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Original article

Effect of genotype and cutting type on the vegetative propagation of the pine hybrid (*Pinus brutia* (Ten) x *Pinus halepensis* (Mill))*

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Summary — Improved methods to propagate vegetatively selected individuals of the promising artificial pine hybrid *Pinus brutia* (Ten) x *P halepensis* (Mill) are required. Repeated spraying with 200 mg•l^{-1} BA or one spraying with 1 000 mg•l^{-1} of the herbicide Arsenal on the stems of 4-year-old seedlings, resulted in the production of the largest possible number of fascicle shoots. The fascicle shoots produced were taken as cuttings and were tested for rooting. In the rooting experiments the effect of genotype, cutting type, cutting size and auxin treatment were investigated. Genotype and cutting type proved to be the most crucial factors for rooting and clones with high rootability.

***Pinus brutia* (Ten) x *P halepensis* (Mill) / induced fascicle shoot / genotype effect / rooting / cutting / vegetative propagation**

Résumé — L'effet du génotype et du type de bouture sur la multiplication végétative de l'hybride de pin (*Pinus brutia* (Ten) x *Pinus halepensis* (Mill)). *L'amélioration des méthodes de multiplication végétative des individus sélectionnés de l'hybride artificiel de pin* Pinus brutia *(Ten) x* Pinus halepensis *(Mill) est nécessaire. Quatre différents régulateurs de croissance (TIBA, Alar, GA_3, BA) ont été appliqués avec différentes combinaisons et concentrations sur la tige de plants de 4 ans de cet hybride artificiel afin d'obtenir l'induction de pousses interfasciculaires (tableau I). L'effet du meilleur traitement (200 mg•l^{-1} BA) de l'expérience a été comparé avec celui de l'herbicide Arsenal. La pulvérisation répétée de 200 mg•l^{-1} BA ou une pulvérisation de 1000 mg•l^{-1} d'Arsenal sur la tige de plants de 4 ans, conduit à la production du plus grand nombre des pousses interfasciculaires (tableau II, fig 1A). Ces pousses ont été utilisées comme boutures et étudiées pour leur enracinement. Dans cette expérience d'enracinement, on a analysé l'effet du génotype, du type de bouture, de la taille de la bouture et du traitement par l'auxine. Parmi les 8 clones testés, on a observé une grande variabilité en ce qui*

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Abbreviations: benzyladenine (BA); gibberelic acid (GA_3); triiodobenzoic acid (TIBA); dimethylaminosuccinamide (Alar); indole-3-butyric acid, potassium salt (K-IBA).

concerne l'enracinement (fig 2). Les boutures interfasciculaires se sont enracinées plus facilement et elles ont développé un meilleur système racinaire que celui des boutures de tige (fig 1B, 1C). En ce qui conceme l'effet du génotype, on a trouvé que quelques clones s'enracinent facilement ou difficilement indépendamment de leur hauteur ou du traitement par l'auxine (tableau III). Les concentrations variées d'auxine (0, 4000, 8000 ppm K-IBA) influent différemment sur les 2 types de bouture (fig 3). Les plantules provenant des pousses interfasciculaires se caractérisent par leur vigueur et leur orthotropie (fig 1D).

P brutia *(Ten) x* P halepensis *(Mill) / pousse interfasciculaire / effet du génotype / enracinement / bouture / multiplication végétative*

INTRODUCTION

The artificial pine hybrid, *Pinus brutia* (Ten) x *P halepensis* (Mill) F_1, is promising for afforestation in Greece because of its successful adaptation in various environments and growth vigour in comparison to its parents (Panetsos *et al*, 1983; Panetsos, 1986b). However, the difficulty in obtaining a large quantity of hybrid seed, especially from parents with high specific combining ability, limits its potential for large-scale planting. Thus, reliable methods for mass vegetative propagation of superior hybrid trees are urgently needed.

Until some years ago, among the vegetative propagation methods (traditional and *in vitro*) only grafting was applied with great success to the above hybrid and its parents (Panetsos, 1986a). Rooting of conifers (especially of mature trees) is generally considered very difficult and success is still too low to allow operational use, except in some cases where young seedlings or juvenile stock plants were used. According to Foster (1990), systems for the large-scale production of rooted cuttings have been developed for many conifer species.

