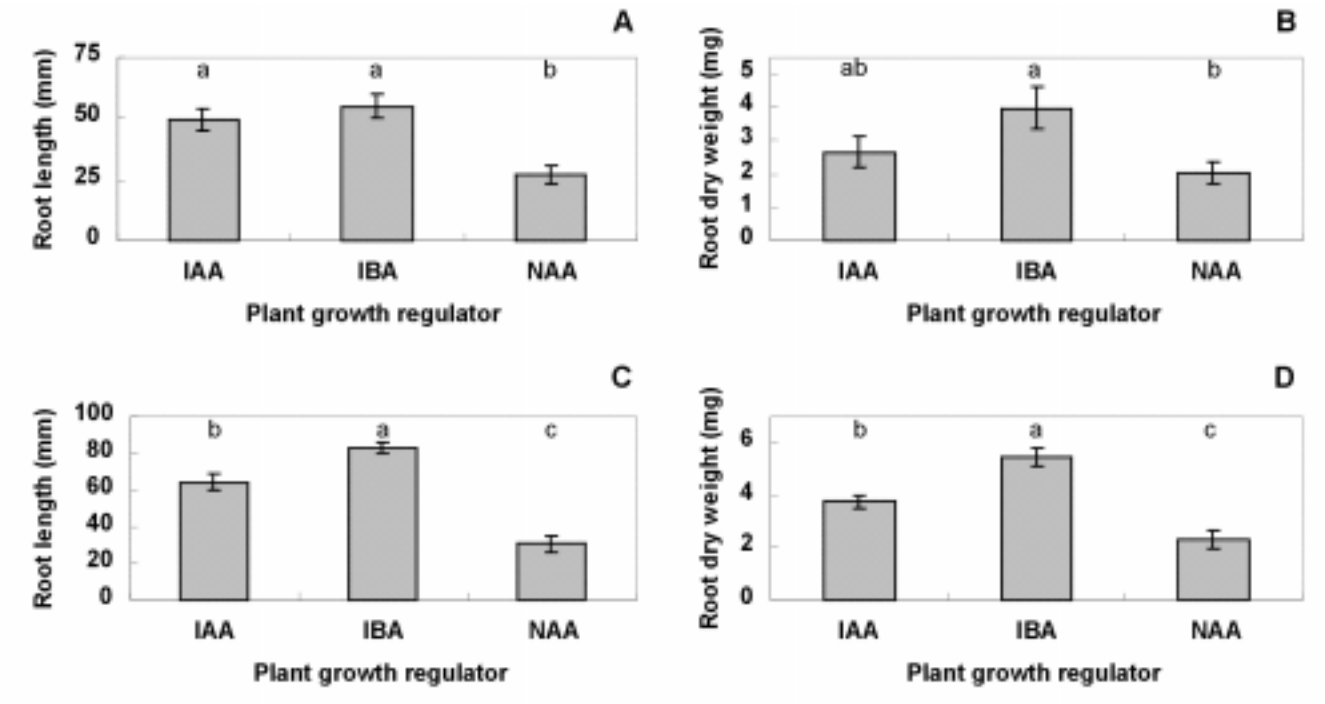

FIGURE 4: **A)** Root length (mm) from juvenile and adult explants cultivated in MS media **B)** Root dry weight (mg) from juvenile and adult explants cultivated in MS media. **C)** Root length (mm) from juvenile and adult explants cultivated in BTMm media. **D)** Root dry weight (mg) from juvenile and adult explants cultivated in BTMm media. Mean values with different letters are significantly different at 0.05 level of probability according to Bonferroni's test. Vertical lines = S.E.

three-way ANOVA analysis, including all data obtained for all factors studied, significant interactions between factors were detected (Table 1). Therefore, for further statistical analysis the results were analyzed using a two-way ANOVA keeping the basal culture medium as a reference factor. The results of this analysis are shown in Table 2 for MS media and in Table 3 for BTMm media.

Rooting was consistently observed in all treatments only in sprouted explants. The auxins IAA and IBA induced better rooting than the auxin NAA ($P < 0.05$, Fig. 2, A and C). Young explants also showed more responsive to obtain plant regeneration than those taken from adult trees ($P < 0.01$, Fig. 2, B and D). These results were consistent in both media. Root initiation commenced 10 days after subculturing, and a high percentage of shoots (about 80% for young material and 56% for adult material) rooted when inoculated in BTMm plus IBA. So, a better plant regeneration response was observed in the explants cultured in BTMm than in MS (Fig. 2).

