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In vitro shoot bud differentiation and plantlet regeneration in *Celastrus paniculatus* Willd.

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Abstract

Multiple shoot buds could be induced directly from internode explants of *Celastrus paniculatus* inoculated on Murashige and Skoog's (MS) medium containing different growth regulators. The best response was obtained when 4.44 μM 6-benzylaminopurine (BAP) was incorporated in the medium. Incorporation of indole-3-acetic acid (IAA) and α-naphthalene acetic acid (NAA) did not improve response, rather promoted callusing. Adventitious shoot buds could be multiplied and elongated on MS medium containing 2.22 μM BAP. Rooting of shoots (80 %) was obtained when their bases were dipped in pre-autoclaved indole-3-butyric acid (IBA) solution (2.45 mM) for 10 min followed by their implantation on medium containing ¼ MS salts, 1.0 % sucrose and 0.6 % agar. Out of 500 plantlets subjected to hardening, 410 were successfully hardened under greenhouse conditions. Twenty plants were established in field while remaining of them were transferred to nursery conditions without any mortality.

Additional key words: adventitious shoot buds, auxins, cytokinins, internode segments, medicinal plant.

Introduction

Celastrus paniculatus Willd. (*Celastraceae*) is a large, woody climbing shrub which occurs naturally in hilly parts of India up to an altitude of 1200 m. The plant is valued for its immense medicinal properties. *In vitro* adventitious shoot bud regeneration has been achieved from mature explants of several woody trees (Swartz *et al*. 1990, Nagori and Purohit 2004, D'Onofrio and Morini 2005), shrubs (Espino *et al.* 2004, Cheepala *et al*. 2004) and herbaceous plant species (Mitra *et al.* 2001, Bacchetta *et al.* 2003). *In vitro* studies in *C. paniculatus* has been carried out (Nair and Seeni 2001, Arya *et al.* 2002, Bilochi 2002) but there is no report on its regeneration through adventitious shoot bud differentiation.

The present paper describes a protocol for high frequency adventitious shoot bud differentiation from culture derived internode explants in *C. paniculatus* and plantlet recovery. This system can prove highly useful in genetic transformation studies.

Materials and methods

Shoot regeneration: *In vitro* shoot cultures were established using nodal explants obtained from mature field grown plants of *Celastrus paniculatus*. Nodal explants were washed in running tap water for about 30 min followed by treatment with 25 % solution of NaOCl (10.0 g dm^{-3} of active chlorine) for 10 min and subsequently washed with autoclaved distilled water for 4 - 5 times. The explants were further surface sterilized with 0.1 % $HgCl_2$ (m/v) for 10 min under *Laminar Flow Clean Air Bench*. After 4 - 5 times thorough washing with autoclaved double distilled water the nodal explants were placed vertically on the medium in culture tubes (25 × 150 mm) containing *ca.* 17 cm^3 of medium. The medium consisted of Murashige and Skoog's (1962; MS) salts, 3.0 % sucrose, 2.22 μM 6-benzylaminopurine (BAP) and gelled with 0.8 % agar as described by Bilochi

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Abbreviations: BAP - 6-benzylaminopurine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; Kn - kinetin; MS - Murashige and Skoog's medium; NAA - α-naphthalene acetic acid.
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(2002). Cultures were kept under controlled conditions of temperature (28 ± 2 ºC), irradiance (45 µmol m^{-2} s^{-1} for 16 h per day provided by fluorescent tubes) and 60 - 70 % relative humidity. Axillary bud proliferation was achieved in *ca.* 90 % explants after 20 - 25 d. Shoot cultures were further multiplied by repeated subculturing onto the same fresh medium after every 21 d. *In vitro* multiplied shoots (*ca.* 4 - 5 cm) were used as the source of explants for the present studies. Internode segments (*ca.* 1.0 cm) harvested aseptically were placed horizontally on the medium in culture tubes that were plugged with non-absorbent cotton. The pH of the medium was adjusted to 5.8 prior to autoclaving at 1.06 kg cm^{-2} for 15 min and the medium was solidified in slanted position.

