academicJournals

Vol. 12(35), pp. 5413-5422, 28 August, 2013
DOI: 10.5897/AJB2012.2960
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Propagation from axillary buds and anatomical study of adventitious roots of *Pinus taeda* L.

Leandro Francisco de Oliveira[1]*, Marguerite Quoirin[1,2], Henrique Soares Koehler[2], Erika Amano[1], Antonio Rioyei Higa[3] and Luciana Lopes Fortes Ribas[1]

[1]Departamento de Botânica, Laboratório de Micropropagação Vegetal, Universidade Federal do Paraná, P.O. Box 19031, 81531-980, Curitiba, PR, Brazil.
[2]Departamento de Fitotecnia e Fitossanitarismo, Universidade Federal do Paraná, P.O. Box 19061, 81531-990, Curitiba, PR, Brazil.
[3]Departamento de Ciências Florestais, Universidade Federal do Paraná, 80210-170, Curitiba, PR, Brazil.

Accepted 7 August, 2013

Micropropagation of *Pinus taeda* by axillary bud proliferation technique and anatomical study of adventitious roots were studied. Apical and basal segments were established *in vitro* in Murashige and Skoog (MS) culture medium. For multiple shoot induction, the explants were transferred to WV3 medium with or without 6-benzylaminopurine (BAP) (0.12, 0.25 or 0.50 µM). The effect of the culture medium and combinations of α-naphthalene acetic acid (NAA) with BAP on adventitious rooting were evaluated. The basal segments exhibited higher multiplication rates (3.7 shoots per explant) than apical segments during establishment of *in vitro* culture. The best rooting rate (40%) was achieved by induction of roots in water-agar (WA) culture medium supplemented with 2.69 µM NAA and 0.44 µM BAP, for 9 days, followed by transfer into GDm/2 medium. Adventitious roots can be derived from the cambium cells, from adventitious meristem, or from a callus developed at the base of the shoots. When formed indirectly, roots can be derived from peripheral cells of the callus. In treatment resulting in the best rooting rate, 58.3% of the roots were formed directly. The callus formation at stem basis did not prevent rooting and did not impair acclimatization. The plantlets were successfully acclimatized with 90% survival.

Key words: Loblolly pine, apical segment, basal segment, rooting, vascular connection, callus.

INTRODUCTION

Pinus taeda L. is an important forest species because of the productivity and quality of its timber, being used primarily at sawmills, rolling mills, manufacturing of particle board, MDF, construction, furniture, crates, pulp and paper (Schultz, 1997). In Brazil, in addition to *Eucalyptus*, *Pinus* species provides raw material for products coming from forests, thus reducing the exploitation of native species such as *Araucaria angustifolia* (Selle et al., 1994; Souza

*Corresponding author. E-mail: lebio.oliveira@gmail.com. Tel: +55 41 33611790. Fax: +55 41 32662042.

Abbreviations: BAP, 6-Benzylaminopurine; **GDm,** Gresshoff and Doy medium modified by Mehra-Palta et al. (1978); **MS,** Murashige and Skoog medium (1962); **NAA,** α-naphthalene-acetic acid; **PGR,** plant growth regulator; **WA,** culture medium composed of water and agar; **WV3,** Coke medium (1996).

et al., 2008).

Research on vegetative propagation of *Pinus* is therefore of great importance and has been applied to improve the quality of its wood and plantation. However, it is frequently still propagated from seed, since the rooting of cuttings depends on the season of the year or on the availability of juvenile material (Schestibratov et al., 2003; Alcantara et al., 2007; Cuesta et al., 2008; Andrejow and Higa, 2009). The micropropagation techniques became a necessary tool for large-scale production of *P. taeda* plants and serve as strategies for tree improvement (Gupta and Durzan, 1991; Govil and Gupta, 1997; Menzies and Aimers-Halliday, 1997).

Research on *P. taeda* is related to direct (Mehra-Palta et al., 1978; Mott and Amerson, 1981; Jang and Tainter, 1991; Frampton et al., 1998; Tang and Guo, 2001) and indirect organogenesis (Tang et al., 1998; Tang and Ouyang, 1999; Tang, 2000). Given the limitations of clonal propagation during the adult phase of trees, most studies used juvenile material as a source of explant, such as various parts of the zygotic embryo, cotyledons, epicotyls and apical meristems. Moreover, in techniques involving a callus phase, such as indirect organogenesis and embryogenesis, the occurrence of somaclonal variation and phenotypic alterations is common, which does not occur in direct organogenesis (DeVerno et al., 1999; Tang, 2000; Cuesta et al., 2008). *Pinus* species have pre-existing axillary buds at the base of their needles that can be induced to develop into shoots. Micropropagation via axillary buds has the advantage of not involving callus formation and avoiding the use of high concentrations of plant growth regulators (PGRs), thus promoting higher genetic stability.

Among the cytokinins, 6-benzylaminopurine (BAP) promoted good responses in the induction of adventitious and axillary shoots of *P. taeda* (Mehra-Palta et al., 1978), *P. lambertiana* (Gupta and Durzan, 1985) and *P. kesiya* (Nandwani et al., 2001).

Adventitious rooting is an essential step for obtaining the economically efficient production system required by clonal forestry (Foster, 1990; George et al., 2008). For conifers such as in *Pinus,* it is considered a difficult step and its success may depend on the species, family and clone (Bergmann and Stomp, 1994).

The use of exogenous auxin is usually required for the induction of adventitious roots in difficult-to-root species. For conifers, α-naphthaleneacetic acid (NAA) is the most commonly used, sometimes in combination with cytokinins (Abdullah et al., 1989). Mehra-Palta et al. (1978) achieved higher percentage of rooting (50%) in *P. taeda* combining 0.54 µM NAA and 0.22 or 0.04 µM BAP in Gresshoff and Doy modified culture medium (GDm), while Mott and Amerson (1981) succeeded in rooting loblolly pine with a combination of 2.69 µM NAA and 0.44 µM BAP added to the same medium.

Depending on the species, the roots may originate from different tissues, either directly or indirectly, which callus can limit rooting and make it difficult transplantation (Hicks, 1987; Apter et al., 1993a, b; Ballester et al., 1999). In *Pinus* species, the callogenesis at the base of explants before the emergence of roots is common (Wagley et al., 1987; Hammann, 1998; Álvarez et al., 2009). When roots emerge from callus, the vascular connection between root and stem is of great importance for the functioning of the vascular system and the survival of plants after transplantation in greenhouse (George et al., 2008). In some *Pinus* species, the high survival rate can be related to vascular connection (Álvarez et al., 2009).

