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Original article

Micropropagation of the pine hybrid *Pinus brutia* (Ten) x *Pinus halepensis* (Mill) by culturing fascicle shoots

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Summary — Fascicle shoots proved to be an ideal plant material for micropropagation of the pine hybrid *P brutia* (Ten) x *P halepensis* (Mill). This could be possibly attributed to their morphological and physiological state. Induced fascicle shoots of 4-yr-old seedlings were used as explants. Their induction was achieved by spraying once with the new herbicide Arsenal (1 000 mg l^{-1}). First, explants were elongated *in vitro* on LP medium and then transferred to the multiplication stage. Multiplication was accomplished by decapitating, quick-dipping in 0.22 mM BA and inoculating the explants on BIMI medium. On the induced first-generation shoots the same procedure was applied, in order to obtain second-generation shoots and then these were proceeded to the rooting and acclimatization stages. In particular, when microcuttings were pretreated with 2.46 μM IBA + 2.7 μM NAA + 0.65% agar w/v + 1.5% sucrose w/v for 7 d and then transferred to greenhouse conditions, a good root system was developed within an 8–12-week period. A large variation in rootability was noted between clones. The above method may be proved to be efficient for clones that exhibit high rooting ability.

***P brutia* (Ten) x *P halepensis* (Mill) / induction of fascicle shoots / micropropagation / clonal variation**

Résumé — Micropropagation de l'hybride du pin *P brutia* (Ten) x *P halepensis* (Mill) au moyen de culture des pousses fasciculaires. *Les pousses fasciculaires constituent un matériel végétal idéal pour la micropropagation de l'hybride artificiel du pin* Pinus brutia *(Ten) x* Pinus halepensis *(Mill). Ceci peut être attribué à leur état morphologique et physiologique. Des pousses fasciculaires induites à partir de plants âgés de 4 ans sont utilisées comme explants. Leur induction est réalisée par une seule pulvérisation avec le nouvel herbicide Arsenal (1 000 mg•l^{-1}) (figs 1, 2). Au début, les explants sont allongés* in vitro *dans le milieu LP puis ils sont transférés pour l'étape de multiplication. Celle-ci résulte de la décapitation, puis l'immersion rapide dans une solution 0,22 mM BA et le repiquage de l'explant dans le milieu BIMI (fig 3, tableau I). Sur pousses induites de la première génération, on applique la même procédure afin d'obtenir des pousses de deuxième génération. Quand ces pousses ont 3–4 cm de haut (fig 4), elles sont soumises aux étapes*

d'enracinement et d'acclimatation (figs 5, 6). En particulier, quand les microboutures sont prétraitées dans un milieu contenant 2,46 μM IBA + 2,7 μM NAA + 0,65% agar w/v + 1,5% saccharose w/v pendant 7 j et puis transférées en serre, un bon système racinaire se développe en 8–12 sem (tableau II). On note une grande variation du pourcentage d'enracinement entre clones (tableau III). La méthode ci-dessus peut être efficace pour les clones qui présentent une capacité d'enracinement élevée.

Pinus brutia *(Ten)* x Pinus halepensis *(Mill)* / *pousses fasciculaires* / *micropropagation* / *variation clonale*

INTRODUCTION

The artificial pine hybrid, *Pinus brutia* (Ten) x *Pinus halepensis* (Mill) F_1, has been proved to be a promising plant material for reforestation in Greece because of its successful adaptation in various environments and growth vigor in relation to its parents (Panetsos, 1975; Panetsos *et al,* 1983; Panetsos, 1986).

The propagation of the hybrid by means of seeds is very difficult since the supply of viable seeds is very low, especially from parents with high specific combining ability. Thus, a reproducible and efficient procedure for mass propagation of outstanding genotypes by *in vitro* culture techniques was investigated as a potential alternative. Although progress in micropropagation of conifers through organogenesis from organ explants has been achieved, rooting of the micropropagated shoots (microcuttings) and the acclimatization of plantlets are still a problem (Jelaska, 1987; Mohammed and Vidaver, 1988; Stiff *et al,* 1989). According to many workers, further research on the influence of factors such as donor age, genotype, type of explant, microcutting quality, auxin treatment, root system and environmental conditions, on rooting and acclimatization is required (Franclet *et al,* 1980; Rancillac *et al,* 1982; Mohammed and Vidaver, 1988).