The medium for all the experiments consisted of MS salts and vitamins plus 3.0 % sucrose, solidified with 0.8 % agar and supplemented with different concentrations of 6-benzylaminopurine (BAP), kinetin (Kn), indole-3-acetic acid (IAA) and α-naphthalene acetic acid (NAA). Effect of different nutrient media: MS (Murashige and Skoog 1962), ½ MS, ¼ MS, B_5 (Gamborg *et al.* 1968), WPM (Lloyd and Mc Cown 1980), BTM (Chalupa 1981) and SH (Schenk and Hildebrandt 1972) was also studied to optimize the nutritional supply for adventitious shoot bud differentiation and plantlet regeneration. All cultures were maintained under the same incubation conditions as have been described above. Explants were subcultured on to the same fresh medium after an interval of 21 d. Single explant was inoculated per culture tube making one replicate. For each treatment six replicates were used and each experiment was repeated thrice. The regeneration frequency, mean numbers of shoots regenerated per explant and mean shoot length was recorded after 9 weeks. For this purpose, morphologically differentiated shoot buds more than 0.5 cm in length were counted as shoots.

Statistical analysis: The experiments were conducted in a completely randomized design (CRD). The data of two representative experiments involving twelve replicates of each treatment were used for statistical analysis. To analyze the effect of various treatments, the data were subjected to analysis of variance (*ANOVA*). Fisher's least significance difference (LSD) test was applied to show statistical significance of differences among the means. The data presented here are the square root transformed ($y = (x + 0.5)^{1/2}$) values and the standard errors of the means.

Shoot elongation, multiplication and rooting: Internode segments with regenerated shoot buds were subsequently transferred on the fresh MS medium supplemented with 2.22 - 4.44 µM BAP for further elongation and multiplication. The cultures were kept under culture room conditions and scored for rate of shoot multiplication and number of elongated shoots after 21 d of culture. Rooting in elongated shoots (*ca.* 3 - 4 cm in length) was induced under *in vitro* conditions on 0.6 % agar-gelled medium containing ¼ MS salts, 1.0 % sucrose and 4.90 µM indole-3-butyric acid (IBA) as described by Bilochi (2002). Also the elongated shoots were inoculated on ¼ MS salt medium without any plant growth regulator. Prior to the inoculation, the bases of the shoots were dipped in pre-autoclaved aqueous solution of IBA (2.45 mM) for pulse treatment for 10 min (Bilochi 2002). The shoot cultures were incubated in culture room conditions for root initiation where shoots produced roots.

Hardening and acclimatization: The rooted plantlets immediately after their removal from agar-gelled medium were subjected to *in vitro* hardening in culture bottles containing autoclaved *Soilrite*™ and covered with polypropylene caps. The *Soilrite*™ was moistened with ¼ MS salts solution. Bottles were subsequently transferred to standard greenhouse conditions (28 ± 2 ºC temperature, a regime of gradually reducing humidity from 80 - 55 % and 400 µmol m^{-2} s^{-1} irradiance) and their caps were opened gradually. The acclimated plantlets were transplanted into polybags containing sand and farmyard manure (1:1) and were allowed to grow under nursery shade conditions. Some of the hardened plants which attained an age of 12 months were transferred in field conditions during monsoon months while remaining of them were kept under nursery shade conditions for further growth and field experiments.

Results

Shoot regeneration: Multiple shoot buds could be induced directly from the internode explants in *C. paniculatus*, on a variety of treatments. On basal MS medium, adventitious shoot buds, without an intervening callus phase, were induced from the internode explants within 12 - 15 d of inoculation in 75 % explants and produced 3 - 4 shoots per explant after 9 weeks. Incorporation of cytokinins (BAP and Kn) and auxins (NAA and IAA) evoked varied response.

