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# Micropropagation of *Pinus taeda* L. from Juvenile Material

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## ABSTRACT

The purpose of this study was to develop a protocol for the micropropagation of *Pinus taeda* from juvenile material. Apical shoots and nodal segments were inoculated into MS, DCR or WV medium. After 90 days, the explants were transferred to WV5 medium supplemented or not with 6-benzyladenine (BA) (2.0 μM) in order to induce multiple shoot formation. For root induction, a medium composed of water and agar and a combination of 1-naphthaleneacetic acid (NAA) (2.69 μM) and BA (0.44 μM) was used for periods of 7, 9 or 12 days followed by transfer to growth regulator-free GDm/2 or GDm/4 medium. During *in vitro* establishment, nodal segments showed better responses than apical shoots, with an average of 4.3 to 5.8 shoots per explant after 90 days of culture. WV5 medium proved better than all other media due to a higher survival rate (86%) and higher elongation percentage (85.2%). BA did not promote better multiplication compared to the control, with approximately 2.4 to 3.0 shoots per explant. The alternate use of BA concentrations (2.00, 0.25 and 1.00 μM in each subculture) in WV5 culture medium can increase the multiplication rate. The estimated production was 7530 shoots from 100 explants in 9 months of culture. The best rooting percentage (47.5%) was obtained when shoots were inoculated in a medium with 2.69 μM NAA and 0.44 μM BA for 12 days. In the roots derived from calluses, the vascular connection was established when roots were longer than 0.6 cm and this size was recommended as the minimum for transplanting. Acclimatized plants showed 90% survival after 90 days. It can be concluded that micropropagation of *P. taeda* from axillary buds excised from seedlings is feasible.

**Keywords:** adventitious roots, apical shoots, axillary buds, loblolly pine, nodal segments, vascular connection
**Abbreviations: AA**, culture medium composed of water and agar; **BA**, 6-benzyladenine; **NAA**, 1-naphthaleneacetic acid; **RED**, root expression and development

## INTRODUCTION

Loblolly pine (*Pinus taeda* L.) is one of the most important species for timber production. In Brazil, most of the forestry products are obtained from *Pinus* and *Eucalyptus* plantations, reducing the pressure imposed on natural forests, especially the Amazon and Araucaria Forests (Selle *et al.* 1994; Souza *et al.* 2008). The main propagation method for *P. taeda* has been by seed, since the rooting of cuttings depends on the season of the year or on the availability of juvenile material (Alcantara *et al.* 2007; Andrejow and Higa 2009). The large-scale production of seedlings in a short period of time is important for breeding programs of forest species (Mott *et al.* 1977; Menzies and Aimers-Halliday 1997; Watt *et al.* 1998). The advantage of micropropagation techniques is the high rate of multiplication, allowing mass propagation of selected genotypes useful for tree improvement and the capture of genetic gains (Gupta and Durzan 1991; Govil and Gupta 1997; Menzies and Aimers-Halliday 1997).

Micropropagation from axillary buds is a simpler technique compared to others, because in addition to using preformed meristems, it allows greater genetic stability, less somaclonal variation and also avoids using high concentrations of cytokinins for the development of axillary buds (Abdullah *et al.* 1986; Baxter *et al.* 1989).

In published research with *P. taeda*, most studies used indirect organogenesis. Use of the direct process was less frequent. The best responses were obtained when juvenile material was used as the explant source, such as: various parts of the embryo (Mehra-Palta *et al.* 1978), zygotic embryos (Tang *et al.* 1998; Tang and Ouyang 1999; Tang 2000; Tang and Guo 2001), cotyledons (Mott and Amerson 1981; Newton *et al.* 1989; Jang and Tainter 1991; Frampton *et al.* 1998; Rahman *et al.* 2003), epicotyls (Frampton *et al.* 1998) and apical meristems (Dhumale and Newton 1996).

The success of micropropagation techniques can be influenced by the choice of salts and organic compounds that compose the culture medium. Some of the most tested culture media for *P. taeda* were modified GD (Mehra-Palta *et al.* 1978), SH (Schenk and Hildebrandt 1972) and DCR (Gupta and Durzan 1985) media. WV5 and WV3 culture media were specially developed with combinations of salts and organic compounds to optimize the *in vitro* responses of *P. taeda* (Coke 1996a, 1996b).