In the last decade, great success in conifer micropropagation was achieved by using the induced fascicle shoots of needle fascicles as explants. In conifers, it was found that a large number of fascicle buds can be induced and developed with the application of foliar spraying of either cytokinin (BA) (Abo El–Nil, 1982; Salonen, 1986; Scaltsoyiannes, 1988; Stiff *et al,* 1989) or a new herbicide imazapyr (Arsenal) (Scaltsoyiannes *et al,* 1993).

The aim of the present work was the development of a reliable micropropagation method for the pine hybrid *P brutia* (Ten) x *P halepensis* (Mill) using fascicle shoots of 4-yr-old seedlings.

MATERIALS AND METHODS

Establishment of cultures

Origin of explant

Four-year-old, potted seedlings, approximately 1.0–1.2 m high of the artificial pine hybrid *P brutia* (Ten) x *P halepensis* (Mill) in a mixture of peat/perlite (1:1 v/v) were placed in greenhouse conditions (14–19°C) (late winter) to be treated for the stimulation of fascicle buds.

The day length was maintained at 18 h by supplementary lighting provided by high-pressure lamps (HPI/T, SON/T, 400 W, light intensity of 65–70 mmol s^{-1} m^{-2}). Stimulation of fascicle buds was achieved with one foliar spray of Arsenal* (ai 250 gl^{-1}) at a concentration of 1 000 mgl^{-1}. Spraying was applied till run off.

* Trademark of American Cyanamid Company.

In about 3–6 weeks, fascicle shoots with juvenile morphology emerged from their fascicles. When the shoots reached the length of 8–15 mm (from the base to the top of the apical meristem), they were excised from the plant and used as explants for the *in vitro* experiments. The explants were characterized for their open apical tip and the lack of apparent axillary buds at the needle bases.

Surface sterilization

After a 2 h wash under running tap water, the explants were disinfected for 16 min in a 1.65% (v/v) sodium hypochlorite solution, containing 0.01% Tween 80. Finally, they were rinsed 3 times with sterile water and were inoculated in glass test tubes containing 10 ml LePoivre medium (LP) (modified according to Aitken-Christie and Thorpe, 1984). The LP medium was chosen because previous experiments indicated that media containing low concentrations of NH_4^+ (like LP) were the most suitable for good growth of fascicle buds (Scaltosoyiannes, 1988). The medium was adjusted to pH 5.65 prior to autoclaving for 15 min at 121°C. Cultures were incubated at 25°C in a growth chamber provided with 18-h photoperiod by cool-white fluorescent light at 80 mmol s^{-1} m^{-2}. The explants were incubated, first, on LP for 4 weeks to elongate and then were treated for the multiplication stage. Subculturing (every 4 weeks) and all experimental work were carried out on this particular medium unless otherwise stated.

Induction and development of axillary shoots

Effect of growth regulators

When the explants reached a length of 3–4 cm they were tested for bud induction by the following treatments. 1) Explants were placed on Bud Induction Medium I (BIMI) with a low concentration of NH_4^+ (Abo El-Nil, 1982) supplemented with 5 μM BA. This concentration of BA proved to be the best for bud formation in previous research (Scaltsoyiannes, 1988). 2) Explants, decapitated or not, were quick-dipped (for 5 s) in 0.22 mM BA and were placed in BIMI. Decapitation was applied to a length of 5 mm from the apex.

In both cases explants remained for 6 weeks in BIMI medium and then were transferred to LP without growth regulators.

