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IN VITRO PROPAGATION OF *SALIX TARRACONENSIS* PAU EX FONT QUER, AN ENDEMIC AND THREATENED PLANT

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SUMMARY

Salix tarraconensis Pau ex Font Quer, an endemic willow species from northeast Spain, was micropropagated with nodal segments. Shoot multiplication was obtained with different cytokinins, either on Murashige and Skoog medium or woody plant medium. Best results for shoot formation were obtained on Murashige and Skoog medium containing 4.9 μ*M* of 6-γ-γ-dimethylallylaminopurine. Shoots showed strong apical dominance, and some cultures displayed apical necrosis. Benzyladenine gave the worst results; shoots displayed very slow growth, deformed leaves, and hyperhydricity. Good rooting of shoots was obtained with different auxins or without plant growth regulators on woody plant medium. The best results (90–100%) were obtained within 20 d. On rooting media with indole-3-butyric acid or indoleacetic acid, shoot elongation was good (35–40 mm length). Apical necrosis was observed in elongating shoots on rooting medium, but this disturbance favored axillary bud sprouting and formation of new shoots. Shoot length and quality of roots decreased gradually as the concentration of naphthaleneacetic acid increased. Plant survival was 90% 4 weeks after removal from *in vitro* conditions.

Key words: apical necrosis; cytokinins; micropropagation; plant conservation; shoot culture; willow.

INTRODUCTION

During the last century, human activities have exerted pressure on natural habitats which threatens some endemic plant species. *In vitro* technology can conserve threatened plant species (Iriondo and Pérez, 1990; Fay, 1992). With micropropagation techniques, many plants can be obtained in a short time from a minimum of original plant material (Cocking, 1986), and thus with minimum impact on threatened populations.

Salix tarraconensis Pau ex Font Quer is a willow species endemic to a small mountain area in eastern Spain. Only a few populations are known, growing on limestone rocks about 1000 m above sea level (Aguilella et al., 1994). This species is classified as endangered by the local Law of the Valencian Community (East of Spain). Reforestation projects, forest fires, and construction of mountain roads can dangerously affect the few known populations.

In vitro culture of different species of the genus *Salix* has been reported: *Salix alba* and *S. fragilis* (Chalupa, 1983), *S. exigua* (Stoehr et al., 1989), *S. viminalis*, and *S. capraea* (Bergman et al., 1985). To our knowledge, there are no reports on the *in vitro* propagation of *S. tarraconensis*. We have developed a practical method for its micropropagation using nodal segments with axillary buds.

MATERIALS AND METHODS

Plant material and sterilization. Plant material was obtained from a natural population growing in Fredes, a mountain area in the northern Valencian Community (Spain). One adult tree was selected for samples, and four short branches 1 or 2 years old were collected in September. Short stem cuttings (6–8 cm long) were removed, and nodal segments 2 cm long with 2–4 axillary buds were used as the initial explants.

Leaves were excised, and the explants were thoroughly washed for 10 min with soap under running tap water. The segments were then treated for 20 min with a mixture of 50% Benomyl and 80% Zineb, each at 10 mg/L, rinsed with distilled water several times, and sterilized by being dipped for 10 min in 70% (vol/vol) ethanol, followed by an immersion for 30 min in a solution containing 7.5% (wt/vol) calcium hypochlorite and a wetting agent (1% Tween 20). Then each was rinsed three times in sterile distilled water for 5 min.

Establishment and culture conditions. To stimulate bud break, sterilized nodal segments were cultured on one of two basal media, either MS (Murashige and Skoog, 1962) or woody plant medium (WPM) salts (Lloyd and McCown, 1980), both with different 6-benzyladenine (BA) concentrations (0, 0.89, 2.22, 4.40, and 8.90 μ*M*). All culture media (establishment, proliferation, and rooting) were supplemented with 30 g sucrose/L, 100 mg myoinositol/L, 10 mg thiamine/L, 0.5 mg nicotinic acid/L, 0.5 mg pyridoxine/L, and 2 mg glycine/L. The media were adjusted to pH 5.7 with KOH before 8 g "Probus" agar/L was added. All media were sterilized by being autoclaved at 121° C for 20 min.