For *Pinus*, the cytokinin 6-benzyladenine (BA) is commonly used in the stage of shoot induction, with good responses. For *P. taeda*, some studies have reported the use of BA at a concentration of 6.66 μM combined with 4.56 μM zeatin with 80 to 100% of explants forming shoots and also the combination of 8.87 μM BA with 2.46 μM AIB resulting in an average of 32 shoots from each embryo used (Mehra-Palta *et al.* 1978; Tang and Ouyang 1999).

Conifers are considered difficult to root and this has been confirmed for *P. taeda*, when traditional techniques of vegetative propagation are used as well as *in vitro* rooting (Mott and Amerson 1981). According to Bergmann and Stomp (1994), in *Pinus*, the success of rooting may depend on species, family and clone, with great variability among the responses.

In *Pinus*, the combination of an auxin with a cytokinin, mainly BA, is commonly used to promote rooting. Mott and Amerson (1981) recommended the combination of 2.69 μM NAA and 0.44 μM BA added to GDm (Mehra-Palta *et al.* 1978) culture medium for *P. taeda* rooting. Bairu *et al.* (2009) reported that cytokinin present in the rooting

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medium can also prevent apical necrosis.

*In vitro*-formed roots often are not functional and die after transplanting. Some roots can be formed directly from the stem or emerge from calluses. In the latter case, 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 (George *et al.* 2008). An anatomical study is needed to monitor the development of these vascular connections between root and shoot and for the selection of shoots rooted at the time of transplantation.

Due to the great importance of this species and the difficulty of obtaining large-scale plants by traditional techniques of vegetative propagation, the purpose of this study was to develop a protocol for the micropropagation of *P. taeda* from juvenile material.

## MATERIALS AND METHODS

### Plant material

Two- to four-month-old seedlings, from a commercial clonal orchard located in Rio Negrinho, Santa Catarina, Brazil, were kept in a greenhouse for 30 days and treated with 1 g $L^{-1}$ Cercobin® (a fungicide) every two days.

### *In vitro* establishment

Apical shoots and nodal segments, approximately 3.0 cm long, were collected from the apical portion of the plants and 90% of the needles were cut. The explants were disinfested by immersion in 0.05% $HgCl_2$ for 5 min, followed by 0.5% NaOCl for 5 min, with constant agitation. Tween 20 CRQ® (Cromato Produtos Químicos) 0.1% was added to the disinfecting solutions. After disinfection, the explants were rinsed three times in sterile distilled water.

The explants were inoculated vertically in 15.0 cm × 2.5 cm test tubes containing 10 mL of MS (Murashige and Skoog 1962), DCR (Gupta and Durzan 1985), WV3 (Coke 1996b) or WV5 (Coke 1996a) culture medium supplemented with 30 g $L^{-1}$ sucrose and solidified with 5.6 g $L^{-1}$ bacteriological Himedia® agar.

After 12 weeks of culture the following parameters were evaluated: the average number of lateral shoots per explant (final number of shoots / initial number of explants), average number of segments (1.0 to 1.5 cm long) per shoot and percentage of elongation rate.

The experimental design was completely randomized in a factorial arrangement of two types of explants and four culture media for the variable average number of shoots per explant. Each treatment consisted of five replications of 10 explants per replicate (n = 50) and one explant per test tube.

### Induction of multiple shoots

After 90 days, the explants established *in vitro* were divided into 1.0 to 1.5 cm long segments and transferred to flasks of 12.5 cm × 6.2 cm, containing 40 mL of WV5 medium, supplemented with BA (0, 0.25, 0.50, 1.00 or 2.00 μM), 30 g $L^{-1}$ sucrose and 5.6 g $L^{-1}$ bacteriological Himedia® agar.

Two subcultures were performed after 8 weeks on the same culture medium with the same concentrations of BA. At the end of each subculture the following parameters were evaluated: percentage of explants with axillary buds, average number of shoots per explant and percentage of elongation rate.

The experimental design was completely randomized, in split plots with four explants per flask, three flasks per replicate and seven replicates per treatment (n = 84). Four BA concentrations were compared in the plots and three subcultures in the subplots.