Addition of cytokinins at varied concentrations (2.22 - 22.2 µM BAP and 2.32 - 23.2 µM Kn each, either singly or in combination) to MS medium caused explant enlargement and induced more shoot buds from entire surface of internode explants within 7 - 9 d of culture. Within 25 - 28 d these buds proliferated profusely on cytokinin containing MS medium. However, the number of shoot buds regenerated per explant, average shoot length and regeneration frequency varied depending upon the kind and concentration of cytokinins used. On addition of BAP in the medium, the number of shoot buds regenerated per explant increased with increasing BAP concentration, reaching a maximum at 4.44 µM BAP and

thereafter decreased with further increase in BAP. BAP at 4.44 µM produced a maximum 14 - 15 shoot buds per explant where all explants responded favourably (Fig. 1*A*,*B*). The length of regenerated shoots showed an inverse relationship with concentration of BAP. MS medium supplemented with Kn did not show better response than any of the BAP concentrations but the shoots were more elongated. The best response to Kn was obtained on 2.32 µM where 92 % explants responded positively and produced 4 - 5 shoots. Any increase in Kn concentration led to decrease in regeneration frequency and number of shoots per explant. However, the highest mean shoot length (*ca.* 3.0 cm) was obtained on 11.62 µM Kn.

A combination of cytokinins (BAP and Kn) at their various concentrations in the medium was not effective in enhancing number of shoot buds per explant as compared to 4.44 µM BAP used alone. The higher concentrations of either of cytokinins (BAP and Kn) in the medium diminished shoot buds induction response of internode explants and caused explant mortality. The best response on combinations, however, was obtained when 4.44 µM BAP was combined with 2.32 µM Kn where 9 - 10 shoot buds regenerated per explant (Table 1).

Incorporation of auxins (IAA and NAA) in different concentrations in the medium individually, either caused callusing or rooting along with callus in some cases. A linear relationship between the callus growth and the concentration of auxins was observed. A compact, nodular and green callus at one or both cut ends of explants was noticed on lower concentrations of IAA (0.057 - 0.57 µM) and NAA (0.054 - 0.54 µM) while concentrations beyond this produced loose, friable and greenish yellow callus from entire surface of the explants. Roots (*ca.* 2 - 3) along with callus were also induced in *ca.* 40 - 50 % explants cultured on medium containing IAA (1.43 - 5.71 µM) and NAA (2.69 - 5.37 µM).

Addition of different concentrations of IAA

Fig. 1. Different stages of *in vitro* adventitious shoot bud differentiation in culture derived internode segments of *Celastrus paniculatus* grown on MS medium containing 4.44 µM BAP. *A* - Induction of adventitious shoot buds after 9 d; *B* - multiple shoot buds on the internode segment; *C* - shoot multiplication; *D* - shoot elongation on lower concentration of BAP (2.22 µM); *E* - induction of roots in pulse treated (2.45 mM IBA for 10 min) shoots after 21 d; *F* - *in vitro* hardening of tissue culture raised plants grown on *Soilrite*™ moistened with 1/4 MS salt solution.

Table 1. Effect of cytokinins (BAP and Kn) on adventitious shoot buds differentiation from internode segments in *C. paniculatus* grown on MS medium. Observations were recorded after 9 weeks. Means of twelve replicates in the same column followed by different letters differ significantly at $P \leq 0.05$. * - transformed values.

BAP [µM]	Kn [µM]	Number of shoot buds* [explant^{-1}]	Shoot length [cm]	Regeneration frequency [%]
0.00	0.00	1.90^{cd}	0.42^{de}	75
	2.32	2.23^{cd}	0.50^{de}	92
	4.65	1.99^{cd}	2.42^{b}	92
	11.62	1.42^{d}	3.12^{a}	58
	23.24	1.38^{d}	1.03^{cd}	50
2.22	0.00	2.84^{bc}	2.28^{d}	100
	2.32	2.93^{bc}	1.90^{bc}	100
	4.65	2.63^{bc}	2.04^{bc}	100
	11.62	2.83^{bc}	1.53^{c}	100
	23.24	2.00^{cd}	0.72^{d}	58
4.44	0.00	3.89^{a}	1.89^{bc}	100
	2.32	3.13^{b}	1.52^{c}	100
	4.65	2.90^{bc}	1.10^{cd}	100
	11.62	2.57^{bc}	0.93^{cd}	83
	23.24	2.09^{cd}	0.50^{de}	50
11.10	0.00	2.75^{bc}	0.63^{de}	100
	2.32	2.96^{b}	0.50^{de}	100
	4.65	2.81^{bc}	0.50^{de}	100
	11.62	1.68^{d}	0.33^{de}	42
	23.24	1.30^{de}	0.25^{de}	25
22.20	0.00	1.82^{cd}	0.42^{de}	83
	2.32	1.61^{d}	0.33^{de}	42
	4.65	2.33^{c}	0.50^{de}	50
	11.62	1.33^{d}	0.25^{de}	25
	23.24	-	-	-
SEM		0.0972	0.103	

Table 2. Effect of auxins (IAA and NAA) incorporated in the MS medium along with 4.44 µM BAP on adventitious shoot bud differentiation from internode segments in *C. paniculatus.* Observations were recorded after 9 weeks. Means of twelve replicates in the same column followed by different letters differ significantly at $P \leq 0.05$. * - transformed values.

