Micropropagation of *Salix humboldtiana* Hild.

Pereira, Ana Maria S.[1], Bertoni, Bianca W.[1], Moraes, Rita M.[2], França, Suzelei C.[1]

[1]Universidade de Ribeirão Preto - Departamento de Biotecnologia Vegetal, 14096-380 Ribeirão Preto, São Paulo, Brazil, [2]Mississippi State University – Department of Agronomy, 38677 Mississippi, USA.

RESUMO: Um protocolo de micropropagação visando a produção em larga escala de *Salix humboldtiana* Hild. foi desenvolvido usando duas fontes de explantes, derivados de plantas adultas e de plantas jovens clonadas. Tanto a proliferação de brotos como a indução de raízes foram influenciadas pela fonte de explante usada. Maior índice de brotação foi obtido em meio MS suplementado com 2.3 µM de cinetina, enquanto que o meio MS com a concentração de macronutrientes reduzida ao meio (MS/2) adicionado com 5.3 µM ANA propiciou o enraizamento. Durante o processo de aclimatação, irrigação com solução de ampicilina 0.005% (v/v) foi essencial para a sobrevivência das plantas.

Palavras chave – cultura de tecido, *Salix humboldtiana* Hild., salgueiro, plantas medicinais

ABSTRACT: Micropropagation of *Salix humboldtiana* Hild.. A micropropagation protocol for large scale production of *Salix humboldtiana* Hild. was carried out using both, explants excised from adult plants and from cloned plantlets. Either shoot proliferation or root induction were influenced by the source of explant used. MS medium supplemented with 2.3 µM kinetin enhanced shoot production while half-strength MS medium added with 5.3 µM NAA enhanced rooting. During the acclimatization process, irrigation with 0.005% (v/v) ampicillin solution was essential for plant maintenance.

Key words: Tissue culture, *Salix humboldtiana* Hild., willow species, salgueiro, medicinal plants

INTRODUCTION

Salix humboldtiana Hild. a medicinal plant that belongs to the Salicaceae family, is indicated as antirheumatic, spasmolytic and antipyretic (Gupta, 1995). According to Pezzuto *et al.* (1991) leaf and flower extracts of this species found to be cytotoxic with cultured KB or P-388 cells. S. *humboldtiana* Hild. is a tree species highly resistant to flood tide and widely recommended for forestation alongside rivers, lakes and other bodies of water. In Brazil this species is popularly known as salgueiro.

Several biotechnological approaches developed with *Salix* species have been reported (Vahala & Eriksson, 1991; Hauth & Beiderbeck, 1991). However, the number of works regarding *in vitro* propagation of this lineage is reduced, probably because most willow species are propagated through cuttings. Several species of the genus *Salix* play an important role because its a woody species which undergo combustion. Grönroos *et al.* (1990) regenerated *S. viminalis* plantlets via shoot culture and established them in the field. The main reason for micropropagating *Salix* species is that explants from a unique elite matrix its possible to produce millions of plantlets while propagation via stalks yields limited number of plants.

The objective of this work was to micropropagate *S. humboldtiana* Hild. achieving large scale clonal production of this medicinal species which can be applied in forestation.

MATERIAL AND METHOD

Plant material

Cuttings (30 cm) excised from *S. humboldtiana* Hild. adult trees, native to Mogi-Guaçú riverbanks, in São Paulo state, Brazil, were transplanted into plastic bags (3 liters) containing soil and sand (1:1) mixture and maintained inside a greenhouse, at 70% humidity, with one daily irrigation, for 30 days. After this period, shoots (5 cm) were removed, providing approximately 6 axillary buds (0.5 cm) as source of explants. Also, one-year-old cloned plants were used as source of explants.