### *In vitro* rooting

Individual shoots (1.5 to 2.0 cm high) with a double bevel cut at the base, were inoculated into induction medium composed of water and 5.6 g $L^{-1}$ agar (AA), supplemented with 2.69 μM NAA and 0.44 μM BA for a period of 7, 9 or 12 days. After the induction period, explants were transferred to RED medium: AA medium or GDm (Gresshoff and Doy modified by Mehra-Palta *et al.* 1978) with salts reduced by half (GDm/2) or a quarter (GDm/4) and plant growth regulator (PGR)-free. The GDm/2 and GDm/4 media contained 20 g $L^{-1}$ sucrose and all media were solidified with 5.6 g $L^{-1}$ bacteriological Himedia® agar. The control group was not transferred to the induction medium and the shoots were inoculated directly into the RED medium, cited above.

The experimental design was completely randomized in a 2 × 4 factorial arrangement (AA medium was not subjected to statistical analysis, because the shoots did not have been root) and four explants in each flask, two flasks per replicate and five replicates per treatment (n = 40).

After 10 weeks of culture, the percentage of rooted shoots, the average number of roots per shoot and the percentage of roots developed from callus or directly from the stem were evaluated.

### Acclimatization

Shoots (1.5 to 2.5 cm high) 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 plastic bag, for 60 days. The plants were irrigated manually 4-5 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.

### Statistical analysis

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

#### 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. The *in vitro* cultures were maintained in a growth room with a temperature of 19 ± 2°C (night) and 28 ± 2°C (day), and a 16-h photoperiod under white fluorescent light (PFD = 40 $\mu mol.m^{-2}.s^{-1}$). For transplant and acclimatization, the plants were maintained in a greenhouse under shade, with temperatures ranging from 20 ± 7°C in October/2010 and 26 ± 8°C during November and December/2010.

### Anatomical study of the roots originated from calluses

Samples were collected from roots obtained *in vitro* 0.2 and 0.6 cm in length, developed from calluses. 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 serial sections were performed on a rotation microtome RM2145 (Leica Microsystems, Germany) with 7 μm thickness 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

### *In vitro* establishment

The explants inoculated in WV5 medium had a higher survival rate for both apical shoots (86%) and nodal segments (76%) compared to those cultured on WV3, DCR and MS. Better results from explants cultured in WV5 were also found for *P. uncinata*, when compared to WPM and MS media (Vejsadová and Lukášová 2010).

According to Coke (1996b), the balance of salts in WV5 and WV3 media favors an optimal development of *in vitro* cultures of *P. taeda*. The WV3 and WV5 media have higher amounts of B, Ca, Mg, S, Cl and P than DCR and MS media, a lower amount of Mn than these media and a lower amount of nitrogen than MS medium. The explants inoculated on MS medium showed the lowest survival rate