Auxins	Conc. [µM]	Number of shoot buds* [explant^{-1}]	Shoot length [cm]	Regeneration frequency [%]
IAA	0.057	2.99^{a}	0.62^{ab}	100
	0.290	2.39^{b}	0.50^{b}	100
	0.570	1.85^{c}	0.75^{ab}	100
NAA	0.054	2.12^{bc}	0.97^{a}	100
	0.270	1.63^{cd}	0.94^{a}	92
	0.540	1.44^{d}	0.48^{b}	75
	1.350	1.03^{e}	0.25^{b}	33
SEM		0.73	0.079	

(0.057 - 5.71 µM) and NAA (0.054 - 5.37 µM) in combination with 4.44 µM BAP in the medium, suppressed regeneration frequency, the number of shoots per explant and mean shoot length as compared to BAP (4.44 µM) used alone. Higher concentrations of IAA (1.43 - 5.71 µM) and NAA (2.69 - 5.37 µM) along with 4.44 µM BAP completely inhibited shoot bud differentiation from internode segments and induced callus only. It was therefore, concluded that the BAP at 4.44 µM concentration was the best for induction of maximum number of shoot buds (Table 2).

Table 3. Effect of different media supplemented with 4.44 µM BAP on adventitious shoot buds differentiation from internode segments in *C. paniculatus.* Observations were recorded after 9 weeks. Means of twelve replicates in the same column followed by different letters differ significantly at $P \leq 0.05$. * - transformed values.

Media	Number of shoot buds [explant $^{-1}$]*	Shoot length [cm]	Regeneration frequency [%]
MS	3.89^{a}	1.89^{ab}	100
½ MS	2.77^{bc}	0.96^{c}	100
¼ MS	2.48^{d}	0.50^{c}	100
WPM	2.91^{b}	1.88^{ab}	100
B5	2.70^{c}	1.50^{b}	100
BTM	2.61^{cd}	2.10^{a}	100
SH	2.54^{cd}	1.00^{bc}	100
SEM	0.058	0.179	

Regeneration of adventitious shoots were obtained from the internode explants on all media tested. However, the type of media significantly influenced the number of shoot buds that regenerated per explant and average shoot length. The best response in terms of shoot buds regenerated per explant was achieved on medium containing basal MS salts and 4.44 µM BAP followed by WPM, ½ MS, B_5, BTM, SH and ¼ MS medium supplemented with the same concentration of BAP. However, BTM medium favoured shoot elongation (Table 3).

Shoot growth, multiplication and rooting: Explants with regenerated shoot buds on MS medium containing 4.44 µM BAP were further subcultured on fresh MS medium supplemented with BAP (2.22 - 4.44 µM). Shoot elongation and multiplication could be achieved on both the treatments tested where shoots attained an average height of 3.0 - 3.5 cm and multiplied further. However, the rate of shoot multiplication (3-fold) and number of elongated shoots (*ca.* 6 - 7) were higher on MS medium containing 2.22 µM BAP (Fig. 1*C,D*). Therefore, all the cultures were subsequently transferred to this medium for further shoot growth and elongation.

Rooting with moderate amount of callus, was achieved in 90 % shoots inoculated on ¼ MS medium containing 0.6 % agar, 1.0 % sucrose and 4.90 µM IBA. A pulse treatment with IBA (2.45 mM) for 10 min could induce 80 % callus free rooting (Fig. 1*E*). Also, the average number (5.0) and length (2.0 cm) of roots were higher in the pulse treated shoots as compared to IBA incorporated in the medium. Therefore, it was concluded

that pulse treatment was a better choice to obtain callus-free rooting.