Nodal segments (three per vessel) were placed upright on the agar in glass flasks 55 × 75 mm containing 30 mL culture medium and sealed with two layers of aluminum foil. Cultures were incubated in a growth chamber and exposed to a 16-h-light photoperiod supplied by Osram cool white fluorescent lamps with a light intensity of 60 $\mu M \cdot m^{-2} \cdot s^{-1}$. Temperature was maintained at 25 ± 1° C during light and 20 ± 1° C during dark. These incubation conditions were also used for shoot multiplication and rooting as described later.

After 10 d, contaminated cultures and necrotic explants were discarded. The percentage of segments with shoots or with bud sprouting was determined 6 weeks later.

Shoot multiplication. To provide a good source of explants for multiplication experiments, shoots were removed from nodal segments on establishment media without BA or with 0.89 μ*M* BA and sectioned into nodal and apical segments. They were then cultured on WPM medium without plant growth regulators, and this basal medium was used for initial maintenance of a shoot stock.

Six one-week-old shoots obtained on basal medium were sectioned into 5–6-mm-long segments (nodal and apical, with 2–3 buds each) and cultured on

TABLE 1

EFFECTS OF DIFFERENT BA CONCENTRATIONS AND CULTURE MEDIUM (MS OR WPM) ON IN VITRO SHOOT PROLIFERATION, SHOOT ELONGATION, AND SHOOT QUALITY OBTAINED WITH NODAL EXPLANTS[a] OF MATURE MATERIAL OF *SALIX TARRACONENSIS* AFTER 6 WEEKS ON ESTABLISHMENT CULTURE[b,c]

Culture Medium	BA (μM)	Number of Shoots per Culture	Shoot Length (mm)	Shoot Quality
MS	0	2.0 ± 0.2 b	20.0 ± 0.8 b	Normal
	0.89	2.8 ± 0.2 a	11.3 ± 0.5 d	Normal
	2.22	1.9 ± 0.2 b	9.9 ± 0.4 de	Chlorotic 30%
	4.40	1.3 ± 0.1 cd	8.4 ± 0.3 e	Hyperhydricity 60%
WPM	0	2.8 ± 0.6 a	24.7 ± 0.7 a	Normal
	0.89	2.7 ± 0.1 a	12.8 ± 0.5 c	Normal
	2.22	1.8 ± 0.2 bc	10.2 ± 0.4 d	Chlorotic 50%
	4.40	1.2 ± 0.1 d	8.6 ± 0.3 e	Hyperhydricity 80%

[a]Three or four buds per explant, and 30 explants per treatment.

[b]Data represent mean ± SE and were compared by Duncan's multiple range test ($P < 0.05$). Data followed by the same letter in the same column are not significantly different.

[c]Culture media were Murashige and Skoog medium (MS) and woody plant medium (WPM), with 6-benzyladenine (BA) added.

different multiplication media. We used two mineral formulations, MS and WPM, in combination with BA, kinetin, or 6-γ-γ-dimethylallylaminopurine (2iP) at different concentrations to study shoot proliferation. Control treatments without cytokinins were included.

After 1 month, the rate of shoot proliferation was determined. Variables recorded were number of new shoots longer than 6 mm, number of shoots suitable for rooting (longer than 12 mm), and mean length of the tallest shoots produced by each explant. We recorded other data to study the quality of shoots and cultures: callus formation at the base of explants, percentage of rooted shoots, apical necrosis on the formed shoots, and morphological aspects of leaves.