Multiplication

Explants from different donors were washed with 1% (w/v) Benomyl for 1 h, 70% (v/v) ethanol for 20 sec. and kept in 0.5% (w/v) NaOCl, under agitation, for 30 min. Surface disinfested explants were inoculated on basic MS (Murashige & Skoog, 1962) medium and after 30 days rates of contamination were determined.

Buds collected from the adult-tree cuttings were then inoculated on basic MS medium supplemented with different concentrations of kinetin (0.9, 2.3, 4.6, 13.9, 23.2 µM), 3% (w/v) sucrose and 0.2% Phytagel (Sigma) while explants collected from cloned plantlets were inoculated on basic MS medium containing varied concentrations of kinetin (0.9, 2.3, 4.6, 13.9, 23.2 µM); 6-benzyladenine (BA) (0.8, 2.2, 4.4, 13.3,

Recebido para publicação em 21/07/99 e aceito para publicação em 05/04/00.

https://doi.org/10.70151/ea5tbg97

22.2 µM); 6-[4-hydroxy-3-methyl-cis-2-butenylamino] purine (zeatin) (0.9, 2.2, 4.5, 13.7, 22.8 µM); 2.3 µM kinetin plus indole-3-acetic-acid (IAA) (0.5, 1.1, 2.8 µM); or 2.3 µM kinetin plus naphthaleneacetic acid (NAA) (0.5, 1.0, 2.3 µM).

After three successive subcultures of 30 days each, number and height of shoots were recorded.

Rooting

Shoots from adult-tree cuttings were inoculated on basic MS or half strength MS (1/2 macronutrients) both supplemented with 5.3 µM NAA and shoots from cloned plantlets were inoculated on basic MS, MS\2 or MS\4 supplemented with (0.5, 2.6, 5.6, 10.7 µM) NAA; or (0.4, 2.4, 4.9, 9.8 µM) indole-3-butyric acid (IBA).

General conditions of cultures

In all experiments the explants were inoculated in glass test tubes (22 x 85 mm) each containing 5 mL of medium and closed with polypropylene closures (Bellco). Test tubes were sealed with plastic film and exposed to a 16-h photoperiod (40 µmol m^{-2} s^{-1}, 85 W cool-white GE fluorescent lamps) at 25 ± 2 ºC.

Either one of the media tested for multiplication or rooting were supplemented with 3% (w/v) sucrose, 0.2% Phytagel, pH adjusted to 5.7 and autoclaved at 121 ºC and 105 kPa for 15 min.

All recorded data, on number and height of shoots as well as percentage of rooted plantlets, were analyzed statistically by analysis of variance followed by Tukey test, with the level of significance set at 5%. All experiments were repeated three times with 10 replicates per treatment.

Transfer to soil

Rooted plantlets (200 unities) 2-2.5 cm high were transferred to 50-hollow styrofoam cases (45 x 90 mm hole) containing a soil/sand substrate (50:50 w/w) and kept in a greenhouse at 80% humidity for 4 weeks. During the first week, every other day 50% of the plantlets were irrigated, three times a day, with 0.005% (v/v) ampicillin solution. After 4 weeks all plantlets were transferred to plastic bags (10 x 15 cm) and maintained under 50% shade with one daily irrigation for 30 days. After this period plantlets were transferred to field.

RESULT

Culture establishment

Both kinds of explants, those collected from adult trees or from cloned plantlets showed same rates of contamination (30%). No shoot induction was noted in explants inoculated on basic MS medium and necrosis was observed in 5% of the inoculated buds.

Multiplication

Explants collected from adult trees inoculated on MS medium supplemented with 13.9 µM kinetin showed increased shoot proliferation, 2.3 shoots per bud. Shoots transferred to MS medium devoid of growth regulator, elongated 26 mm. However, explants from cloned plantlets inoculated on MS supplemented with 2.3 µM kinetin produced 3.8 shoots per bud and when transferred to MS basic medium shoots elongated 27 mm (Table 1).