The purpose of this study was to establish an efficient method for micropropagation of *P. taeda* by axillary buds, comparing the presence and absence of apical buds, combinations of plant growth regulators to induce adventitious rooting, transplantation and performing anatomical studies in order to ascertain the origin of *in vitro*-formed roots.

MATERIALS AND METHODS

Establishment of *in vitro* cultures

Two to four-month-old seedlings from a commercial clonal orchard were provided by the Battistella Forest Company located at Rio Negrinho, Santa Catarina, Brazil. The seedlings were kept in a greenhouse for 20 days and treated with 1 g L^{-1} Cercobin® (a fungicide) every two days, making a total of 10 sprays.

For establishment of *in vitro* cultures, apical and basal segment, 3.5 cm long, were collected from the apical portion of the plants, one explant per seedling, and 90% of the needles were cut. The explants were disinfested by immersion in 0.05% (w/v) $HgCl_2$ for 5 min, followed by 0.5% (v/v) NaOCl for 5 min, under constant agitation. Tween 20 (CRQ® Cromato Produtos Químicos) 0.1% (v/v) was added to the disinfestation solutions. After disinfestation, the explants were rinsed three times in sterile distilled water.

The explants were inoculated vertically in 15.0 × 2.5 cm test tubes containing 10 mL of MS (Murashige and Skoog, 1962) culture medium supplemented with 30 g L^{-1} sucrose and solidified with 5.6 g L^{-1} bacteriological Himedia® agar.

After 6 weeks of culture, microbial contamination, the necrosis and survival rates, the percentage of explants with shoots and the average number of axillary shoots per explant (final number of shoots/initial number of explants) were evaluated. The experimental design was completely randomized. For each explant type, there were 30 replications of 10 explants per replicate and one explant per test tube.

Shoot multiplication

For induction of multiple shoots, apical and basal segments established *in vitro* were transferred to flasks of 12.5 × 6.2 cm, containing 40 mL of WV3 (Coke, 1996) culture medium, supplemented with BAP (0, 0.12, 0.25 or 0.50 µM), 30 g L^{-1} sucrose and 5.6 g L^{-1} bacteriological Himedia® agar. Apical segments longer than 4 cm were subdivided into two segments, with the upper segment containing the apical bud and the lower segment being devoid of apical buds. Two subcultures were performed on the same culture medium with the same concentrations of BAP. The culture period was four

weeks for the initial culture and eight weeks for the two subsequent subcultures. At the end of each subculture, the following parameters were evaluated: percentage of explants with shoots, average number of axillary shoots per explant (final number of shoots / initial number of explants) and percentage of elongation rate.

The experimental design was completely randomized, in split plots with three explants per flask, five flasks per replicate and five replicates per treatment. Four BAP concentrations were compared in the plots and three subcultures in the subplots. The data were analyzed separately for each type of explant.

Adventitious rooting

For adventitious rooting, individual shoots were inoculated into plant growth regulator (PGR)-free WV5 medium supplemented with 2 g L^{-1} activated charcoal 20 days before rooting induction. Then, shoots (1.5 to 2.0 cm high) with a double bevel cut at the base were inoculated into GDm/2 medium (Gresshoff and Doy modified by Mehra-Palta et al., 1978) with half-strength salts. The control was a PGR-free medium and the treatments were: 0.54 µM NAA and 0.04 µM BAP, 0.54 µM NAA and 0.22 µM BAP, 0.54 µM NAA and 0.44 µM BAP, 2.69 µM NAA and 0.44 µM BAP. We also used a medium composed of water and agar (WA) supplemented with 2.69 µM NAA and 0.44 µM BAP. In the two later treatments, an induction period of 9 days was tested, followed by transfer of the shoots into PGR-free GDm/2. In the other treatments, the shoots remained in the same culture medium for ten weeks. The GDm/2 medium contained 20 g L^{-1} sucrose and all media were solidified with 5.6 g L^{-1} bacteriological Himedia® agar.

The experimental design was completely randomized, with four explants in each flask, two flasks per replicate and six replicates per treatment. After 10 weeks of culture, the following parameters were evaluated: percentage of rooted shoots, average number of roots per shoot and the percentage of roots developed from a callus or directly from the stem.

Transplanting and acclimatization

Plants 1.5 to 2.5 cm high and with roots longer than 0.6 cm were used for transplanting and acclimatization. The roots were washed in running water and plantlets planted in Plantmax Forests® substrate in plastic bags of 600 cm^3, one plant per bag, for 60 days. The plants were manually irrigated 4 to 5 times daily during the first 20 days. After 90 days, plants were evaluated for the percentage of survival and average number of roots per plant.

Culture conditions

All media had their pH adjusted to 5.8 with NaOH 0.1 N or HCl 0.1 N and were autoclaved for 20 min at 121°C. *In vitro* cultures were maintained in a growth room with temperatures of 19 ± 2°C (night) and 28 ± 2°C (day), a 16-h photoperiod under white fluorescent light (PFD = 40 $\mu molm^{-2}s^{-1}$). For transplantation and acclimatization, the plants were maintained in a greenhouse under shade, with temperatures ranging from 25 ± 7°C.

The data were submitted to Bartlett's test and analysis of variance (ANOVA) and means compared by Tukey's test ($P \leq 0.05$) using the statistical software MSTAT-C® (Department of Crop and Soil Sciences, Michigan State University).

Anatomical study of adventitious roots

The anatomical study was realized according to de Oliveira et al. (2012). Samples were collected from shoots with roots approximately 0.2 cm long, developed directly or indirectly from stem basis. The material was fixed in FAA (Berlyn and Miksche, 1976), dehydrated in alcohol series, then infiltrated and embedded in Historesin® following the manufacturer's instructions (Leica Microsystems, Germany). Longitudinal series sections (7 µm thick) were performed on a rotation microtome RM2145 (Leica Microsystems, Germany) and stained with toluidine blue (O'Brien et al., 1965). The characteristics of the material were recorded on digital equipment connected to a Zeiss microscope®.

RESULTS AND DISCUSSION

Explants of 2- to 4-month-old seedlings (Figure 1a) were used, because in preliminary tests, the older explants (6-month-old) showed slower responses and higher microbial contamination. For conifers and woody plants in general, juvenile explants have shown better responses *in vitro* (El-Nil, 1982; Lapp et al., 1996; Bonga et al., 2010) and better responses to rooting in mini-cuttings (Majada et al., 2011). Disinfestation treatment allowed a survival of around 60% of the plantlets, with no influence of the explant type. The percentage of necrosis was low for both apical and basal segments (Table 1).