Shoot mean length (SL) of the explants grown in BTMm were higher than in MS (24 mm and 17 mm,

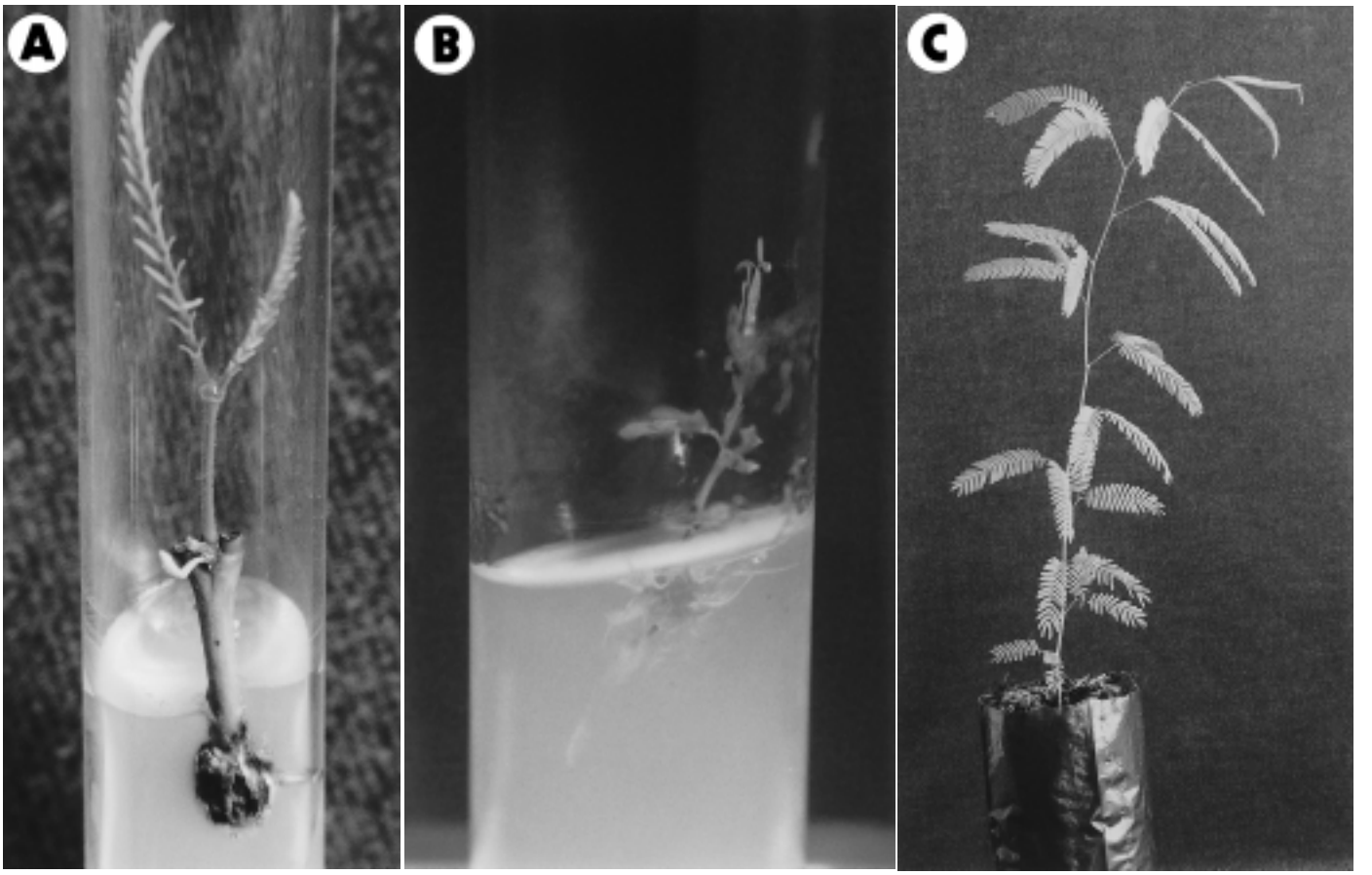

FIGURE 5: Transverse section of the stem from explants growing in BTMm-IBA after 21 days. An adventitious root (**Ar**) is seen originated on the cambial zone (**Ca**) in front of a vascular radius (**r**). **Ct**: non-embryogenic callous tissue, **Cr**: cortex, **Ph**: phloem, **Pi**: pith, **X**: xylem.