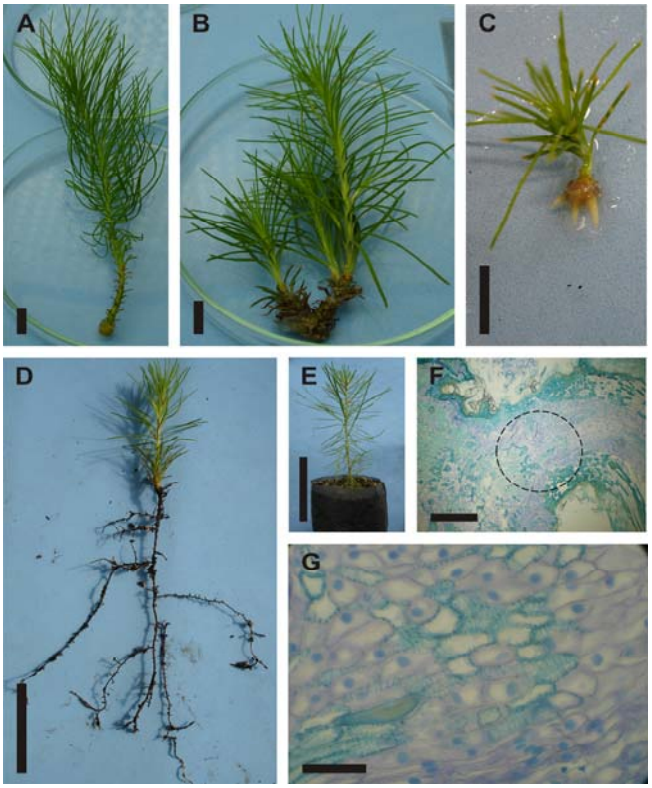


**Fig. 1 Micropropagation of *Pinus taeda* L. from juvenile material.** (**A**) Elongated apical shoot after 12 weeks of culture in WV5 medium (Bar: 1 cm). (**B**) Nodal segment after 8 weeks of culture on WV5 medium supplemented with 2.0 µM BA (Bar: 1 cm). (**C**) *In vitro* rooting showing roots derived from callus at the shoot base (Bar: 1 cm). (**D**) Acclimatized plants with well-developed roots after 90 days in greenhouse (Bar: 5 cm). (**E**) Transplanted plants growing in plastic bag with substrate in a greenhouse (Bar: 10 cm). (**F**) Root developed *in vitro* (0.6 cm long) with vascular connection established between root and callus (Bar: 1 mm). (**G**) Detail of the tracheids differentiated from parenchyma cells forming the vascular connection between root and callus (Bar: 100 µm).

**Table 1** Effect of culture medium and explant type on axillary shoots formation in *Pinus taeda*, after 90 days of *in vitro* culture.

| Culture medium | Average number of shoots per explant | |
|---|---|---|
| | Nodal segments* | Apical shoots* |
| WV5 | 5.0 ± 0.5 b A | 1.9 ± 0.4 a B |
| WV3 | 5.8 ± 0.5 a A | 2.0 ± 0.3 a B |
| DCR | 4.3 ± 0.3 c A | 1.2 ± 0.3 b B |
| MS | 5.5 ± 0.6 ab A | 1.6 ± 0.4 ab B |

* 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 at p≤0.05

**Table 2** Effect of different culture media on the elongation of apical shoots of *Pinus taeda*, after 90 days of culture.

| Culture medium | Elongation (%)* | Average number of segments per shoot* |
|---|---|---|
| WV5 | 85.2 ± 19.9 a | 5.8 ± 0.7 a |
| WV3 | 54.5 ± 7.8 b | 4.4 ± 0.6 b |
| DCR | 45.3 ± 14.0 bc | 3.6 ± 0.6 bc |
| MS | 26.8 ± 7.9 c | 3.3 ± 0.3 c |

* Means within a column followed by the same do not differ significantly according to Tukey's test at p≤0.05

**Table 3** Percentage of explants with axillary buds of *Pinus taeda* in WV5 medium supplemented or not with BA, after 8 weeks in each subculture.

| BA (µM) | Initial culture* | 1st subculture* | 2nd subculture* |
|---|---|---|---|
| 0.00 | 43.5 ± 24.1 ab A | 61.5 ± 13.6 a A | 62.0 ± 12.0 a A |
| 0.25 | 30.3 ± 22.8 b B | 77.5 ± 15.7 a A | 71.7 ± 15.9 a A |
| 0.50 | 56.5 ± 34.7 ab A | 68.0 ± 13.2 a A | 72.6 ± 19.5 a A |
| 1.00 | 61.2 ± 11.8 a A | 58.8 ± 14.1 a A | 62.6 ± 14.1 a A |
| 2.00 | 72.7 ± 13.5 a A | 72.3 ± 21.3 a A | 64.3 ± 26.2 a A |

* 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 at p≤0.05

for both types of explants, so this formulation was not used in later stages. High concentrations of N in MS medium may have a toxic effect in some species (George *et al.* 2008).

For the average number of shoots per explant there was a significant interaction between the "culture medium" and "explant type" factors. For nodal segments the average number of shoots per explant on WV3, WV5 and MS media was superior to the value obtained on DCR medium and, for apical shoots, WV3, WV5 media gave better results (**Table 1**). As to the explant type, the average number of shoots per nodal segment (4.3 to 5.8) was significantly higher than the number per apical shoots (1.2 to 2.0) for all culture media tested (**Table 1**). A similar result was also obtained by McKellar *et al.* (1994), when nodal segments and apical shoots of *P. patula* were compared. This suggests that *P. taeda* may contain some level of endogenous cytokinin that overcomes apical dominance and induces organogenesis of shoots from pre-formed meristems without the application of a growth regulator.