Traditionally, environmental factors affecting rootability of conifer cuttings have received the most attention when developing production systems, but, as Foster (1990) states, genetic differences in rooting ability among clones seem to be an almost universal phenomenon for forest tree species. One of the most important physiological factors affecting rootability of conifer cuttings is considered to be juvenility, associated with the age of the ortet. On the other hand, one major aim of vegetative propagation is to multiply trees old enough to have demonstrated their superior characteristics (Girouard, 1974). In the last few decades, multiplication of conifers through micropropagation techniques has been an attractive possibility. However, most conifers have been so difficult to propagate *in vitro*, especially from mature tissues (except for a few cases (*eg*, Horgan and Holland, 1989; Scaltsoyiannes *et al*, 1994)) that it does not permit the commercial application of this process.

According to Mergen and Simpson (1964), pine fascicle shoots could provide a large number of propagules. The fact that this material rooted more readily than stem cuttings from long shoots (Libby *et al*, 1972; Whitehill and Schwabe, 1975; Inglis, 1984) is probably due to its juvenile state (Sacher, 1954; Abo El-Nil, 1982) and the higher level of endogenous root promoters (Hong, 1969).

In 1959, Reines and MacAlpine demonstrated stimulation of fascicle buds of *P eliottii* by the removal of terminal buds. Kummerow and Hoffmann in 1963, reported that kinetin could stimulate the growth of fascicle buds. Other workers later reported that removal of terminal buds combined with treatment of plants with cytokinin-like substances could stimulate fascicle bud development and also that this type of material could root more readily than stem cuttings

(Hong, 1969; Kiang *et al,* 1974; Whitehill and Schwabe, 1975; Inglis, 1984; Scaltsoyiannes, 1988).

The objective of the present study is the investigation of: a) the stimulation and development of fascicle shoots of the pine hybrid *P brutia* (Ten) x *P halepensis* (Mill); and b) the vegetative propagation of selected individuals of the hybrid by using fascicle shoots as cuttings.

MATERIALS AND METHODS

Induction and development of fascicle shoots

Experiment 1. Effect of growth regulators

Four-year-old seedlings of *P brutia* (Ten) x *P halepensis* (Mill) < 1.2 m in height, were selected in January, for their uniform growth and were placed in a greenhouse (14–29°C) to stimulate fascicle shoot development with growth regulators after excision of terminal and lateral buds. Daylength was maintained at 18 h by supplementary lighting, provided by high pressure lamps (HPI/T, SON/T, 400 W). The seedlings were fertilised at 3-month intervals by applying Compresal Supra (12 N + 12 P + 17 K + 2 Mg + micronutrients). Chemical treatments and their concentrations are shown in table I. Twelve seedlings per treatment were used. The above treatments were applied 5 times, every 7 d, on the whole foliage of the seedlings with a compressed air sprayer and 80 ml solution was enough to run-off the entire plant. The solutions were prepared from stock solutions stored at 5°C. A mixture of Triton 100 and Triton 20 at 0.01% and 0.005% (V/V), respectively, was used as surfactant. To provide uniformity in our measurements, the assessment of fascicle shoot number referred to a length of 20 cm on the main stem and were recorded 8 weeks after the first application of the chemical. Fascicle shoots greater than 10 mm were considered as induced shoots.

Experiment 2. Effect of the herbicide imazapyr (Arsenal, CYANAMID)

The following spring, selected plant material of the same origin and of about the same age and

Table I. Effect of different growth regulators on the number of induced fascicle shoots*.

Treatment ($mg \cdot l^{-1}$)	*Number of induced fascicle shoots (per 20 cm length of stem)*
Control (bud excision without any chemical treatment)	6.9 ± 0.6
Wetting agents only (0.01% Triton 10 + 0.005% Triton 20)	7.1 ± 0.6
BA (100)	21.7 ± 1.2
BA (200)	23.5 ± 1.4
BA (400)	26.3 ± 1.5
GA_3 (50)	4.2 ± 0.6
TIBA (50)	7.6 ± 0.7
TIBA (100)	12.2 ± 1.2
Alar (500)	10.4 ± 0.8
Alar (1000)	11.4 ± 0.8
BA (200) + TIBA (50) + Alar (500)	23.3 ± 1.1
BA (200) + TIBA (100) + Alar (1 000)	20.1 ± 1.1
BA (100) + GA_3 (50)	16.8 ± 1.8

* Fascicle shoot length ≥ 10 mm; results are given as means ± SE; sample size 12.

Table II. Effect of the cytokinin BA and the herbicide (Arsenal) on the number of induced fascicle shoots*.