The explant type influenced the formation of axillary shoots. Basal segments showed a higher percentage of explants forming axillary shoots (90 to 100%) and average number of shoots per explant (3.7), compared to apical segments (Table 1; Figure 1b and c). The presence of the apical bud can induce a greater elongation of the shoots, while its absence enables axillary shoots to develop, as observed for some species of *Pinus* (McKellar et al., 1994; Scaltsoyiannes et al., 1994). A similar response was obtained for *P. brutia* × *P. halepensis*, probably due to overcoming apical dominance when the apical bud was removed (Scaltsoyiannes et al., 1994; George et al., 2008). McKellar et al. (1994) also observed better results in basal segments than in apical segments of *P. patula*, comparing the average number of shoots in both explant types.

In the induction of multiple shoots, there was no interaction between the number of subcultures and the concentrations of BAP tested. However, the number of subcultures influenced the induction of axillary shoots. In the second subculture, basal segments showed a significant decrease in the percentage of explants with axillary shoots, from 98.4% to 41.2% (Table 2) and in the average number of shoots per explant (from 1.4 to 1.2) (Table 3). According to the study of George (1996), in woody plants, it is common to note a decrease in the rate of multiplication during the subcultures, after reaching a maximum value. Apical segments inoculated into culture medium containing BAP showed a higher percentage of explants forming shoots than in the control (Figure 1d), while there was no significant differences between treatments for basal segments (Table 2).

The explant type initially used influenced the response

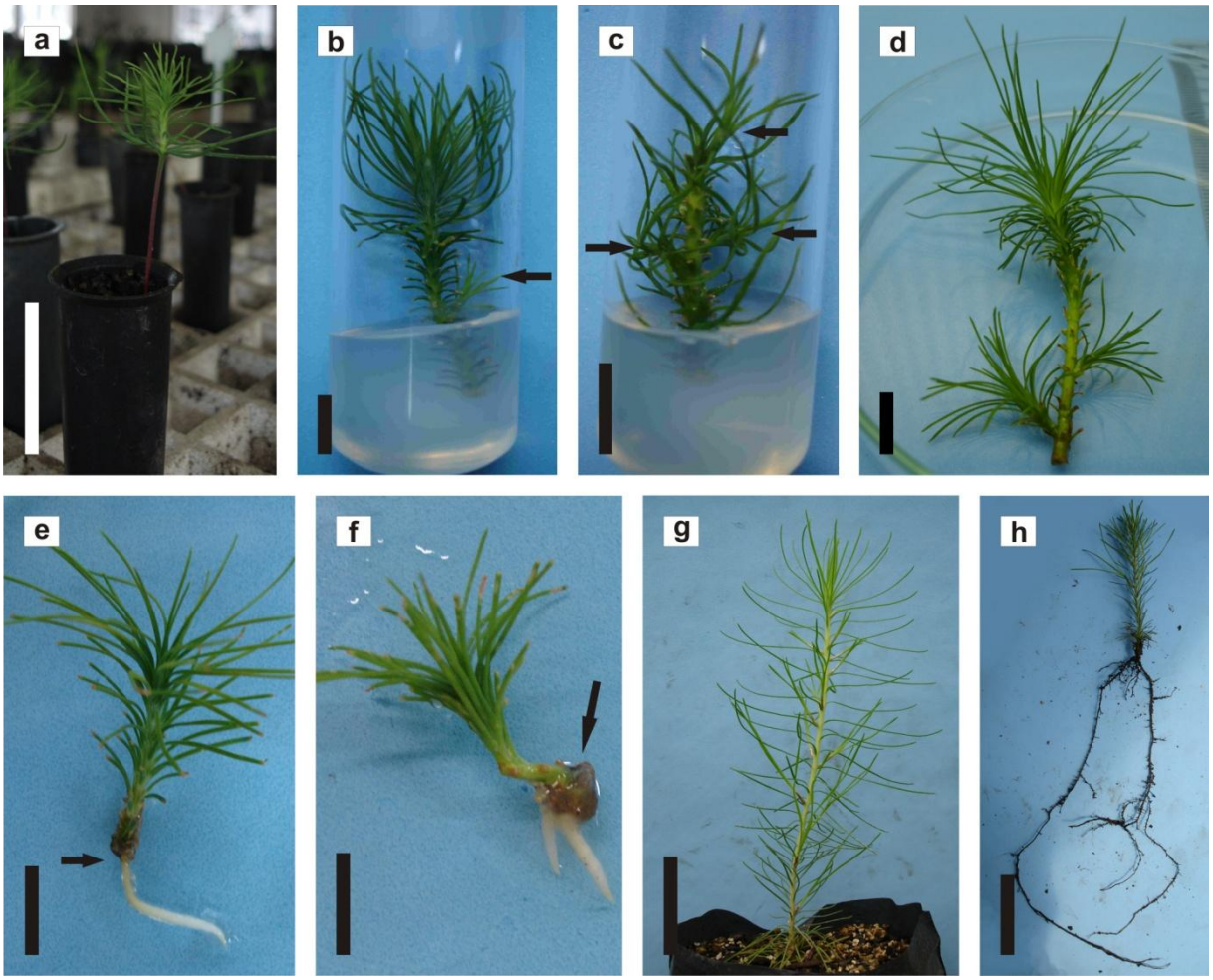

Figure 1. Micropropagation of *Pinus taeda* L. by axillary buds. (**a)** Two to four-month-old seedlings kept in a greenhouse used as explant source; (**b**) apical segments and (**c)** basal segments inoculated into MS culture medium, after 6 weeks with axillary shoots (arrows); (**d)** apical segment with axillary shoots developed in WV3 culture medium with 0.25 µM BAP at the initial culture; (**e)** rooted shoots with the root derived directly from the stem (arrow) and (**f)** rooted shoots with the roots derived from a callus at the base (arrow); (**g)** micropropagated plantlet after 2 months of acclimatization; (**h)** plantlet with developed roots after 2 months of acclimatization. *Bars* **a, g** and **h** = 5 cm; **b-f** = 1 cm.

of multiple shoot induction during the subcultures. Unlike the results of establishment of *in vitro* culture, the average number of shoots per explant from basal segments was not superior to the number obtained with explants from apical segments, with approximately 1.2 shoots per explant being obtained at the end of the two subcultures (Table 3). Although the overcoming apical dominance has led to the formation of up to three axillary shoots per explant during establishment of *in vitro* culture, at the multiplication step some of these axillary shoots did not elongate or could not be individualized, maintaining the same multiplication rate.