In addition to a greater survival rate, the shoots grown on WV5 medium had an elongation rate of 85.2% (**Fig. 1A**) which was significantly superior to the values obtained onWV3, DCR and MS media (**Table 2**). This made it possible to section the shoots into various segments (1.0 to 1.5 cm long), resulting in an average of six segments per shoot and consequently a larger number of explants at the end of multiplication stage. Thus, both types of explants were selected and used during the stage of multiple shoot induction. This strategy of sectioning the shoots proved efficient and was also described by Baxter *et al.* (1989), who achieved the elongation of the shoots in six weeks, followed by division into two or more nodal segments of 1.5 cm.

The highest percentage of elongation may have been promoted by the higher concentration of *myo*-inositol present in the WV5 and WV3 media, ten times higher than in the MS medium (Coke 1996a). In addition to being a source of carbohydrate, *myo*-inositol is also known to stimulate the growth of shoots (George *et al.* 2008). Moreover, the WV3 and WV5 media contain higher concentrations of thiamine (0.4 mg $L^{-1}$) than the DCR and MS media (0.1 mg $L^{-1}$). Thiamine is a cofactor in carbohydrate metabolism and is directly involved in the biosynthesis of amino acids, being the basic vitamin required by all plant tissues (Razdan 2003; George *et al.* 2008). The need for thiamine is particularly evident in low levels of cytokinins (Dodds and Roberts 1995).

## Induction of multiple shoots

In the initial culture, the highest percentages of explants with shoots were obtained with concentrations of 1.00 and 2.00 µM BA in WV5 medium, and these results only differed significantly from those obtained with 0.25 µM BA (**Table 3**). In the first and second subcultures, the percentages of explants with shoots were similar for the control and the four BA concentrations (**Table 3**). Similar responses were obtained in studies of other pine species, albeit with BA concentrations higher than those used in this work (up to 50 µM) (Gupta and Durzan 1985; Žel *et al.* 1988; Lin *et al.* 1991; McKellar *et al.* 1994; Lapp *et al.* 1996; Watt *et al.* 1998; Nandwani *et al.* 2001). In the control treatment 40 to 60% of *P. taeda* explants formed shoots during the two subcultures in WV5 medium. This again emphasizes the presence of an endogenous level of cytokinin in *P. taeda* sufficient to promote shoot induction. According to Vejsadová and Lukášová (2010), similar responses were obtained only with 22.19 µM BA in WV5 medium for *P. uncinata* explants.

The average number of shoots per control explant did

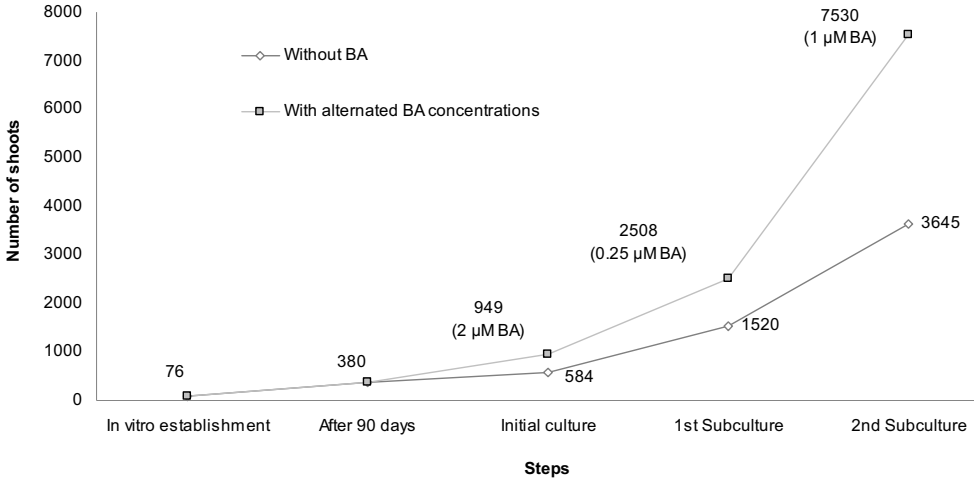


**Fig. 2 Estimated production of *Pinus taeda* shoots.** Number of shoots produced at the stage of *in vitro* establishment of induction of axillary shoots and during several subcultures in WV5 medium, with different concentrations of BA or without.