Treatment (mg•l⁻¹)	*Number of induced fascicle shoots (corresponding to a stem length of 20 cm)*
BA (200)	22.26 ± 0.13
Arsenal (1000)	22.05 ± 0.16

* Fascicle shoot length ≥ 10 mm; results are given as mean ± SE. Sample size 25.

height as in *Experiment 1* was sprayed (table II) with: a) 1 000 $mg \cdot l^{-1}$ herbicide imazapyr (Arsenal) (ai 250 $g \cdot l^{-1}$) once for induction and development of fascicle shoots; and b) 200 $mg \cdot l^{-1}$ BA, which proved to be one of the best treatments from *Experiment 1*.

It is noteworthy that in the Arsenal treatment no bud excision occurred, in contrast to BA treatment which was applied as in *Experiment 1*. Growth conditions (environment, nutrition, *etc*) of the present experiment were as similar to those in *Experiment* 1 as possible.

Rooting

Experiment 3

In order to test clonal and cutting type effects on rooting ability of the pine hybrid *P brutia* (ten) x *P halepensis* (Mill), the following experiment was carried out.

Two types of cutting, 12–15 cm long, were tested for rooting: a) induced fascicle shoots, derived from the application (in January) with Arsenal (1 000 $mg \cdot l^{-1}$) on 8 clones 4–5 years old (height > 1.5 m) growing in the field; and b) stem cuttings (normal) from the same clones. The induced fascicle cuttings were collected at the end of May and were semi-lignified to succulent with both juvenile leaves and needle fascicles. On the same ortet we could find simultaneously induced fascicle shoots and non-induced shoots (normal) after the Arsenal application.

The following treatments were applied to each type of cutting and each of the 8 clones: a) control (no treatment); b) K-IBA 4 000 $mg \cdot l^{-1}$ (potassium salt); and c) K-IBA 8 000 $mg \cdot l^{-1}$ (potassium salt).

The cutting base was quick-dipped in auxin solution for 5 s, and allowed to dry before insertion into the heated rooting bench (temperature adjusted to 24°C) equipped with a special intermittent mist system (3 s/15 min) for 12 h per day. Rooting medium consisted of perlite and peat 1:1 v/v, in a mixture. Photoperiod, light quality and ambient temperature remained the same as in *Experiment 1*. Cuttings were watered with a solution of fungicide Captan at 2-week intervals. The experimental design was a randomised complete block design (treatment x cutting type x clone x replication) with 4 replications and 5 cuttings per plot (*ie* a total number of 120 cuttings/clone). Rooting was recorded 4 months after insertion of the cuttings.

Experiment 4

Because the induced fascicle cuttings of the previous experiment reached the length of 12–15 cm the second year after Arsenal treatment, the following year we tested shorter cuttings 4–5 cm long which were collected 2–3 months after the application with Arsenal.

Induced fascicle cuttings derived from the best and the worst clone (3 and 1, respectively) of the previous experiment were treated with 2 000 $mg \cdot l^{-1}$ K-IBA (potassium salt). The experimental design and all other conditions were the same as in *Experiment 3*.

RESULTS

Induction and development of fascicle shoots

As shown in table I, there were significant differences in the number of fascicle shoots induced after application of the various growth regulators. The untreated plants produced fewer shoots which are longer close