Axillary shoots developed along the whole axis of the explant; however, in apical segments, they developed preferably in the middle region and more frequently in the basal region of the segment. This effect may be related to a gradient of juvenility starting near the basal region of the explant (Figure 1d), as it occurs in mature trees or in seedlings (Hartmann et al., 2002; George et al., 2008). In this study, the highest average numbers of axillary shoots per explant (2 to 3) were obtained in apical segments (Figure 1d), with no difference between the control and explants cultured on media containing BAP (0.12, 0.25 and 0.50 µM) (Table 3). A similar result was obtained with *P. lambertiana*, after 8 weeks in DCR culture medium supplemented with a higher concentration of BAP (2.22 µM) (Gupta and Durzan, 1985).

These results indicate that induction of axillary shoots is feasible on culture medium containing low concentrations of BAP or without BAP. This second alternative is interesting for a commercial protocol because it reduces the cost of the medium. However, the juvenile shoots

Table 1. Effect of explant type on establishment of *in vitro* culture and axillary shoot development in *Pinus taeda*, after 6 weeks of culture.

Explant type	Contamination by		Necrosis (%)	Survival (%)	Explants with axillary shoots (%)	Average number of shoots per explant
	Bacterium (%)	Fungus (%)				
Apical segment	7.0	31.0	0.3	61.7	38.3 ± 18.3b	1.5 ± 0.3b
Basal segment	8.3	30.7	0.0	61.0	92.0 ± 13.0a	3.7 ± 0.4a

Values are means ± SD, n = 10. Means within a column followed by different letters differ significantly according to Tukey's test (P ≤ 0.05).

Table 2. Percentage of explants with axillary shoots that developed in *Pinus taeda* in WV3 medium supplemented or not with BAP, after ([a]) four weeks and ([b]) eight of culture.

Explant type	BAP [µM]	Initial culture [a]	1st Subculture[b]	2nd Subculture[b]	Mean
Apical segment	0.00	48.9	63.9	56.4	56.4±14.8b
	0.12	57.8	64.8	66.7	63.1±16.8ab
	0.25	70.8	80.0	66.5	72.4±11.5a
	0.50	60.0	84.5	62.7	69.1±19.1ab
Mean		59.4 ± 19.5B	73.3 ± 14.0A	63.1 ± 13.0AB	
Basal segments	0.00	96.7	100.0	38.1	78.3±37.3a
	0.12	96.7	100.0	35.3	77.3±36.8a
	0.25	100.0	100.0	42.8	80.9±37.3a
	0.50	100.0	98.3	48.6	82.3±38.3a
Mean		98.4±26.4A	99.6±30.1A	41.2±14.0B	

Values are means ± SD, n = 5. Means within a column followed by the same lower case letter and means within a line followed by the same upper case letter do not differ significantly according to Tukey's test (P ≤ 0.05).

Table 3. Average number of axillary shoots developed per explant of *Pinus taeda* in WV3 medium supplemented or not with BAP, after ([a]) four weeks and ([b]) eight of culture.

Explant type	BAP (µM)	Initial culture[a]	1st Subculture[b]	2nd Subculture[b]	Mean
Apicalsegments	0.00	1.7	2.7	2.4	2.3±0.7a
	0.12	1.8	3.0	2.7	2.5±0.9a
	0.25	2.2	3.3	3.4	3.0±0.6a
	0.50	2.1	3.6	3.0	2.9±0.8a
Mean		2.0±0.5B	3.2±0.7A	2.9±0.6A	
Basalsegments	0.00	1.5	1.2	1.2	1.3±0.2a
	0.12	1.5	1.3	1.2	1.3±0.2a
	0.25	1.4	1.2	1.2	1.3±0.2a
	0.50	1.3	1.2	1.3	1.3±0.3a
Mean		1.4±0.3A	1.2±0.1B	1.2±0.2B	

Means ± SD, n = 5. Means within a column followed by the same lower case letter and means within a line followed by the same upper case letter do not differ significantly according to Tukey's test (P ≤ 0.05).

of *P. taeda* must have an endogenous level of cytokinins sufficient to promote induction. Addition of high concentrations of cytokinins to the culture medium can cause necrosis or even a low rate of development of axillary shoots, as was observed by Lapp et al. (1996) in *P. monticola*. According to the study of Baxter et al. (1989) for *P. oocarpa*, *P. caribaea* and *P. tecunumanii*, there was multiplication of axillary buds even in low concentrations of cytokinins (0.1 to 5.0 µM BAP) and levels above 1 µM were not recommended, thus avoiding high concentrations such as those used in other micropropagation techniques such as organogenesis.

In general, the low concentrations of BAP tested in this study did not influence elongation (Table 4). The high con-

Table 4. Percentage of elongation of apical segments of *Pinus taeda* inoculated into culture medium WV3 supplemented or not with BAP, after ([a]) four weeks and ([b]) eight of culture.

BAP(µM)	Initial culture[a]	1st Subculture[b]	2nd Subculture[b]	Mean
0.00	31.2	23.6	39.1	31.3±9.2a
0.12	30.9	24.4	33.8	29.7±6.7a
0.25	29.8	19.7	27.4	25.6±7.3a
0.50	29.9	23.6	31.5	28.4±5.1a
Mean	30.4±5.2A	22.8±6.0B	33.0±6.8A	

Means ± SD, n = 5. Means within a column followed by the same lower case letter

Table 5. Effect of culture medium and concentrations of NAA and BAP on adventitious rooting of *Pinus taeda* shoots after 10 weeks of culture.

Root medium	Rooting (%)	Average number of roots per shoot
GDm/2 – control group (without PGR)	16.7±15.1a	1.3
GDm/2 – 0.54 µM NAA and 0.04 µM BAP	18.8±17.2a	1.5
GDm/2 – 0.54 µM NAA and 0.22 µM BAP	14.6±9.4a	1.4
GDm/2 – 0.54 µM NAA and 0.44 µM BAP	22.9±16.6a	1.7
GDm/2 – 2.69 µM NAA and 0.44 µM BAP*	10.4±12.3a	1.3
WA – 2.69 µM NAA and 0.44 µM BAP*	40.0±22.9a	1.6

*Period of induction of 9 days followed by transfer to GDm/2 culture PGR-free medium. Means ± SD, n = 6. Means in the column followed by different letters are different according to Tukey's test ($P \leq 0.05$).

concentrations of cytokinins can promote high development of buds, but inhibit shoot elongation (Žel et al., 1988).