Rooting. Proliferating axillary shoots 12 mm or longer were excised from cultures growing on the best multiplication medium and transferred to glass flasks with 30 mL MS or WPM medium for rooting. These culture media, either lacking growth regulators or containing an auxin (indole-3-butyric acid, IBA; indoleacetic acid, IAA; naphthaleneacetic acid, NAA), at different concentrations, were tested for rooting. All shoots used were cut to 12-mm length before being cultured.

After 1 month on the rooting media, we recorded percentage of shoots producing roots, number of roots per rooted shoot, length of longest root, and number and length of new shoots formed. The day of rooting of each shoot was recorded.

Rooted plantlets were then transferred to 60 × 80-mm plastic pots containing vermiculite and perlite (3:1 by volume) and kept in the growth chamber under a day:night temperature regime of 28 ± 2° C:25 ± 2° C and 16-h photoperiod of about 120 $\mu M \cdot m^{-2} \cdot s^{-1}$ supplied by Osram fluorescent tubes. To maintain cultures at high humidity, pots were covered with clear plastic bags for 3 weeks. Then the plastic cover was gradually removed to adapt the plantlets to ambient humidity conditions.

Statistical design. Thirty shoots were tested on each multiplication and rooting medium, and the experiments were conducted at least twice with a completely randomized design. Results were analyzed by analysis of variance and the means compared with Duncan's multiple-range test at $P < 0.05$.

Results and Discussion

Initiation of shoot cultures. Of the nodal explants, 56.6% remained uncontaminated and sterile cultures were obtained. After about 10–12 d of culture, nodal explants showed bud break, and after 1 month, 100% of the explants had one to three buds sprouted.

Shoot elongation, however, depended on the medium used (Table 1). The best elongation after 6 weeks was obtained on media without BA, especially on WPM (24.7 ± 0.7 mm long). Shoots obtained on media with BA presented short internodes and quickly lost their ability to elongate. At 2.22–4.40 μM BA, the appearance of shoots was not good, and leaves were curled. With 4.40 μM BA, 60% of the shoots showed hyperhydricity. Leaves formed on media without BA were normal and no apparent difference was observed between shoots obtained on the two mineral media, MS or WPM.

Shoot multiplication. The effect of three cytokinins (BA, kinetin, and 2iP) and two culture media (MS and WPM) on shoot multiplication was examined. On media without growth regulators, nodal segments produced only one shoot, and the best shoot elongation was obtained with WPM.

The best results for multiplication were obtained with MS medium and 4.90 μM 2iP (Table 2, Fig. 1 *A*). The longest shoots were obtained with 0.98 μM 2iP in both media. At concentrations of 2iP higher than 0.98 μM multiplication was better on MS medium than on WPM. During this multiplication phase, some treatments also induced root formation after 1 month of culture (Table 2), and the best rooting percentage was obtained on WPM without cytokinin.

The smallest number of shoots were obtained with BA (a mean of 1.15 shoots >6 mm long and shoots >12 mm long in all concentrations tested). Also, as BA concentration increased, shoot growth became distorted, showing major abnormal developmental traits—deformed leaves, pale color, and often, hyperhydricity. The same abnormal shoot growth on media containing BA were observed by Read et al. (1989) for some clones of *Salix viminalis* cultured in presence of 1.0 μM BA. Our results with *Salix tarraconensis* are in contrast to those of previous micropropagation studies with different willows (Bhjowani, 1980; Chalupa, 1987), but in agreement with others (Bergman et al., 1985; Read et al., 1989). In these two studies, BA at low concentrations (0.44–0.10 μM) was found to be a good cytokinin for shoot proliferation for some *Salix* species.

Salix tarraconensis shoots cultured with 2iP showed strong apical dominance, and growth of axillary buds was suppressed. The shoot apex or the first axillary bud (on nodal segment explants) greatly inhibited growth of the other axillary buds. Thus, the number of shoots longer than 6 or 12 mm was low on all culture media. Cytokinins generally enhance *in vitro* axillary bud growth, whereas auxins are considered important for establishing and maintaining apical dominance (De Klerk, 1992). However, although our culture media contained a cytokinin and no auxin, the shoots displayed a notable apical dominance.