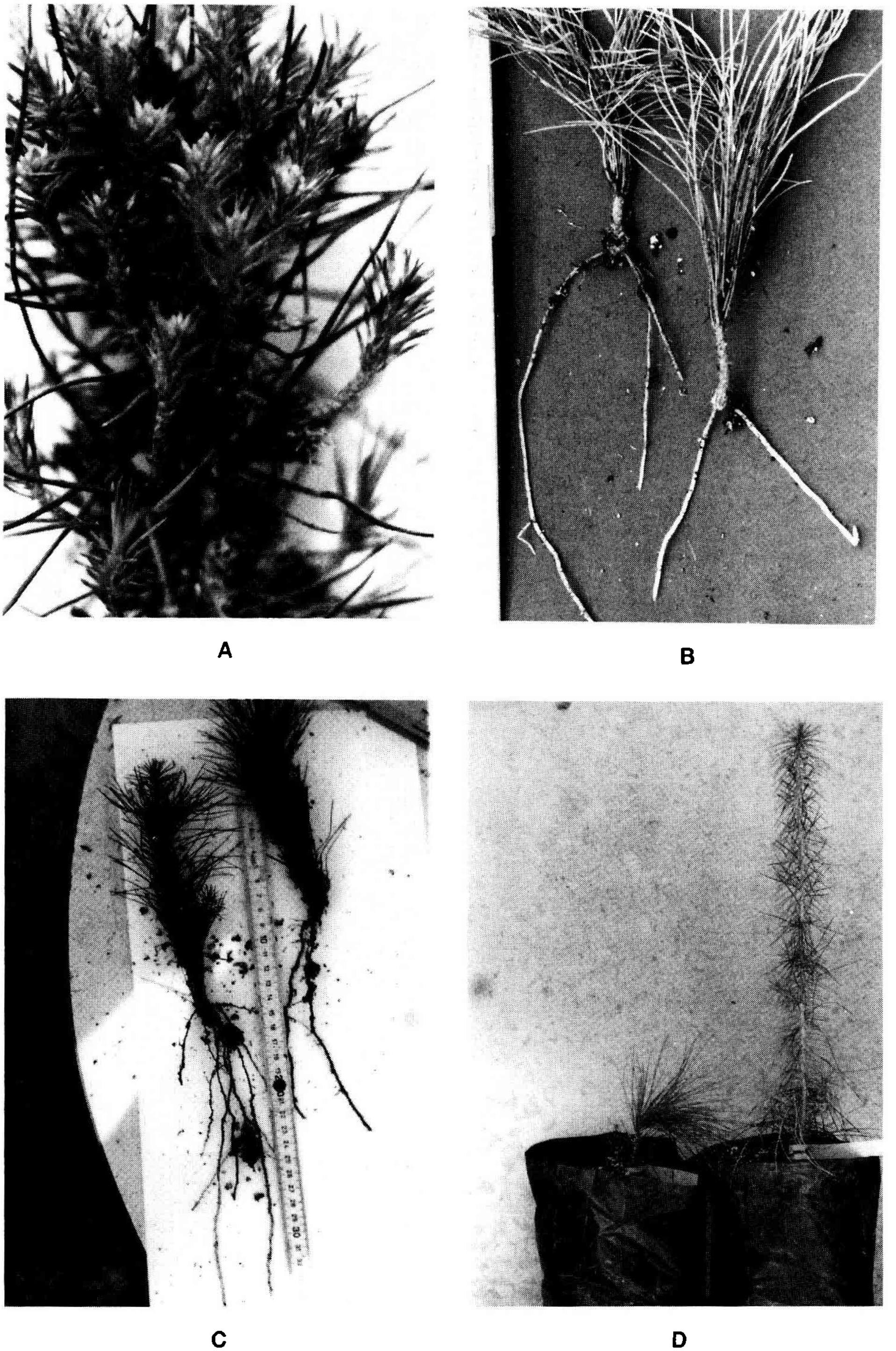

Fig 1. A. Outgrowth of induced fascicle shoots (—>) after the application of the herbicide (1 000 mg•l^{-1} Arsenal) on 4-year-old seedlings. **B.** Rooted stem cuttings (4 000 ppm K-IBA). **C.** Rooting of induced fascicle cuttings (without auxin treatment). **D.** Plantlets of the same clone derived from induced fascicle (left) and normal (right) cuttings.

to the bud excision site, compared with the treated ones.

Cytokinin-treated plants (100–400 mg•l^{-1} BA) produced the largest number of fascicle shoots which appeared earlier (3rd week after bud excision) than those of the other treatments. TIBA and Alar applications were found to be ineffective when applied alone as well as in combination with BA. The application of GA_3 alone or in combination with BA appeared to depress bud induction.

The herbicide (Arsenal) effect on shoot induction and their outgrowth (table II, fig 1A), appeared to be similar to that caused by the BA treatment, which proved to be the most promising treatment in *Experiment 1*.

In all the above cases the induced fascicle shoots were characterized by vigorous growth and open apical buds.

Rooting experiments

Clones exhibited great variation in their rooting percentages, regardless of cutting type (stimulated or normal) (fig 2), which ranged from 82% to 3%. Figures 1B and 1C also indicate that cutting type is another important factor affecting rootability regardless of clone. Moreover, it should be pointed out that plantlets derived from fascicle shoots were orthotropic, fast growing, and exhibited good acclimatization in contrast to those originating from normal cuttings (fig 1D).

The three levels of K-IBA (fig 3) affected the 2 cutting types differently. Normal cuttings responded positively to the increase of the auxin level, while stimulated ones responded negatively.

The results in table III can be compared with the performances of clones 1 and 3 in figure 2, which shows that cutting length had no effect on rooting and also that genotype remains the most important factor.

Table III. Rooting percentages of small-sized cuttings (4–5 cm long) from 2 clones*.

	Rooting %	
	Control 0 ppm	*K-IBA 2 000 ppm*
Clone 3	90	80
Clone 1	25	25

* Sample size of 20 cuttings.