The highest rooting rate (40%) was observed in WA culture medium supplemented with 2.69 µM NAA and 0.44 µM BAP for 9 days, followed by transfer into GDm/2 medium for 10 weeks, although Tukey's test showed that this average did not differ significantly from those obtained with other combinations of plant growth regulators (Table 5). For *P. sylvestris* root induction, Žel et al. (1988) also recommended a medium composed of water and agar, but in pulse treatment with 53.8 µM NAA for 24h. For *P. virginiana,* Chang et al. (1991) obtained lower rooting rates (29.2%) than in this study using the same combination of plant growth regulators.

GDm/2 culture medium supplemented with 0.54 µM NAA and 0.04-0.44 µM BAP was not efficient for root induction (14-23% rooting) (Table 5). However, Mehra-Palta et al. (1978) obtained 50% rooting with the same combinations in *P. taeda*. It is possible that these differences were due to the use of different families or different genotypes. In this work the sources of explants were seedlings from a clonal commercial orchard. Some genotypes tend to have better responses than others and rooting of forest species usually presents a great variability among different selected families (Foster, 1990; Scaltsoyiannes et al., 1994; Tang and Ouyang, 1999; Cuesta et al., 2008).

Within four and five weeks of culture, roots emerged at the base of some shoots while other shoots rooted after ten weeks of culture (Figure 3). This rooting rate in this period of time is relatively good when compared to other studies with *Pinus* that showed rooting varying between 40 and 50% in the same period (Cuesta et al., 2008; Stojičić et al., 1999; Watt et al., 1998; Gladfelter and Phillips, 1987; Scaltsoyiannes et al., 1994; Stiff et al., 1989). The mean number of roots per shoot was similar for all treatments, around 1.5 (Table 5).

Adventitious rooting occurred directly from the stem (Figure 1e) or indirectly from the callus (Figure 1f). The roots originated either from the cambium cells (Figure 2a), as observed in *Castanea sativa* (Ballester et al., 1999), from meristematic cells (Figure 2c and d), or from callus developed at the base of the shoots (Figures 2e and f), which seems to be common in *Pinus* species, as reported in *P. monticola* (Stiff et al., 1989), *P. pinaster* (Alvarez et al., 2009) and *P. pinea* (Cuesta et al., 2008).

The percentage of roots formed directly from the stem or indirectly from a callus (Figure 1e and f) varied according to rooting treatment (Figure 4). In the most efficient treatment, 58.3% of the roots were formed directly and 41.7% indirectly. This can be due to the duration of auxin treatment, which can promote or inhibit rooting for some species or even induce the development of structures that look swollen or calluses at the shoot base (Gladfelter and Phillips, 1987; Lin et al., 1991). Mott and Amerson

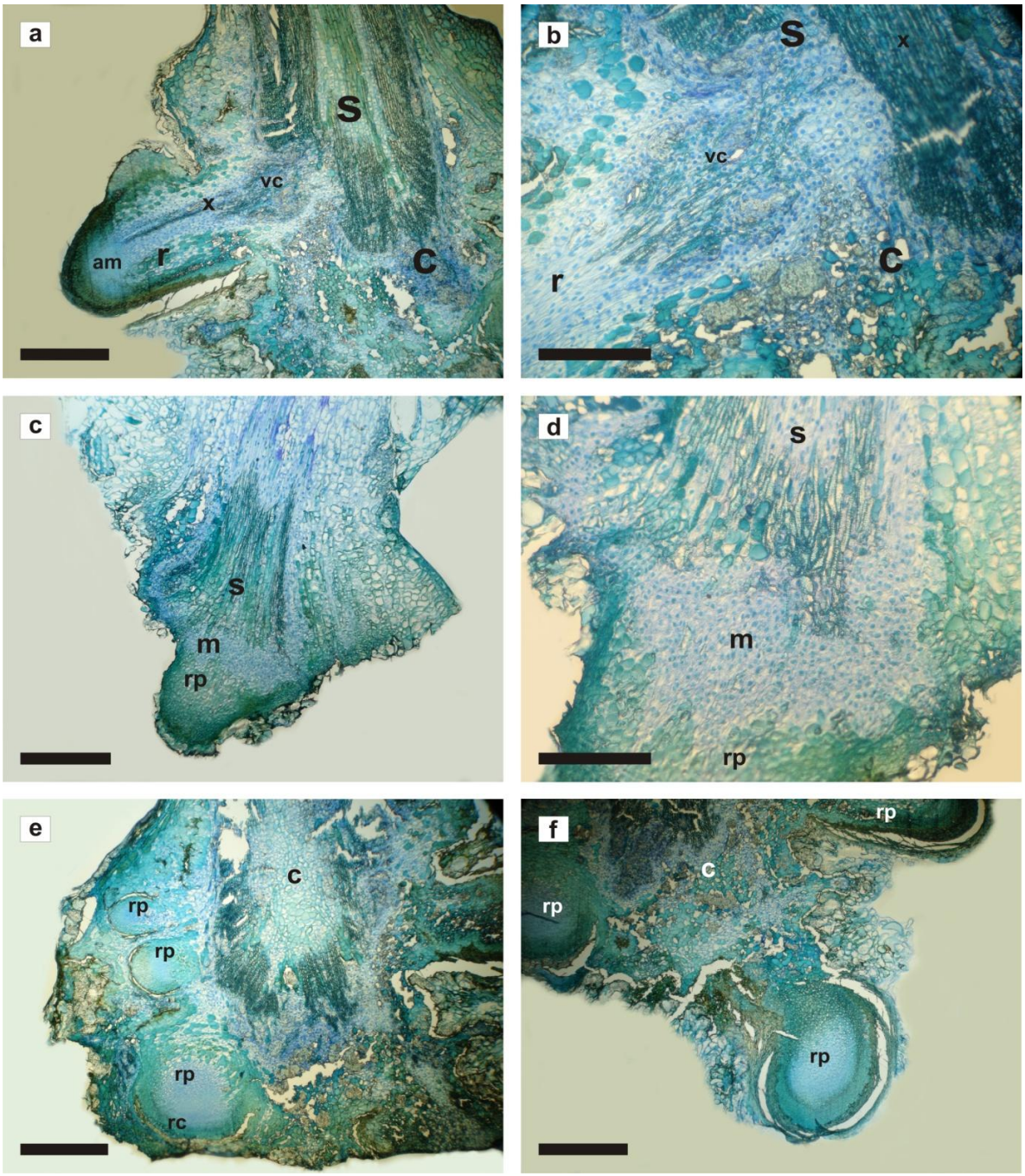

Figure 2. Anatomical study of *Pinus taeda* L. adventitious root formation *in vitro*. (**a**) and (**b**) root developed *in vitro* formed from cambium cells with vascular connection; (**c**) and (**d**) root formed directly from stem with an adventitious meristem that precedes the root; (**e**) and (**f**) roots formed indirectly from a callus. *Bars* **a**, **c**, **e** and **f** = 1 mm; **b** and **d** = 500 µm. *am* apical meristem, *c* callus, *m* meristematic cells, *r* root, *rc* root cap, *rp* root primordium, *s* stem, *vc* vascular connection, *x* xylem