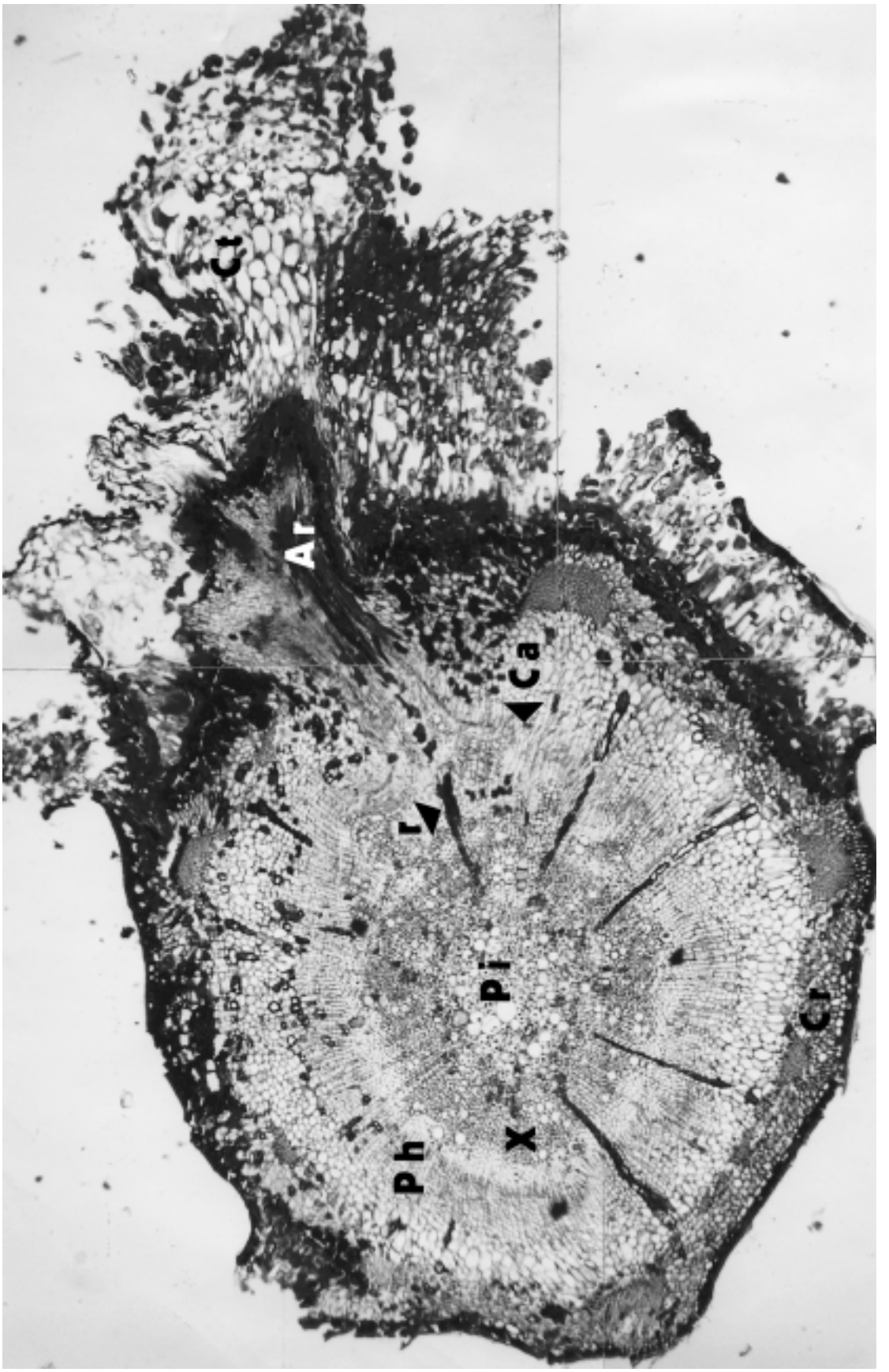

FIGURE 6: **A)** Rooted cutting coming from an adult tree. **B)** Rooted cutting coming from a 4 months old plant. **C)** A rusticated plant *ex vitro* of *Prosopis chilensis* attained after *in vitro* culturing coming from **B)**.

respectively). However, when the results were statistically analyzed in a separate way, it was observed that shoot length (SL) did not show significant differences between auxins in MS (Table 2 and Fig. 3 A), but in BTMm IBA induced longer SL than IAA and NAA (Table 3 and Fig. 3 C).