Elongation of buds

Shoots 8–10 mm long were isolated and transferred to LP medium with and without activated charcoal (1.5% w/v) (Merck) for elongation. In the case that elongated shoots were intended for rooting, secondary needle removal was conducted in order to facilitate elongation of microcuttings.

Second generation shoot formation *in vitro*

When the first generation of elongated shoots reached a length of 3–4 cm they were decapitated and transferred to BIMI after a quick-dip in 0.22 mM BA. The newly induced shoots (second generation) were elongated on LP medium supplemented with 1.5% w/v activated charcoal.

Rooting of shoots

Taking into consideration previous experiments (Scaltsoyiannes, 1988), the following procedure was applied for rooting. Some 10- to 12-week-old second-generation shoots (3–4 cm) originating from various clones (bulk material) were treated for rooting by incubating the shoots *in vitro* in the following:

1) water + 0.65% agar, washed (Sigma) w/v + 1.5% sucrose w/v (control);

2) 4.9 μM IBA + 2.7 μM NAA + 0.65% agar, washed (Sigma) w/v + 1.5% sucrose w/v; and

3) 2.46 μM IBA + 2.7 μM NAA + 0.65% agar, washed (Sigma) w/v + 1.5% sucrose w/v.

After 7 d, the microcuttings were transferred to greenhouse conditions in a mixture of peat/perlite (1:1 v/v). Rooting results were recorded within 8–12 weeks.

In order to study the clonal variation in rootability, the third treatment was applied to 2 clones (A, B) (original codes: K_3, K_4). These 2 clones were selected for rooting experiments because from preliminary trials they exhibit great variation in rooting ability (Scaltsoyiannes, 1988). Rooting results were recorded within 10–16 weeks.

RESULTS AND DISCUSSION

The research on micropropagation of conifers was restricted mainly to juvenile tissues (embryos, cotyledons, or a few-week-old seedlings) (Aitken *et al,* 1981; Horgan and Aitken, 1981; David *et al,* 1982; Rancillac *et al,* 1982; Aitken-Christie and Thorpe, 1984). In our research, micropropagation of the artificial hybrid *P brutia* (Ten) x *P halepensis* (Mill) was succeeded from fascicle shoots of a 4-yr-old plant. This is in agreement with other researchers working with the same type of explants on other conifer species (Abo El-Nil, 1982; Salonen, 1986; Inglis, 1988; Scaltsoyiannes, 1988; Stiff *et al,* 1989).

According to Abo El-Nil (1982), the juvenile morphology of explants is considered to be of major importance for satisfactory *in vitro* reaction. The induction of fascicle shoots by the herbicide treatment proved very successful (figs 1, 2). The herbicide (Arsenal) effect on induction of fascicle shoots was demonstrated for the first time by Christensen (1988) when he tested its effectiveness on a conifer plantation. The low percentage (12–20%) of infection was propably due to the type of explants and the fact that these explants derived from plants grown indoors.

The quick-dip treatments of explants in 0.22 mM BA proved to be beneficial for induction and development of buds (table I) (fig 3). These results are in agreement with the findings of Abo El-Nil (1982) and, moreover, in our case the application of quick-dip treatment to decapitated explants was even better (100% reaction), probably due to lack of apical dominance.

The elongation of shoots was satisfactory (figs 4, 5) and in a period of 8–12 weeks an average of 25–30 axillary shoots per

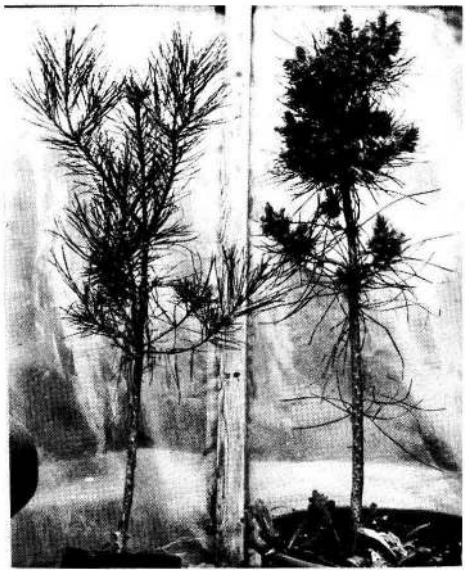

Fig 1. Untreated (left) and herbicide (Arsenal)-treated (right) 4-yr-old trees of the pine hybrid *P brutia* (Ten) x *Pinus halepensis* (Mill) 6 weeks after the application of foliar spraying.