(1981) also found a protuberance at the base of *P. taeda* explants, a disruption of the epidermis and the formation of a callus before the emergence of roots (Figure 2e and f). Callus formation at the stem basis did not prevent rooting.

When the roots formed directly from the stem, cells with meristematic characteristics were observed (Figure 2c and d). They have prominent nuclei, dense cytoplasm and

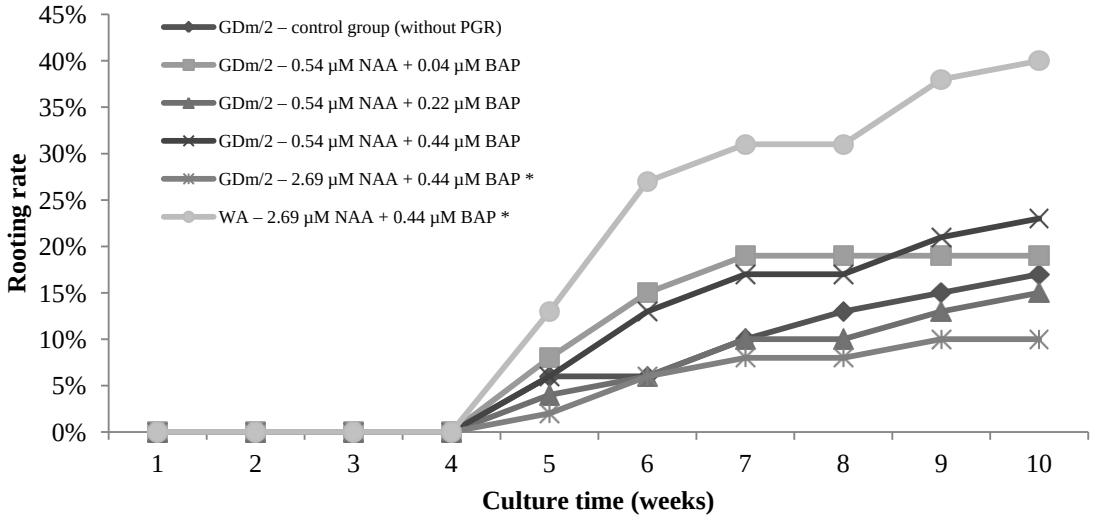

Figure 3. Effect of combinations of NAA and BAP and culture medium on adventitious rooting of *Pinus taeda* shoots. GDm/2, Gresshoff and Doy modified by Mehra-Palta et al. (1978), with salts reduced by half; WA, water and agar 5.6 g L^{-1}. *Induction period of 9 days followed by transfer to growth-regulator-free GDm/2 medium.

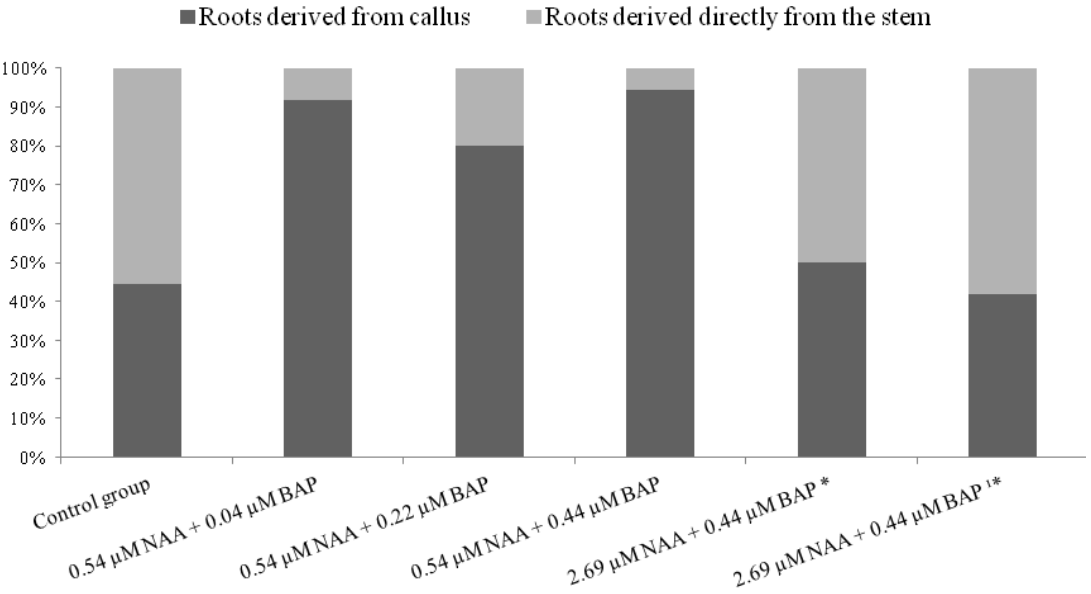

Figure 4. Effect of combinations of NAA and BAP and culture medium on the percentage of roots formed from a basal callus or directly from the stem of *Pinus taeda* shoots. The explants were inoculated into GDm/2, Gresshoff and Doy modified by Mehra-Palta et al. (1978), with salts reduced by half, except ([1]), cultured in WA (water and agar 5.6 g L^{-1}). *Induction period of 9 days followed by transfer to growth-regulator-free GDm/2 medium.

thin cell walls. The presence of the root cap and apical meristem is also observed. The vascular connection between root and stem is not present in the initial stage of root formation, but only in most developed roots (de Oliveira et al., 2012). A continuous connection of the vascular system between root and shoot is essential for the survival of micropropagated plants, as reported for *P. eldarica* (Wagley et al., 1987).