TABLE 2

SHOOT PRODUCTION FROM 6-MM-LONG NODAL SEGMENTS OF *SALIX TARRACONENSIS* WITH 2iP IN TWO CULTURE MEDIA[a,b]

Culture Medium	2iP (μM)[c]	Number of Shoots >6 mm long (±SE)	Number of Shoots >12 mm long (±SE)	Shoot Length Mean (mm ± SE)	Shoot Rooting (%)	Apical Necrosis (%)
MS	0	1.00 ± 0 e	0.55 ± 0.1 ef	15.53 ± 0.8 e	66.6	0
	0.98	2.00 ± 0.2 b	1.50 ± 0.1 b	28.83 ± 1.6 ab	6.6	36.6
	2.46	1.86 ± 0.2 bc	1.53 ± 0.1 b	27.83 ± 1.4 b	6.6	23.3
	4.90	2.43 ± 0.2 a	2.03 ± 0.2 a	27.73 ± 1.7 b	23.3	30.0
	9.80	2.03 ± 0.2 b	1.56 ± 0.2 b	22.83 ± 1.3 c	6.6	23.3
WPM	0	1.00 ± 0.0 e	1.00 ± 0.1 def	22.33 ± 1.4 cd	90.0	0
	0.98	1.50 ± 0.1 cd	1.43 ± 0.1 bc	32.56 ± 1.5 a	10.0	50.0
	2.46	1.43 ± 0.1 d	1.06 ± 0.1 cd	18.10 ± 1.3 de	26.6	6.6
	4.90	1.36 ± 0.1 de	0.76 ± 0.1 def	15.83 ± 1.5 e	6.6	6.6
	9.80	1.23 ± 0.1 de	0.43 ± 0.1 f	14.00 ± 1.8 e	30.0	0

[a]Data were recorded at 30 d.
[b]Data were compared by Duncan's multiple-range test ($P < 0.05$). Data followed by the same letter in the same column are not significantly different.
[c]Culture medium was treated with 6-γ-γ-dimethylallylaminopurine (2iP).

Shoot-tip necrosis occurred in some cultures during the study on multiplication (Table 2). This physiological disorder was observed only in cultures on 2iP, especially on MS medium. The symptoms were observed after 20 d of culture, when shoots were actively growing. This disturbance developed first at the tip of the apical bud and advanced to the youngest leaves before a more general necrosis developed in the whole apical bud, which subsequently died.

Various factors have been proposed as the cause of apical necrosis. Sha et al. (1985) reported calcium deficiency as the most important cause for shoot-tip necrosis in several temperate trees. Inefficient