Fig 2. Close view of induced shoots on a branch of an Arsenal-treated 4-yr-old tree of the pine hybrid.

Table I. Reaction of explants of the artificial hybrid pine *P brutia* (Ten) x *P halepensis* (Mill) to different treatments for shoot induction *in vitro*[a]

	Non-decapitated explants			*Decapitated explants*
	BIMI (control)	*BIMI + 5 μM BA*	*BIMI + 0.22 mM BA (quick-dip)*	*BIMI + 0.22 mM BA (quick-dip)*
% of reacted explants	2 ± 3[b]	45 ± 11	62.5 ± 1	100
Remarks	Well-developed shoots		shoots were not well developed	well-developed shoots

[a] Formation of axillary shoots in > 50% of the primary needles; [b] results are given as mean ± SE, sample size 20.

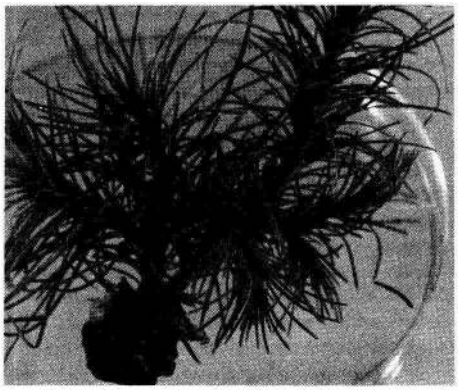

Fig 3. Induction of axillary shoots *(in vitro)* on a decapitated explant of the pine hybrid *P brutia* (Ten) x *P halepensis* (Mill) 6 weeks after its quick-dip in 0.22 mM BA and establishment on BIMI.

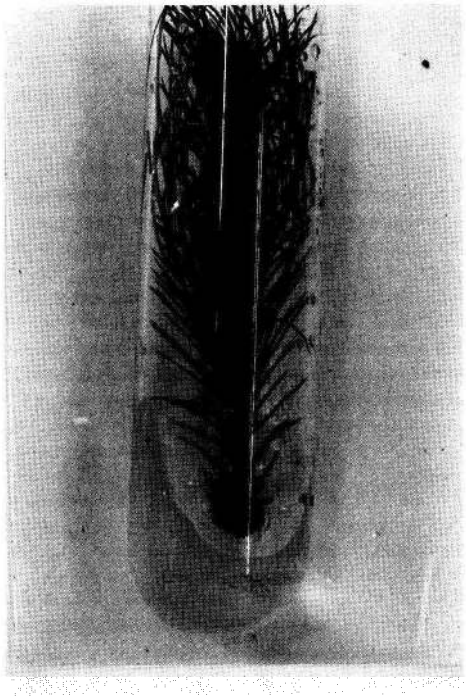

Fig 4. An elongated 10-week-old microcutting (second-generation shoots *in vitro*) of the hybrid.

explant were produced. Moreover, the elongated shoots had very good appearance without any signs of vitrification.

Activated charcoal (1.5% w/v) reduced the elongation time to almost 5–6 weeks (instead of 8 weeks) to reach the desired length (3 cm) for rooting or for other treatments. The positive effect of activated charcoal on shoots has also been found by many other workers (Mehra-Palta *et al*, 1978; Von Arnold and Eriksson, 1981; David *et al*, 1982). The unexpected bud formation on BIMI free of growth regulators (table I) was concluded to be an after-effect of foliar spraying on mother plants (Scaltsoyiannes, 1988).