**Table 4** Average number of shoots per explant of *Pinus taeda* inoculated into culture medium WV5 supplemented or not with BA after 8 weeks in each subculture.

| BA (µM) | Initial culture | 1st subculture | 2nd subculture | Mean* |
|---|---|---|---|---|
| 0.00 | 2.0 | 2.8 | 2.6 | 2.4 ± 0.6 a |
| 0.25 | 1.6 | 3.0 | 3.0 | 2.5 ± 0.9 a |
| 0.50 | 2.2 | 2.9 | 3.1 | 2.7 ± 0.9 a |
| 1.00 | 2.3 | 2.4 | 3.2 | 2.6 ± 0.7 a |
| 2.00 | 2.8 | 3.0 | 3.2 | 3.0 ± 0.9 a |
| Mean* | 2.2 ± 0.7 B | 2.8 ± 0.6 A | 3.0 ± 0.8 A | |

* 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 at p≤0.05

**Table 5** Percentage of elongation of the apical shoots of *Pinus taeda* inoculated into culture medium WV5 supplemented or not with BA after 8 weeks in each subculture.

| BA (µM) | Initial culture* | 1st subculture* | 2nd subculture* |
|---|---|---|---|
| 0.00 | 47.8 ± 27.2 a B | 48.3 ± 19.3 a B | 155.3 ± 41.6 a A |
| 0.25 | 51.8 ± 21.3 a B | 49.7 ± 33.7 a B | 121.7 ± 28.2 a A |
| 0.50 | 54.7 ± 16.4 a B | 43.1 ± 27.7 a B | 133.7 ± 26.4 a A |
| 1.00 | 44.5 ± 18.9 a B | 48.0 ± 17.6 a B | 120.6 ± 25.9 a A |
| 2.00 | 45.7 ± 13.5 a AB | 37.1 ± 11.9 a B | 74.7 ± 23.6 b A |

* 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 at p≤0.05

not differ significantly from that obtained with BA concentrations (0.25 to 2.00 µM) in WV5 medium (**Fig. 1B**). However, the average number of shoots of the first and second subcultures was statistically higher than that of the initial culture (**Table 4**).

Although there was no significant difference between the mean numbers of shoots per explant in the different treatments, from a practical standpoint, with alternate use of BA (2.00, 0.25 and 1.00 µM) in each subculture, the production of shoots increased and reached an estimated value of 7350 shoots from 100 explants, in nine months of culture (**Fig. 2**). In this system, the number of shoots that can be obtained is twice the number obtained in the growth regulator-free WV5 medium (3645 shoots). These results are higher than those found by Baxter *et al.* (1989) who obtained until 1000 explants from 13 clones of *P. oocarpa* in one year.

Shoot elongation was obtained during multiplication and there was an interaction between the concentrations of BA and subcultures. The number of subcultures influenced the elongation of shoots, with the highest rates being obtained in the second subculture. However, in this subculture, some effect of BA was observed when comparing the concentration of 2.00 µM to other concentrations (0.25 to 1.00 µM) and control, as there was less elongation at 2 µM (**Table 5**). According to Žel *et al.* (1988), the highest concentrations of BA promote development of buds, but these do not elongate. Exposure to BA for long periods or at high concentrations can also inhibit the growth or development of shoots (Amerson *et al.* 1985; Halos and Go 1993; García-Férriz *et al.* 1994).

## *In vitro* rooting

No significant interaction was found between the period of root induction and the RED media tested. The first rooted shoots had formed their roots after five weeks of culture. The shoots submitted to induction treatment for 12 days in medium composed of water and agar plus 2.69 µM NAA and 0.44 µM BA followed by transfer to the GDm/2 medium, showed 47.5% rooting at the end of ten weeks of culture (**Table 6**). This result was similar to those described in other studies with *P. taeda*, which ranged from 30 to 50%. However, some authors only obtained roots by adding other regulators to the medium, such as $GA_3$ and IBA in addition to BA (Mehra-Palta *et al.* 1978; Tang *et al.* 1998; Tang and Ouyang 1999; Tang 2000; Tang and Guo 2001).