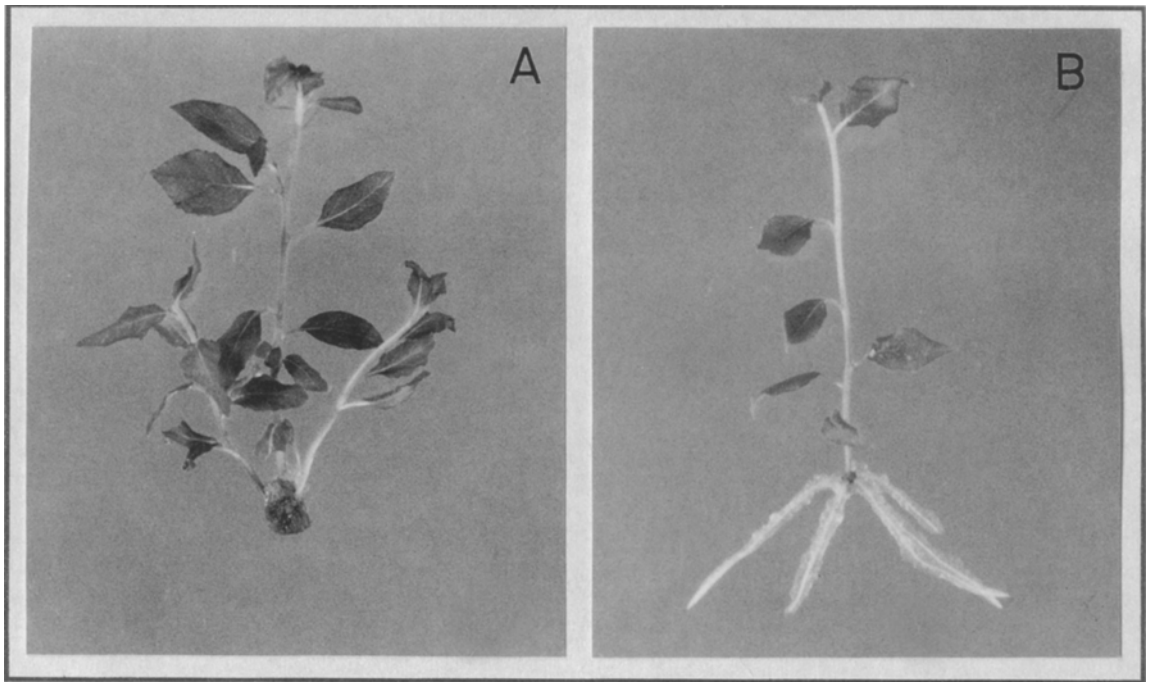

FIG. 1. *A*, *Salix tarraconensis* shoot development from an explant cultured on multiplication medium based on Murashige and Skoog mineral formulation in combination with 6-γ-γ-dimethylallylaminopurine (2iP). *B*, Root development on *Salix tarraconensis* shoots after 20 d in rooting medium (woody plant medium mineral formulation without growth regulators or with indoleacetic acid or indole-3-butyric acid).

TABLE 3

EFFECTS OF DIFFERENT AUXINS ON ROOTING PERCENTAGE, SHOOT PRODUCTION AND ELONGATION, AND SHOOT-TIP NECROSIS OF 12-MM-LONG SHOOTS OF *SALIX TARRACONENSIS* ON WPM MEDIUM[a,b]

Auxin μM	Percent Rooting At: 10 d	15 d	20 d	25 d	Shoot Length Mean (mm ± SE)	Number of Explants with: 1 Shoot	2 Shoots	≥3 Shoots	Apical Necrosis (%)
None	76	90	96	96	31.50 ± 1.3 c	23	5	2	6.6
IAA 1.14	90	96	100	100	37.03 ± 1.4 ab	17	13	0	20.0
2.85	86	100	100	100	38.30 ± 1.2 ab	20	9	1	13.3
5.71	80	93	96	96	40.06 ± 1.6 a	19	8	3	26.6
IBA 0.98	73	93	93	96	35.06 ± 1.6 bc	19	10	1	26.6
2.46	86	96	96	100	38.44 ± 1.5 ab	14	10	6	53.3
4.90	76	96	96	96	41.44 ± 2.2 a	15	12	3	60.0
NAA 1.07	40	83	93	96	24.96 ± 1.7 d	15	15	0	6.6
2.69	33	76	90	90	17.56 ± 1.6 e	21	9	0	3.3
5.37	43	90	100	100	12.63 ± 0.8 f	29	1	0	0

[a]At 30 d, shoot length, number of explants with shoots, and apical necrosis was recorded from cultures on woody plant medium (WPM) treated with different levels of indoleacetic acid (IAA), indole-3-butyric acid (IBA), and naphthaleneacetic acid (NAA).

[b]Data were compared by Duncan's multiple-range test ($P < 0.05$). Data followed by the same letter in the same column are not significantly different.

distribution of calcium to apical parts of longer shoots rather than poor uptake of this ion was proposed as the main cause for this physiological disorder (Bangerth, 1979). Also, a low transpiration rate caused by high humidity in the culture vessel and a high demand for assimilates have been related to calcium deficiency in organs and tissues (Barghchi and Alderson, 1985). However, preventing excess humidity in the vessels and doubling calcium strength in MS and WPM media did not appreciably improve shoot-tip necrosis on rosewood (Lakshmi Sita and Raghava Swamy, 1993). In this case, modifications of WPM (proportion of NH_4^+ to NO_3^- ions and enriched sulfur concentration) controlled this physiological disorder.