Roots formed from cambium cells have vascular connections through tracheids arising from the procambium and cambium (Figure 2a and b), unlike roots formed from peripheral cells of the callus, where the tracheids are formed

from the differentiation of parenchyma and cells with a high division rate, as described by Hamann (1998) and de Oliveira et al. (2012) in cuttings and microcuttings of *P. taeda*, respectively. This differentiation of parenchyma cells in tracheids, also observed in *P. pinea* hypocotyl (Kalev and Aloni, 1998), may be due to the action of auxin.

When formed indirectly (Figure 2e and f), roots can be derived from peripheral cells of the callus, as observed by de Oliveira et al. (2012) or from cambium cells, after emerging from callus, as described by Hamann (1998).

The micropropagated plants showed a high survival rate, irrespective of root origin, with 90% of transplanted seedlings surviving after 60 days in the greenhouse (Figure 1g). The plants showed good adaptation to acclimatization and the results were better than those found by Leach (1979), where 38% of micropropagated plants of *P. taeda* died after 5 weeks of acclimatization.

The acclimatized plants showed a satisfying number of roots per plant (4 secondary roots larger than 1.5 cm per plant) (Figure 1h), as observed by de Oliveira et al. (2012). According to the study of Anderson et al. (1992), this result is an essential factor for plant survival during the acclimatization, because plants with fine or little branched roots may not absorb nutrients well.

In this study, it can be concluded that the micropropagation of *P. taeda* by axillary buds is feasible. The explant type affected the establishment of *in vitro* cultures and induction of multiple shoots. Use of both explant types is recommended for increasing the multiplication rate. In general, the addition of BAP to culture medium did not affect the shoot induction or elongation responses. For adventitious rooting, the culture of shoots for 9 days in a culture medium composed of water and agar supplemented with 2.69 µM NAA and 0.44 µM BAP is recommended, followed by transfer to GDm/2 culture medium for root development. The callus presence did not inhibit rooting as the formation of the roots can occur from various parts of the callus or directly from the shoot base, and it did not affect acclimatization.

ACKNOWLEDGMENTS

The authors are grateful to Battistella Florestal Company for financial assistance, to FINEP (Financiadora de Estudos e Projetos) and to REUNI Program (Reestruturação e Expansão das Universidades Federais) for providing a grant to Leandro de Oliveira, to Tatiana Mazon Cezar for help and suggestions in the adventitious rooting step and Eileen Bagyary for editing the manuscript.

REFERENCES

Abdullah AA, Grace J, Yeoman MM (1989). Rooting and establishment of Calabrian pine plantlets propagated *in vitro*: Influence of growth substances, rooting medium and origin of explant. New Phytol. 113:193-202.

Alcantara GB, Ribas LLF, Higa AR, Ribas KCZ, Koehler HS (2007). Effect of seedling age and season on rooting of *Pinus taeda* L. minicuttings. Revista Árvore 31:399-404.

Álvarez JM, Majada J, Ordás J (2009). An improved micropropagation protocol for maritime pine (*Pinus pinaster* Ait.) isolated cotyledons. For. 82:175-184.

Anderson AB, Frampton LJJr, McKeand SE, Hodges JF (1992). Tissue-culture shoot and root system effects on field performance of loblolly pine. Can. J. For. Res. 22:56-61.

Andrejow GMP, Higa AR (2009). Rooting potentiality of *Pinus taeda* L. mini-cuttings collected from apical coppicing of young seedlings. Floresta 39:897-903.

Apter RC, Davies FTJr, McWilliams EL (1993a). *In vitro* and *ex vitro* adventitious root formation in Asian Jasmine (*Trachelospermum asiaticum*) II. Physiological comparisons. J. Am. Soc. Hort. Sci. 118:906-909.

Apter RC, McWilliams EL, Davies FTJr (1993b). *In vitro* and *ex vitro* adventitious root formation in Asian Jasmine (*Trachelospermum asiaticum*) I. Comparative morphology. J. Am. Soc. Hort. Sci. 118:902-905.

Ballester A, San-José MC, Vidal N, Fernández-Lorenzo JL, Vieitez AM (1999). Anatomical and biochemical events during *in vitro* rooting of microcuttings from juvenile and mature phases of chestnut. Ann. Bot. 83:619-629.

Baxter R, Brown SN, England NF, Ludlow CHM, Taylor SL, Womack RW (1989). Production of clonal plantlets of tropical pine in tissue culture via axillary shoot activation. Can. J. For. Res. 19:1338-1342.

Bergmann BA, Stomp AM (1994). Effect of genotype on rooting of hypocotyls and *in vitro*-produced shoots of *Pinus radiata.* Plant Cell Tiss. Org. Cult. 39:195-202.

Berlyn GP, Miksche JP (1976). Botanical microtechnique and cytochemistry. Iowa State University Press, Iowa.

Bonga JM, Klimaszewska KK, Aderkas PV (2010). Recalcitrance in clonal propagation, in particular of conifers. Plant Cell Tiss. Org. Cult. 100:241-254.

Chang S, Sen S, McKinley CR, Aimers-Halliday J, Newton RJ (1991). Clonal propagation of Virginia Pine (*Pinus virginiana* Mill.) by organogenesis. Plant Cell Rep. 10: 131-134.

Coke JE (1996). Basal nutrient medium for *in vitro* cultures of loblolly pines. USA Patent 5.534.434. http://www.freepatentsonline.com/5534434.pdf. Accessed 20 June 2011.

Cuesta C, Ordás RJ, Fernández B, Rodríguez A (2008). Clonal micropropagation of six selected half-sibling families of *Pinus pinea* and somaclonal variation analysis. Plant Cell Tiss. Org. Cult. 95:125-130.

de Oliveira LF, Ribas LLF, Quoirin M, Kehler HS, Amano E, Higa AR (2012). Micropropagation of *Pinus taeda* L. from Juvenile Material. Tree For. Sci. Biotech. 6:96-101.

DeVerno LL, Park YS, Bonga JM, Barret JD (1999). Somaclonal variation in cryopreserved embryogenic clones of white spruce (*Picea glauca* (Moench) Voss.). Plant Cell Rep. 18: 948-953.

El-Nil AMM (1982). Method for asexual reproduction of coniferous trees 1982. U.S. Patent 5.353.184. http://www.freepatentsonline.com/4353184.pdf. Accessed 10 August 2011.

Foster GS (1990). Genetic control of rooting ability of stem cuttings from loblolly pine. Can. J. Bot. 20:1361-1368.

Frampton LJJr, Amerson HV, Leach GN (1998). Tissue culture method affects *ex vitro* growth and development of loblolly pine. New For. 16:125-138.