Hardening and acclimatization: None of the plantlets directly transferred from rooting medium to the potting mix and grown under natural conditions survived. However, 25 % of such plants survived if the potted plants were kept in polythene tents under high humidity (70 - 80 %). The survival percentage rose to 80 % if the plantlets were hardened under aseptic conditions before being transferred to the polybags. During the *in vitro* hardening, shoots elongated, leaves turned greener and expanded. Consequently, the plants seemed much healthier after *in vitro* hardening. Such plants also grew more vigorously in the greenhouse (Fig. 1*F*). More than 410 plantlets out of 500 were successfully hardened and 20 plants were established in field while remaining of them were maintained in nursery without any mortality.

Discussion

The data presented above demonstrated that high frequency adventitious shoot bud development and plantlet regeneration is possible in internode segments obtained from *in vitro* multiplying shoot cultures of *C. paniculatus*. BAP appeared to be more effective than Kn for inducing adventitious shoot buds in internode segments of *C. paniculatus* in agreement with the results described by various authors (Mishra 2002, Espino *et al.* 2004, Purohit *et al*. 2004). Kn was effective over BAP on shoot elongation as has also been reported in *Rosa hybrida* L. (Ibrahim and Debergh 2001). The positive effect of combined cytokinins on adventitious shoot bud differentiation has been reported in *Picea glauca* and *P. mariana* (Rumary and Thorpe 1984). In the present study combination of BAP and Kn was significant, however it could not supercede the effect of 4.44 µM BAP used alone. Similar results were also observed in shoot tip explants of *Sterculia foetida* (Anitha and Pullaiah 2002). As observed with other species (Thorpe *et al.* 1991, Gomez and Segura 1994) exogenous auxin supported callus formation than shoot formation in cultured internode segments of *C. paniculatus*.

The beneficial effect of reduced salts and sucrose concentrations during rooting phase has been described in several reports (Barve and Mehta 1993, Purohit and Singhvi 1998). Rooting by dip treatments of auxins has been recommended by Harry and Thorpe (1991). It is supposed to eliminate the inhibitory effect on root growth when IBA is incorporated in the medium (Hartmann *et al.* 1990). Purohit and Singhvi (1998) have recommended IBA pulse treatment for rooting in *A. sapota* shoots. This method of root induction has been successfully employed in the present studies also.

In vitro hardening was a pre-requisite for successful transfer of plants in soil in the present case as has been recommended in *Boswellia serrata* (Purohit *et al.* 1995). Development of *in vitro* regeneration system has served a useful purpose of transformation of plants by direct gene transfer (Khurana and Khurana 1999). In conclusion, the development of highly reproducible regeneration system in *C. paniculatus* using internode explants during present investigation promises its use in genetic improvement programmes of this useful medicinal plant.

References

Anitha, S., Pullaiah, T.: Shoot regeneration from hypocotyl and shoot tip explants of *Sterculia foetida* L. derived from seedlings. - Taiwania **47**: 62-69, 2002.

Arya, V., Singh, R.P., Shekhwat, N.S.: A micropropagation protocol for mass multiplication and off-site conservation of *Celastrus paniculatus* - a vulnerable medicinal plant of India. - J. sustain. Forest. **14**: 107-120, 2002.

Bacchetta, L., Remotti, P.C., Bernardinin, C., Saccardo, F.: Adventitious shoot regeneration from leaf explants and stem nodes of *Lilium*. - Plant Cell Tissue Organ Cult. **74**: 37-44, 2003.

Barve, D.M., Mehta, A.R.: Clonal propagation of mature elite trees of *Commiphora wightii*. - Plant cell tissue Organ Cult. **35**: 237-244, 1993.

Bilochi, G.: *In vitro* studies on some medicinal plants of Aravallis in Rajasthan. - Ph.D. Thesis, M.L. Sukhadia University, Udaipur 2002.

Chalupa, V.: Clonal propagation of broad-leaved forest trees *in vitro*. - Commun. Inst. Forest Czech. **12**: 255-271, 1981.

Cheepala, S.B., Sharma, N.C., Sahi, S.V.: Rapid *in vitro* regeneration of *Sesbania drumondii*. - Biol. Plant. **48**: 13-18, 2004.