Culturing in MS, the shoot dry weight was significantly higher with NAA (1.7 mg) and lower with IAA (1.1 mg) (Fig. 3 B), but no significant differences were found between both types of explants used (Table 2). Neither there were any significant differences in shoot dry weight in explants grown in BTMm when comparing between explant age or between auxins (Table 3; Fig. 3 D).

For all explants cultured in MS, root length (RL) was significantly lower in treatments containing NAA (Fig. 4 A). In BTMm, RL was significantly higher in treatments with IBA (Fig. 3 C). Explants cultured with IBA, so much in BTMm as MS showed a significant increase in root dry weight (Fig. 4 B and D). On the other hand, for the last four variables analysed BTMm showed better results than MS (Fig. 3 and 4, A and B vs C and D).

Arce and Balboa (1991) found that rooting of *P. chilensis* cuttings obtained from adult plants grown in the Southern Hemisphere was only observed when cuttings were taken during the spring-summer season (September to March). This period corresponds to the largest vegetative growth and reproductive activity. Percentages of rooting did not exceed 15% (Arce and Balboa, 1991). When they used cuttings from young plants grown in a greenhouse, rooting exceeding 80% was only observed in liquid aerated media (Arce and Balboa, 1991). We also ended up with simmilar results only when explants were taken from the nearest terminal zone of mature tree branches. Nevertheless, as was mentioned before, the percentage of rooting obtained in our study was greater than in previous work for other species of *Prosopis* (Bovo *et al*., 1991; Castillo de Meier and Bovo, 2000) and represents a significant advance in the asexual propagation of this species.

The improved response of explants grown on BTMm was probably produced by the lower osmotic strength of this medium compared with MS; MS is one of the most saline media formulated for tissue culture (Krikorian, 1991). For RN both basal media gave similar responses without significant differences between adult and juvenile material, although the percentage for adult material was slightly lower. The number of primary roots per explant ranged from one to five.

Calli were induced in both basal media that were treated with NAA as growth regulator. These calli were non-typical embryogenic or organogenic, and were mainly cotton- like and non-morphogenic. Calli also developed with IBA and IAA, but the relative development was not significant. Avoiding callus formation minimizes the risk of somaclonal variation (Larkin and Scowcroft, 1981). No adventitious rooting was observed in the callus tissue formed at the base of the cuttings.

Observation of transverse sections of shoots revealed the shoot cuttings used as explants were in primary stage of growth with xylem and sclerenchyma tissues slightly lignified as the weak reaction of these tissues to phloroglucinol-hydrocloric acid revealed. After 21 days of *in vitro* culture some secondary growth was produced but epidermal and related primary tissue were still present (Fig. 5). *In vitro* formed roots had an endogenous origin and arose close to the vascular cambium (Fig. 5). These roots were connected with the parent axis vascular system. No adventitious rooting was observed in the callus tissue formed at the base of the cuttings. As it has been found in other ready rooting shoots the cortex was internally delimited by a discontinuous ring of perivascular fiber (Fig. 5). The degree of sclerification of primary phloem is negatively correlated to the ability of one-year-old stems to form adventitious roots (Beakbane, 1961). So, as the propagation capacity falls, the continuity of the sclerinchymatosous ring increases (Beakbane, 1961; Esau, 1977). This suggest that it could be necessary the presence of discontinuity in the sclerinchymatosous ring for the adventitious roots to develop.

When working with adult trees, contamination by fungus can be of significant interference with rooting responses. This interference could be a limiting factor for asexual propagation of material derived from elite individuals. Working with *P. nigra,* Bovo *et al*. (1991) reported contamination of 19% to 57% of samples. We observed a maximum contamination of 17% for adult explants and 7% for juvenile explants, which indicates that contamination is less of an obstacle to *P. chilensis* propagation and demonstrates the efficacy of the way the explants were dissinfected in this work.

Explants cultivated under our protocol developed into normal youthful plants in a period of 40 to 60 days showing enough elongation to identify 3 to 5 internodes. This plant morphology would also provide a source of news explants.

It is important to mention that the differential response to establish culture *in vitro* sometimes observed when using explants coming from different adult individuals can be positively correlated with the excellent macrovegetative multiplication performance observed

of the same donor plants (V. Lauric and L. Caro, unpublished).