George EF (1996) Plant propagation by tissue culture. Exegetics Press, Edington, England.

George EF, Hall MH, De Klerk GJ (2008) Plant Propagation by Tissue Culture. Springer Press, Dordrecht, The Netherlands.

Gladfelter HJ, Phillips GC (1987). De novo shoot organogenesis of *Pinus eldarica* Medw. *in vitro* I. Reproducible regeneration from long-term callus cultures. Plant Cell Rep. 6: 163-166.

Govil S, Gupta SC (1997). Commercialization of plant tissue culture in India. Plant Cell Tiss. Org. Cult. 51 65-73.
Gupta PK, Durzan DJ (1985). Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). Plant Cell Rep. 4:177-179.
Gupta PK, Durzan DJ (1991). Loblolly pine (*Pinus taeda* L.). In: Bajaj, Y.P.S. (ed): Biotechnology in Agriculture and Forestry, Trees III, vol 16, Springer, Verlag Press, Berlin, pp. 387-407.
Hamann A (1998). Adventitious root formation in cuttings of loblolly pine (*Pinus taeda* L.): developmental sequence and effects of maturation. Trees 12:175-180.
Hartmann HT, Kester DE, Davies FTJr, Geneve RL (2002). Plant Propagation: Principles and practices. Prentice Hall, Pennsylvania.
Hicks GS (1987). Adventitious rooting of apple microcuttings *in vitro*: an anatomical study. Can. J. Bot. 65: 1913-1920.
Jang JC, Tainter FH (1991). Micropropagation of shortleaf, Virginia and loblolly pine x shortleaf pine hybrids via organogenesis. Plant Cell Tiss. Org. Cult. 25:61-67.
Kalev N, Aloni R (1998). Role of auxin and gibberelin in regenerative differentiation of tracheids in *Pinus pinea* seedlings. New Phytol. 138:461-468.
Lapp MS, Malinek J, Coffey M (1996). Microculture of western white pine (*Pinus monticola*) by induction of shoots on bud explants from 1-to-7-year-old-trees. Tree Physiol. 16:447-451.
Leach GN (1979). Growth in soil of plantlets produced by tissue culture. Tappi J. 62:59-61.
Lin Y, Wagner MR, Heidmann LJ (1991). *In vitro* formation of axillary buds by immature shoots of Ponderosa pine. Plant Cell Tiss. Org. Cult. 26:161-166.
Majada J, Martínez-Alonso C, Feito I, Kidelman A, Aranda I, Alía R (2011). Mini-cuttings: an effective technique for the propagation of *Pinus pinaster* Ait. New For. 41:399-412.
McKellar DS, Herman B, White MP (1994). Towards a protocol for the micropropagation of *Pinus patula*. South Afr. For. J. 171:33-41.
Mehra-Palta A, Smeltzer RH, Mott RL (1978). Hormonal control of induced organogenesis experiments with excised plant parts of loblolly pine. Tappi J. 61:37-40.
Menzies MI, Aimers-Halliday JA (1997). Propagation options for clonal forestry with *Pinus radiata*. In: IUFRO '97 Genetics of Radiata Pine, 1-5 December, Rotorua, New Zealand, pp. 256-263.
Mott RL, Amerson HV (1981). A tissue culture process for the clonal production of loblolly pine plantlets. Tech. Bull. North Carolina Agric. Res. Serv. 271:3-14.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
Nandwani D, Kumaria S, Tandon P (2001). Micropropagation of *Pinus kesiya* Royle *ex* Gord (Khasi pine). Gartenbauwissenschaft 66: 68-71.
O'Brien TP, Feder N, McCully ME (1965). Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59:368-373.
Scaltsoyiannes S, Panetsos K, Economou A, Tsoulpha P (1994). Micropropagation of the pine hybrid *Pinus brutia* (Ten) x *Pinus halepensis* (Mill) by culturing fascicle shoots. Ann. For. Sci. 51:175-182.
Schestibratov KA, Mikhailov RV, Dolgov SV (2003). Plantlet regeneration from subculturable nodular callus of *Pinus radiata*. Plant Cell Tiss. Org. Cult. 72:139-146.
Schultz RI (1997). Genetics and tree improvement. In: Schultz, R.I. (ed) Loblolly pine: the ecology and culture of loblolly pine (*Pinus taeda* L.). U.S. Department of Agriculture, Forest Service, New Orleans, pp.1-50.
Selle GL, Schneider PR, Finger CAG (1994). Site classification for *Pinus taeda* L., across of the dominant height, by the region of Cambará do Sul, RS, Brazil. Ciência Florestal 4:77-95.
Souza CAM, Chassot T, Finger CAG, Schneider PR, Fleig FD (2008). Taper function for assortment of *Pinus taeda* L. stem. Ciência Rural 38:2506-2511.
Stiff CM, Wenny DL, Dumroese RK (1989). Establishment of western white pine shoots *in vitro* using needle fascicles. Can. J. For. Res. 19:1330-1333.
Stojičić D, Budimir S, Ćulafić L (1999). Micropropagation of *Pinus heldreichii.* Plant Cell Tiss. Org. Cult. 59:147-150.
Tang W (2000). Micropropagation of loblolly pine by somatic organogenesis and RAPD analysis of regenerated plantlets. J. For. Res. 11:1-6.
Tang W, Guo Z (2001). *In vitro* propagation of loblolly pine via direct somatic organogenesis from mature cotyledons and hypocotyls. Plant Growth Regul. 33:25-31.
Tang W, Ouyang F (1999). Plant regeneration via organogenesis from six families of loblolly pine. Plant Cell Tiss. Org. Cult. 58:223-226.
Tang W, Ouyang F, Guo Z (1998). Plant regeneration through organogenesis from callus induced from mature zygotic embryos of loblolly pine. Plant Cell Rep. 17:557-560.
Wagley LM, Gladfelter HJ, Philips GC (1987). De novo shoot organogenesis of *Pinus eldarica* Medw. *in vitro* II. Macro- and micro-photographic evidence of de novo regeneration. Plant Cell Rep. 6:167-171.
Watt MP, Ramgareeb S, Hope B, Blakeway FC, Denison NP (1998). Micropropagation via axillary bud proliferation from seedlings and juvenile shoots of *Pinus patula* Schiede *et* Deppe. Southern Afr. For. J. 181:1-5.
Žel J, Gogala N, Camloh M (1988). Micropropagation of *Pinus sylvestris*. Plant Cell Tiss. Org. Cult.14:169-175.