For RED of *P. taeda*, the salts of the GDm medium were necessary because shoots did not root when the culture medium consisted only of water and agar at this stage. The rooting percentages obtained in the GDm/2 and GDm/4 media were similar. However, when transferred to GDm/4,

**Table 6** Percentage of rooting *in vitro* of shoots of *Pinus taeda* after different periods of induction and culture in two media of root expression and development after 10 weeks.

| | Induction period [b] | | | | |
|---|---|---|---|---|---|
| RED medium [c] | Control [a] (%) | 7 days (%) | 9 days (%) | 12 days (%) | Mean (%)* |
| GDm/2 | 5.0 | 20.0 | 27.5 | 47.5 | 25.0 ± 21.8 a |
| GDm/4 | 15.0 | 30.0 | 22.5 | 27.5 | 23.8 ± 16.2 a |
| Mean (%)* | 10.0 ± 9.9 B | 25.0 ± 15.6 AB | 25.0 ± 14.4 AB | 37.5 ± 24.3 A | |

[a] Control treatment: without regulators
[b] Treatment with 2.69 µM NAA and 0.44 µM BA on induction medium with water and agar
[c] After the induction period, explants were transferred to the medium of expression and development, except the control treatment
* 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 at p≤0.05

**Table 7** Average number of roots per shoot in *Pinus taeda*, after different periods of induction and culture in two media of root expression and development, after 10 weeks.

| | Induction period[b] | | | | |
|---|---|---|---|---|---|
| **RED medium [c]** | **Control [a]** | **7 days** | **9 days** | **12 days** | **Mean** |
| GDm/2 | 1.5 | 1.4 | 1.8 | 1.6 | 1.6 |
| GDm/4 | 1.0 | 1.9 | 1.2 | 2.6 | 1.7 |
| Mean | 1.3 | 1.7 | 1.5 | 2.1 | |

[a] Control treatment: without regulators
[b] Treatment with 2.69 µM NAA and 0.44 µM BA on induction medium with water and agar
[c] After the induction period, explants were transferred to the root expression and development medium, except the control treatment

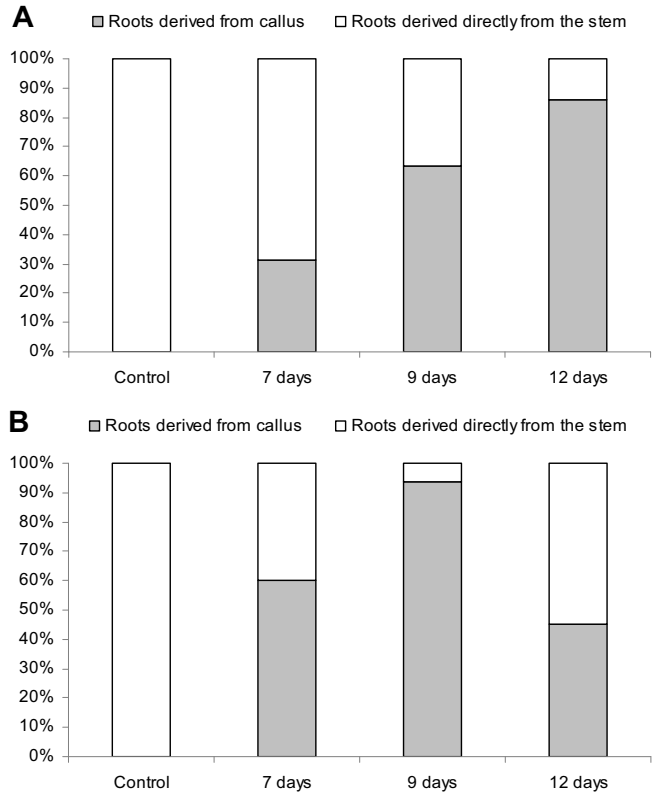


**Fig. 3 Effect of induction period and culture medium on the percentage of roots formed from basal callus or directly from the stem of *Pinus taeda* shoots.** After the induction period in medium composed of water, agar, 2.69 µM NAA and 0.44 µM BA, shoots were transferred into GDm/2 (**A**) or GDm/4 (**B**), for 10 weeks.

the RED culture medium, the shoots had an average of 2.6 roots per plant (**Table 7**). This result was better than those found in some studies with loblolly pine and other pine species, which showed average of one root per shoot (Tang *et al.* 1998; Tang and Ouyang 1999; Tang 2000; Schestibratov *et al.* 2003).