D'Onofrio, C., Morini, S.: Development of adventitious shoots from *in vitro* grown *Cydonia obloga* leaves as influenced by different cytokinins and treatment duration. - Biol. Plant. **49**: 17-21, 2005.

Espino, F.J., Linacero, R., Rueda, J., Vazquez, A.M.: Shoot regeneration in four *Begonia* genotypes. - Biol. Plant. **48**: 101-104, 2004.

Gamborg, O.L., Miller, R.A., Ojima, K.: Nutrient requirement of suspensions cultures of soybean root cells. - Exp. Cell Res. **50**: 151-158, 1968.

Gomez, M.P., Segura, J.: Factors controlling adventitious bud induction and plant regeneration in mature *Juniperus oxycedrus* leaves cultured *in vitro*. - *In Vitro* cell. dev. Biol. Plant **30**: 210-218, 1994.

Harry, I.S., Thorpe, T.A.: Engelmann spruce (*Picea engelmannii* Parry *ex*. Engelm.). - In: Bajaj, Y.P.S. (ed.): Biotechnology in Agriculture and Forestry. Vol. 16. Pp. 408-22. Springer-Verlag, Berlin - Heidelberg 1991.

Hartmann, H.T., Kester, D.E., Davies, F.T., Jr. (ed.): Plant Propagation - Principles and Practices. 5th Ed. - Prentice Hall, Englewood Cliffs - New Jersey 1990.

Ibrahim, R., Debergh, P.C.: Factors controlling high efficiency

adventitious bud formation and plant regeneration from *in vitro* leaf explants of roses (*Rosa hybrida* L.). - Sci. Hort. **88**: 41-57, 2001.

Khurana, P., Khurana, J.: Application of genetic transformation to tree biotechnology: a review. - Indian J. exp. Biol. **37**: 627- 638, 1999.

Lloyd, G.G., McCown, B.H.: Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. - Int. Plant Propagator's Soc. Combined Proc. **30**: 421-427, 1980.

Mishra, P.: Direct differentiation of shoot buds from leaf explants of *Cajanus cajan* L. - Biol. Plant. **45**: 347-351, 2002.

Mitra, S.K., Mukherjee, K.K.: Direct organogenesis in Indian spinash. - Plant Cell Tissue Organ Cult. **67**: 191-194, 2001.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue culture. - Physiol. Plant. **15**: 473-497, 1962.

Nagori, R., Purohit, S.D.: *In vitro* plantlet regeneration in *Annona squamosa* through direct shoot bud differentiation on hypocotyl segments. - Sci. Hort. **99**: 89-98, 2004.

Nair, L.G., Seeni, S.: Rapid *in vitro* multiplication and restoration of *Celastrus paniculatus* (*Celastraceae*), a medicinal woody climber. - Indian J. exp. Biol. **39**: 697-704, 2001.

Purohit, S.D., Singhvi, A.: Micropropagation of *Achras sapota* through enhanched axillary branching. - Sci. Hort. **76**: 219-229, 1998.

Purohit, S.D., Singhvi, A., Nagori, R.: *In vitro* shoot bud differentiation from leaf segments of *Achras sapota*. - Biol. Plant. **48**: 109-112, 2004.

Purohit, S.D., Tak, K., Kukda, G.: *In vitro* propagation of *Boswellia serrata* Roxb. - Biol. Plant. **37**: 335-340, 1995.

Rumary, C., Thorpe, T.A.: Plantlet formation in white and black spruce. I. *In vitro* techniques. - Can. J. Forest. Res. **14**: 10-16, 1984.

Schenk, R.V., Hildebrandt, A.C.: Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. - Can. J. Bot. **50**: 199-204, 1972.

Swartz, H., Bors, R., Mohamed, F., Naess, K.: The effect of *in vitro* pre-treatments on subsequent shoot organogenesis from excised *Rubus* and *Malus* leaves. - Plant Cell Tissue Organ Cult. **21**:179-184, 1990.

Thorpe, T.A., Harry, I.S., Kumar, P.P.: Application of Micropropagation to Forestry. - In: Debergh, P.C., Zimmerman, R.H. (ed.): Micropropagation, Technology and Application. Pp. 311-336. Kluwer Academic Publishers, Dordrecht 1991.