Explants from cloned plantlets cultured on MS medium supplemented with kinetin associated to NAA or kinetin and IAA showed no significant differences on shoot proliferation (Table 2). Better results was achieved with MS medium added with kinetin and devoid of auxin supplement.

Rooting

Plantlets from explants derived of adult trees inoculated on half strength MS medium supplemented with 5.3 µM NAA showed 93% rooting whereas plantlets inoculated on basic MS medium supplemented with the same concentration of NAA exhibited only 31% of rooting (Table 3).

Shoots from cloned plantlets cultured on MS medium with different concentrations of macronutrients supplemented with various concentrations of NAA and IBA rooted well, although some difference in rooting rates was observed (Table 3). MS medium supplemented with IBA stimulated red pigment formation on roots maybe due to antocyanin accumulation. Half strength MS medium supplemented with 0.4 µM IBA enhanced rooting but reduced aerial parts development. Inferior growth of aerial parts was a typical response of explants cultured on medium supplemented with IBA. Half strength MS medium supplemented with 5.3 µM NAA showed better results on rooting *S. humboldtiana* Hild. (80%) promoting continuous growth of aerial parts. Rooting was increased on MS/4 medium supplemented with 10.7 µM NAA (86%) but shoot elongation was decreased influencing negatively the acclimatization process.

TABLE 1. Effects of different types and concentrations of cytokinins on number and length of shoots from adult and juvenile plant material of *S. humboldtiana* Hild.

Cytokinin	Concentration µM	Adult explant		Juvenile explant	
		Number of shoots	Shoot height (mm)	Number of shoots	Shoot height (mm)
kinetin	0.0	1.0 b	26 a	1.0 C	27 A
	0.9	1.2 ab	26 a	3.0 B	17 B
	2.3	2.0 ab	17 ab	3.8 A	15 B
	4.6	1.6 ab	18 b	3.6 AB	11 C
	13.9	2.3 a	10 bc	1.2 C	6 D
	23.2	1.7 ab	7 c	1.0 C	5 D
BA	0.8			2.7 A	10 B
	2.2			1.2 B	10 B
	4.4			1.0 B	7 C
	13.3			1.0 B	5 C
	22.2			1.0 B	6 C
zeatin	0.9			1.4 B	22 A
	2.2			1.7 B	15 B
	4.5			1.6 B	10 C
	13.7			1.8 A	15 B
	22.8			1.7 B	6 C

Data represent means of three replicate experiments with ten explants per treatment. Means followed by the same letter do not differ statistically at p = 0.05 according the Tukey test. Mean comparisons are valid for growth regulator combinations

TABLE 2. Effects of association of kinetin plus NAA and kinetin plus IAA on height and number of shoots from juvenile plant material of *S. humboldtiana* Hild.

Growth regulator concentration (µM)	Number of shoots	Shoot height (mm)
NAA + kinetin		
0.5 + 2.3	3.5 A	15 A
1.0 + 2.3	2.9 B	15 A
2.6 + 2.3	1.3 C	19 A
IAA + kinetin		
0.5 + 2.3	3.1 A	15 A
1.1 + 2.3	2.7 B	14 A
2.8 + 2.3	2.7 B	15 A

Data represent means of three replicate experiments with ten explants per treatment. Means followed by the same letter do not differ statistically at p = 0.05 according the Tukey test. Mean comparisons are valid for growth regulator combinations.

TABLE 3. Effects of different types and concentrations of auxins and macronutrients in basic MS on rooting of *S. humboldtiana* Hild.

Auxin	Concentration (μM)	% of rooted plantlets MS	MS\2	MS\4
Adult material				
NAA	5.3	31 B	93 A	
Juvenile material				
NAA	0	0	0	0
	0.5	33 B	60 A	73 B
	2.6	60 A	60 A	60 AB
	5.3	53 AB	80 A	46 B
	10.7	40 AB	80 A	86 A
IBA	0.4	73 A	93 A	46 B
	2.4	53 A	73 AB	73 A
	4.9	60 A	53 B	40 B
	9.8	86 A	80 A	80 A

Data represent means of three replicate experiments with ten explants per treatment. Means followed by the same letter do not differ statistically at p = 0.05 according the Tukey test. Mean comparisons are valid for growth regulator combinations.