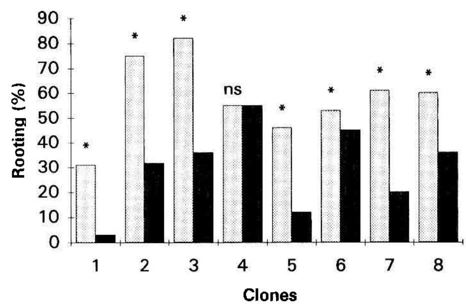

Fig 2. Effect of cutting type (stimulated ▨ or normal ☐) and genotype on rooting of 8 clones. * Statistically significant differences (χ^2 test, P = 0.05) between stimulated and normal cuttings for each clone; ns: not significant.

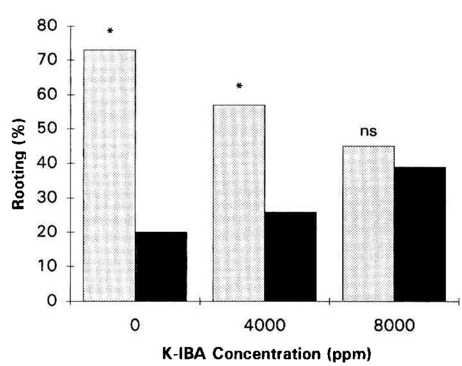

Fig 3. Effect of K-IBA concentration and cutting type (stimulated ▨ or normal ☐) on rooting. * Statistically significant; ns: not significant differences (χ^2 test, P = 0.05).

DISCUSSION

The most successful treatments for the induction and development of fascicle shoots on *P brutia* (Ten) x *P halepensis* (Mill) seedlings were: a) the application of BA (100–400 $mg \cdot l^{-1}$) in conjunction with the removal of buds; and b) spraying with the herbicide imazapyr (1 000 $mg \cdot l^{-1}$ Arsenal) without bud removal. The cytokinin effect is an agreement with results obtained from similar work on 3-year-old *P caribaea* (Inglis, 1984) and *P sylvestris* (Whitehill and Schwabe, 1975; Phillion *et al*, 1983). The herbicide (Arsenal) effect on induction of fascicle shoots was first demonstrated by Christensen (1988). Although the herbicide interference in the plant regulation balance is unknown, it was observed that its application caused terminal bud decaying and fascicle shoot induction.

According to our results and those mentioned by Wickson and Thimann (1958) it is assumed that an antagonism exists within buds between uxin and endogenous cytokinin, which on several occasions results in the dominance of the apical bud (higher auxin level). Growth of the inhibited fascicle shoots seems to be accomplished when endogenous cytokinin levels are increased, either by lateral and terminal bud excision or cytokinin treatment.

TIBA, Alar and GA_3 treatments reduced significantly the number of fascicle shoots induced, which is consistent with the findings of Inglis (1984).

According to Libby *et al* (1972), 2 requirements must be met for large-scale propagation: a) production of a large number of propagules; and b) development of conditions for their successful rooting and establishment. In our case, fascicle shoots seem to satisfy the above-mentioned prerequisites, since they can provide almost as many propagules as the number of brachyblasts, and also root more readily than cuttings originating from stem shoots.

From our results it is proved that the more critical factors affecting rooting of fascicle shoots are the genotype and the type of cuttings. As regards genotype, some clones root readily and others poorly, regardless of the cutting type and hormone level. Several other workers have also noticed the effect of genotype on rooting of conifer cuttings (Thulin and Faulds, 1968; Kleinschmit and Schmidt, 1977; Donald, 1987; Foster, 1990).

The higher rootability of fascicle shoot cuttings compared with that of normal ones was also demonstrated by many other workers (Inglis, 1984; Donald, 1987), a fact that could be attributed to their possible juvenile state (Sacher, 1954; Libby *et al*, 1972; Abo El-Nil, 1982) and the higher level of endogenous root promoters of these shoots (Hong, 1969). Plants derived from rooted fascicle shoot cuttings, one year after transplanting, remained orthotropic and exhibited growth vigour and good acclimatization. Orthotropic behaviour exhibited in plantlets derived from fascicle cuttings was also noticed by Alazard and Kadio (1983) and Scaltsoyiannes *et al* (1992).

Fascicle shoots that are 4–5 cm long can be used for the full exploitation of plant propagules and time could be saved as they reach that size in only 2 months from shoot induction. This makes this technique productive and economically successful.

Finally, preliminary experiments conducted with mature tissues (scions) of a pine hybrid *P brutia* (Ten) x *P halepensis* (Mill) that is 23 years old, gave us encouraging results on induction and rooting of fascicle shoots.

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