Shoot-tip necrosis was a problem also observed during *in vitro* shoot culture of other plants, such as almond (Rugini et al., 1986), chestnut (Vieitez et al., 1989), common ash (Hammatt and Ridout, 1992), and *Pistacia vera* (Abousalim and Mantell, 1994). However, in our species, this disorder was not a major problem because when the shoot tip was necrotic, apical dominance disappeared, some axillary buds sprouted, and some short shoots developed until the first axillary bud again resumed apical dominance. In this way, a large number of shoots longer than 6 mm was obtained.

Rooting. Rooting ability was studied with shoots excised from cultures growing on MS medium and 4.90 μM 2iP. Results obtained on rooting media (WPM, Table 3; MS, data not shown) were very similar. A high percentage of shoots (90–100%) rooted within a month on the majority of media tested.

Microcuttings cultured on basal medium devoid of growth regulators or with IBA or IAA produced normal roots and formed very little callus on the base of the explant after 1 month of culture (Fig. 1 *B*). However, with NAA, the roots were not normal. They were thick and some grew out onto the surface of the agar. Moreover, rooting of microcuttings with this auxin gave rise to an excessive amount of callus formed at the basal end of the shoots, although this did not affect the final percentage of shoots rooted. Elongation of shoots rooted with NAA also was not acceptable.

Three or four days after culture on rooting medium, the basal ends of microcuttings swelled, and roots began to emerge after 7–8 d of culture. Rooting on media with IBA, IAA, or without growth regulators was very rapid. A high percentage of microcuttings rooted at 10 d on rooting media (except with NAA), and within 15–20 d it was over 90% for most media. The presence of NAA in the medium delayed root differentiation.

Shoots cultured in rooting media elongated 35–40 mm with IBA or IAA. Rooting capacity, root quality, and shoot elongation were poorest in media with NAA. Shoot length on basal medium plus NAA decreased as this auxin concentration increased.

During the rooting phase, shoot-tip necrosis was observed, as it was during the proliferation phase. This disturbance appeared within 20–25 d of culture on rooting media. In some media, this symptom was more frequent and appeared on rooted shoots with a high elongation rate, especially at high concentrations of IBA (Table 3). In media with NAA, shoot-tip necrosis was very low.

Apical necrosis often occurred during the rooting stage, when shoots were transferred to a medium without cytokinins. In this case, this physiological disorder could be due to total absence of exogenous cytokinin in rooting media (Kataeva et al., 1991). This cytokinin deficiency, associated with the rapid elongation of the rooted shoots, leads to cessation of divisions in the apical meristem and to cellular necrosis. However, on rooting media without growth regulators (Table 3), apical necrosis was very low (6.6%), and shoots rooted and also elongated. This result indicates that factors other than cytokinins can be implicated in this abnormality.

As in the multiplication phase, however, this physiological disorder was not a major problem because the dead apical bud was replaced by the closest axillary bud. The shoot that formed from this bud maintained growth and resumed apical dominance. When these plantlets were transferred from *in vitro* culture to pots, tissue with apical necrosis was cut and shoots grew without problems.

Woody plant medium with IAA (1.14 μM–5.71 μM) or 0.98 μM IBA were selected as the best media for rooting because they did not result in high levels of shoot-tip necrosis.

With the method used to adapt *in vitro* rooted plantlets to nonsterile conditions, 90% of the plantlets survived the transfer to ambient conditions. We conclude that *Salix tarraconensis* can be micropropagated easily, which can rapidly increase the number of plants *ex situ* and thus help to preserve this threatened species.

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