Acclimatization

During the acclimatization process, the incidence of necrosis was efficiently decreased, from 75% to 5%, when plantlets were irrigated with ampicillin solution.

DISCUSSION

Following described procedures, 3000 plantlets of *Salix humboldtiana* Hild. were produced and employed in a reforestation program, in the region of Brotas a city in São Paulo state, carried out by CETESB – Companhia de Tecnologia de Saneamento Ambiental, the public agency of environmental sanity of the state of São Paulo.

Proliferation of shoots obtained with explants from cloned plantlets cultured on medium with decreased kinetin concentration justify the manipulation of one-year old micropropagated plants for the *in vitro* propagation. Reduced levels of exogenous growth regulators are always indicated for micropropagation in order to minimize clonal variation.

The most suitable auxin for rooting *S. humboldtiana* Hild. was NAA. Neuner & Beiderbeck (1993) also used NAA when manipulating clones of *Salix caprea* and achieved satisfactory results on rooting even though most clones rooted in basic MS medium.

Half strength MS medium enhanced rooting. Reduced salt concentration may inhibit the production of substances considered harmful to rooting (Skirvin *et al.*, 1980; Lineberger, 1983). According to Lisková *et al.* (1989), the production or lack of antocyanins as well as phenoliccompounds in tissue culture of willow is related to the concentration of macronutrients in the culture medium.

Tissue cultured plantlets of *Salix viminalis* were compared in a field trial with conventionally propagated cuttings of the same clone. No morphological abnormalities were observed in plants from tissue culture. The survival rate of tissue culture derived plants was higher than that of conventional cuttings, however the plantlets grew slower (Grönroos *et al.*, 1990). Micropropagated plantlets of *S. humboldtiana* Hild. also presented no morphological changes during the period of one year, in which they were kept in the experimental field at the University of Ribeirão Preto - UNAERP. The survival percentage of micropropagated plants was superior to cuttings while their development have been similar. Multiplication ratio was 50 plants per explant.

Based on these results, a scheme for the multiplication of *Salix humboldtiana* Hild. was assembled as shown in Figure 1.

The protocol described above is effective for the production of vigorous plants to restore degraded regions. Besides, this procedure is essential to forestation programs where the provision of cuttings for large scale production of plants represents the only option for recovering the area with this economically viable medicinal species.

FIGURE 1. Scheme for the multiplication of *Salix humboldtiana* Hild.