Root formation was direct and indirect. In the indirect process, callus formation preceded the emergence of roots, depending on the induction time and the RED medium used (**Fig. 1C, Figs. 3A-B**). This may be due to the time of induction of roots, or to the long treatment with auxin, which can increase or inhibit rooting, sometimes forming structures that look swollen or calluses at the shoot base (Gladfelter and Phillips 1987; Lin *et al.* 1991). In this study, that type of callus was observed; however, this translucent appearance occurred only at the beginning of its formation. These calluses darkened after a few weeks but did not prevent the formation of roots.

Mott and Amerson (1981) used the same regulator concentrations for 10 to 12 days in GDm/2 medium and 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. Callogenesis seems common in *Pinus* species. Stiff *et al.* (1989) reported that after 30 days of treatment with NAA, protuberances smaller than 0.5 cm without a defined cap emerged from a translucent white callus that formed at the base of *P. monticola* shoots and this occurred in 33% of explants. For *P. pinaster*, Álvarez *et al.* (2009) also reported that rooting was indirect, with the formation of calluses at the base of the shoots, as well as for *P. pinea* (Cuesta *et al.* 2008).

*In vitro* developed roots, when derived from the callus, were originated from peripheral cells. In micropropagated plants of *P. taeda* the vascular connection between stem and root, when derived from callus, is not formed in the early stage of development of the roots (0.2 cm long). This vascular connection was established only in roots longer than 0.6 cm (**Figs. 1F, 1G**). Wagley *et al.* (1987) reported that a continuous connection of the vascular system between root and shoot was essential for the survival of micropropagated plants of *P. eldarica*. According to Cuesta *et al.* (2008), the *in vitro* rooted shoots of *P. pinea* showed normal morphology with a well-developed root system and a vascular connection between stem and root.

Parenchyma cells differentiating and differentiated into tracheids were found between the vascular system of the callus and of the root (**Fig. 1G**). This type of differentiation of parenchyma cells was also observed in *P. pinea* hypocotyl under the action of auxin (Kalev and Aloni 1998). The tracheids differentiated from parenchyma showed reduced size and walls with thickening spiraled and bordered pits (**Fig. 1G**), as observed by Kalev and Aloni (1998). They may appear singly and not necessarily in a continuous pattern and develop laterally, as observed in this study.

## Acclimatization

Micropropagated plants of *P. taeda* reached an average of 90% of survival after 90 days in the greenhouse (**Fig. 1E**). This demonstrates that *P. taeda* has adapted well to transplantation and acclimatization in conditions used in this study when compared to other pine species whose survival is not always high (Stojičić *et al.* 1999). Leach (1979) reported that 38% of *P. taeda* plants usually died three to five weeks after transplantation.

The acclimatized plants showed a satisfying mean number of roots per plant with 4.6 roots longer than 1.5 cm per plant (**Fig. 1D**). This factor is essential for plant survival during acclimatization, because plants with fine or less branched roots may not be well suited for the absorption of nutrients (Anderson *et al.* 1992). This result is superior to that found by Abdullah *et al.* (1989) for *P. brutia* who obtained approximately 2 to 2.5 roots per plant. According to these authors, the low number of roots per plant may be due to the residual effect of high concentrations of BA, which was not observed in this study with *P. taeda*.

## CONCLUSION

In this study, a protocol for micropropagation of *P. taeda* via axillary buds was described. The WV5 culture medium was the best for *in vitro* establishment and induction of axillary shoots, both in apical and nodal segments, in addition to promoting higher elongation. Alternate concentrations of BA tend to increase the production of shoots. Rooting was achieved in two steps: root induction (on a medium composed of water and agar plus 2.69 µM NAA and 0.44 µM BA for 12 days) and root expression and development (on growth regulator-free GDm/2). The connection between the vascular system of root and callus is present in roots longer than 0.6 cm, this minimum size being recommended for transplanting, since plant acclimatization allowed a survival rate of 90%.

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