Our micropropagation protocol proved to be simple and reliable and it allows cloning of juvenile or adult trees of *P. chilensis*. Healthy plants obtained by this way from adult or young explants can develop normally under *ex vitro* conditions (Fig. 6 A-C). Increasing substantially its multiplication rate using the *in vitro* protocol described in this work makes it potentially useful for large-scale production.

Acknowledgements

We gratefully acknowledge Ing. Agr. O. Bovo for his helpful suggestions. This research was supported by the Secretaría de Ciencia y Tecnología - UNS and the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC, La Plata, Argentina). The valuable comments on the manuscript of Dr. M.H. Ebinger, Los Alamos National Laboratory, are deeply appreciated.

References

ARCE P, BALBOA O (1991). Seasonality on rooting of *Prosopis chilensis* cuttings and *in vitro* micropropagation. Forest Ecology and Management 40: 163-173.

BEAKBANE AB (1961). Structure of the plant stem in relation to adventitious rooting. Nature 192: 954-955.

BOVO OA, CASTILLO G, ZIBETTI G, MUZZIO P (1991). Cultivo *in vitro* de tejidos del género Prosopis (Leguminosae). Actas Jornadas Taller Com. Nac. de Prosopis. Sgo. del Estero, pág. 28.

CASTILLO DE MEIER G, BOVO OA (2000). Plant regeneration from single-nodal-stem explants of legume tree *Prosopis alba* Griseb. Biocell 24: 89-95.

CHALUPA V (1983). Microprogation of conifer and broadleaved forest trees. Commun Inst Forest Czechosl 13: 7-39.

ESAU K (1977). Anatomy of Seed Plants. John Wiley and Sons, Inc., New York. 550 pp.

FELKER P (1992). Capturing and managing the genetic variation in Prosopis spp. For economically useful characters. In: Tropical Trees: the potential for domestication and rebuilding of forest resources. ITE Symposium and ECTF Symposium Procs. R.R.B. Leakey and A. C. Newton (Eds.) pp. 98-104.

GUPTA PK, MASCARENHAS AF, JAGANNATHAN V (1981). Tissue culture of forest trees: Clonal multiplication of *Tectona grandis* (Teak) by tissue culture. Plant Sci Let 20: 195-201.

JORDAN M, BALBOA O (1985). *In vitro* regeneration of *Prosopis tamarugo* Phil. and (Mol.) Stuntz from nodal sections. Gartenbauwissenschaft 50: 138-142.

JORDAN M (1987). *In vitro* culture of *Prosopis* species. In: Cell and Tissue Culture in Forestry. Bonga, J.M. and Durzan, D.J. (Eds.), pp. 301-316. Martinus Nijhoff Pubs., Boston.

KRIKORIAN AD (1991). Medios de cultivo: Generalidades, composición y prepararción. In: Cultivo de tejidos en la Agricultura. Fundamentos y aplicaciones. Roca, W. and Mroginski, L. (Eds.). CIAT. Colombia, pp. 15-45.

LARKIN PI, SCOWCROFT WR (1981). Somaclonal variation. A novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60: 197-214.

MURASHIGE T, SKOOG F (1962). A revised medium for rapid growth and biosassays with tobacco cultures. Physiology Plant 15: 473-497.

NANDWANI D, RAMAWAT KG (1991). Callus culture and plantlets formation from nodal explants of *Prosopis juliflora* (Swartz) DC. Indian J Exp Biol 29: 523-527.

O'BRIEN TP, McCULLY ME (1981). The study of Plant Structure. Principles and Selected Methods. Termacarphi Pty. Ltd., Melbourne, Australia. 432 pp.

SHEKHAWAT NS, RATHORE TS, SINGH RP, DEORA NS, RAO SR (1993). Factors affecting *in vitro* clonal propagation of *Prosopis cineraria*. Plant Growth Reg 12: 273-280.

STEEL RGD, TORRIE JH, DICKEY DA (1996). Principles and Procedures of Statistics: A Biometrical approach. 3rd ed. McGraw-Hill, New York. 312 pp.

SPSS, Inc. (1997). SYSTAT 7.0: Statistics, Chicago, USA. 547 pp.

YANG J, CHUNG J, CHEN Z (1995). Vegetative propagation of adult *Eucaliptus grandis* x *urophylla* and comparison of growth between micropropagated plants and rooted cuttings. Plant Cell Reports 15: 170-173.