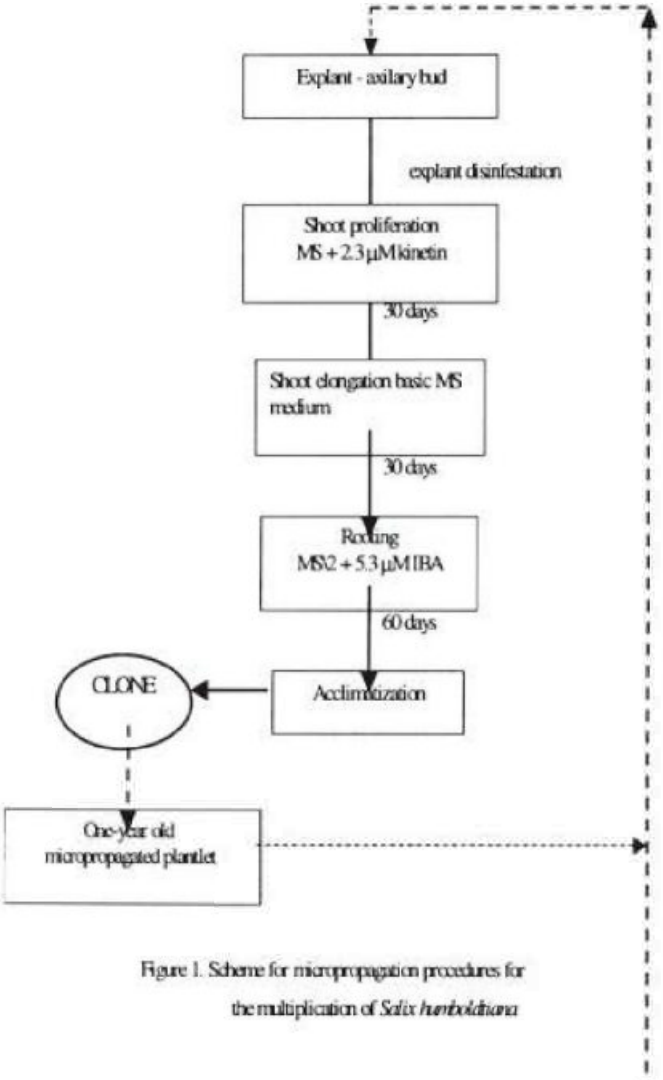

Figure 1. Scheme for micropropagation procedures for the multiplication of *Salix humboldtiana*

ACKNOWLEDGMENT

The authors acknowledge Companhia de Tecnologia de Saneamento Ambiental - CETESB for financial support.

REFERÊNCIA BIBLIOGRÁFICA

GRÖNROOS, L., HARDNER, C., GULLBER, U. Field performance of *Salix* clone propagated via shoot culture *in vitro*. **Scandinavian Journal of Forest Research,** v. 5, p. 487-92, 1990.

GUPTA, M.P. **270 plantas medicinales iberoamericanas.** Santafé de Bogotá: Presencia, p.495, 1995.

HAUTH, S., BEIDERBECK, R. *In vitro* culture of *agrobacterium rhizogenes*-induced hairy roots of *Salix alba* L. **Silvae Genetica** ,v.41, n.1, p.46-8, 1991.

LINEBERGER, R.D. Shoot proliferation, rooting, and transplant survival of tissue cultured *Hally jolivette* cherry. **Hortscience,** v.18, p.182-5, 1983.

LISKOVÁ, D., ZAKUTNÁ, L., KÁKONIOVÁ, D. Pigment formation in *Willow* tissue culture. **Biologia (Bratislava)** ,v.44 , n.11, p.1039-45, 1989.

MURASHIGE, T., SKOOG, F. A revised medium for rapid growth and bio assays with tabacco tissue cultures. **Physiologia Plantarum**, v.15, p.473-97, 1962.

NEUNER, H., BEIDERBECK, R. *In vitro* propagation of *Salix caprea* L. single node explant. **Silvae Genetica** , v.42, n.6, p.308-10, 1993.

PEZZUTO, J.M., CHE. C.T., McPHERSON, D.D., ZHU, J.P., TOPCU, G., ERDELMEIER, C.A.J., CORDELL, G.A. DNA as an affinity probe useful in the detection and isolation of biologically active natural products. **Journal of Natural Products,** v.54, n.6, p.1522-30, 1991.

SKIRVIN, R.M., CHU, M.C., RUKAN, H. Tissue culture of peach, sweet and sour cherry, and apricot shoot tips. **Proceedings of State Horticultural Society,** v.113, p.30-8, 1980.

STOEHR, M.U., MANTANG, C., ZSUFFA, L. *In vitro* plant regeneration via callus culture of mature *Salix exigua*. **Canadian Journal of Forest Research**, v.19, p.1634-8, 1989.

VAHALA, T., ERIKSSON, T. Callus production from *Willow* (*Salix viminalis* L.) protoplasts. **Plant Cell Tissue and Organ Culture**, v.27, p.243-8, 1991.