It is generally considered that the rooting and acclimatization stages of microcuttings

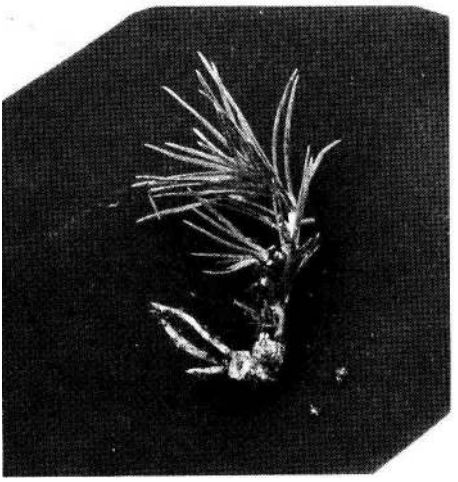

Fig 5. A rooted microcutting of the pine hybrid 8 weeks after its transfer to greenhouse conditions.

Fig 6. A plantlet of the pine hybrid after the acclimatization stage 1 yr after rooting.

are the most critical steps in conifer micropropagation (Franclet *et al,* 1980; Rancillac *et al,* 1982; Mohammed and Vidaver, 1988). In our case the microcuttings which derived from this type of explant (fascicle shoots) exhibited excellent behavior in the rooting and acclimatization stages (figs 5, 6).

In the first rooting experiment (bulk material) the presence of auxin was necessary for rooting, as shown in table II. There was no significant difference between the 2 auxin treatments tested for root induction. Auxin pretreatment of microcuttings in conifers was also reported by Aitken-Christie and Thorpe (1984). Microcuttings formed well-developed root system with many hairy roots and the acclimatization occurred with no problems (fig 6).

From the rooting experiments with 2 clones (A, B) a large variation in rooting ability was observed, 84% and 32%, respecti-

Table II. Effect of auxin treatment on survival and rooting percentage of microcuttings (bulk material) of the artificial hybrid pine *P brutia* (Ten) x *P halepensis* (Mill) *in vivo.*

	% survival	*% rooting*
Control	81.25 ± 9.75[a]	0
4.9 μM IBA + 2.7 μM NAA	62.5 ± 12	25 ± 11
2.46 μM IBA + 2.7 μM NAA	75 ± 11	31.25 ± 11

[a] Results are given as mean ± SE, sample size 16.

Table III. Effect of clonal variation on rooting ability of 2 clones A, B (original codes K_3, K_4).

	% rooting	
	Clone A (K_3)	*Clone B* (K_4)
Control	52 ± 10[a]	12 ± 6.5
2.46 μM IBA + 2.7 μM NAA	84 ± 7.3	32 ± 9.32

[a] Results are given as mean ± SE, sample size 25.

vely (table III). The above verifies previous rooting experiments conducted on these 2 clones (Scaltsoyiannes, 1988). A large variation among clones was also observed by Aitken-Christie and Thorpe (1984) who worked on rooting of *P radiata* microcuttings and also by Kleinschmidt and Schmidt (1977) and Zobel and Talbert (1987) who indicated that there was generally a large clonal variability in the rooting ability of forest trees. Rooting experiments are currently under way on different clones of the hybrid and its parental species, using various growth regulators and different techniques, *ie* with or without *in vitro* pretreatment and quick-dip auxin treatment.

CONCLUSIONS

Fascicle shoots proved to be an ideal material as explants for micropropagation of 4-yr-old artificial hybrid pine *P brutia* (Ten) x *P halepensis* (Mill), probably due to their morphological and physiological state.

The combination of decapitation and quick-dip in high concentrations of BA (0.22 mM) was beneficial for shoot induction and multiplication *in vitro.*

Pretreatment of microcuttings (second generation shoots *in vitro*) with growth regulators and the clone genotype proved to be crucial prerequisites for successful rooting in non-aseptic (greenhouse) conditions and subsequent acclimatization of plantlets. This is an efficient method for the micropropagation of the hybrid clones that exhibit high rooting ability.

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