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INFLUENCE OF CULTURE MEDIUM, EXPLANT LENGTH AND GENOTYPE ON MICROPROPAGATION OF *Pinus taeda* L.

INFLUÊNCIA DO MEIO DE CULTURA, COMPRIMENTO DO EXPLANTE E GENÓTIPO NA MICROPROPAGAÇÃO DE *Pinus taeda* L.

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ABSTRACT

This work aimed to establish a micropropagation protocol for *Pinus taeda* L. Apical shoots from 5-day seedlings, of different genotypes (F27, B05 and PC), were cultured on WV_5 medium supplemented with 44 µM 6-benzylaminopurine (BAP) and 0.05 µM α-naphtaleneacetic acid (NAA), for 14 days, followed by subcultures on growth regulator-free medium. Explants were sectioned into apical shoots and nodal segments for multiplication. Different lengths of explants, BAP concentrations and cultivation periods were tested. Rooting was induced on WV_5/2, WV_3/2, GDm/2 and agar-water culture media supplemented with 2.68 µM NAA and 0.44 µM BAP for nine days, followed by a 4-week subculture on growth regulator-free medium. It was verified that genotype influenced shoot formation and rooting. The length of 1.0 cm is recommended for nodal segment explants to obtain a high number of axillary shoots. For apical shoots, 0.5 cm explant and WV_5 medium formulation allowed the best results for elongation. The best period of subculture was eight weeks, both for nodal segments and for apical shoots. The higher percentage of nodal segments with shoots (99.2%) and the higher average number of shoots per explant (4.0) were obtained with F27 genotype in medium culture containing 2.5 µM BAP. For apical shoots, the best result for elongation was observed for the shorter explants (0.5 cm) on WV_5 culture medium (218.3%). The maintenance of *in vitro* clonal micro garden of vigorous shoots was obtained for two years of 8-week subcultures on WV_5 culture medium. The best rooting rate (55.6%) was obtained when shoots were inoculated in agar-water medium with 2.68 µM NAA and 0.44 µM BAP for nine days. Plantlets were successfully acclimatized (85% of survival), so a micropropagation protocol was established.

Keywords: loblolly pine; *in vitro* culture; forestry species; agar-water.

RESUMO

O presente trabalho teve como objetivo estabelecer um protocolo de micropropagação de *Pinus taeda* L. Brotações apicais de plântulas de cinco dias de germinação, de diferentes genótipos (F27, B05 e PC) foram cultivadas em meio de cultura WV_5, acrescido de 44 µM de 6-benzilaminopurina (BAP) e de 0,05 µM de ácido α-naftalenoacético (ANA) durante 14 dias, seguido de quatro subcultivos para meio sem reguladores de crescimento. Explantes foram seccionados em brotações apicais e segmentos nodais para a multiplicação. Foram testados comprimentos diferentes dos explantes, concentrações de BAP e períodos de cultivo. O enraizamento foi induzido em meios de cultura WV_5/2, WV_3/2, GDm/2 e ágar-água, acrescidos

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de 2,68 µM de ANA e 0,44 µM de BAP, pelo período de nove dias, seguido de subcultivo para as mesmas formulações, sem reguladores por quatro semanas. Verificou-se que o genótipo influenciou a formação de brotações e enraizamento. O comprimento de 1,0 cm é recomendado para os segmentos nodais produzirem o maior número de brotações axilares. Para brotações apicais, os explantes de 0,5 cm cultivados em meio de cultura WV5 apresentaram melhores resultados de alongamento. O melhor período de subcultivo foi oito semanas, tanto para os segmentos nodais como brotações apicais. A maior percentagem de segmentos nodais com brotações (99,2%) e o maior número médio de brotações por explante (4,0) foram obtidos com o genótipo F27, em meio de cultura contendo 2,5 µM de BAP. Para brotações apicais, o melhor resultado para alongamento foi observado com explantes menores (0.5 cm) em meio de cultura WV_5 (218,3%). A manutenção de microjardim clonal *in vitro* de brotações vigorosas foi obtida por dois anos em meio de cultura WV5, com subcultivos de oito semanas. O melhor resultado de enraizamento (55,6%) ocorreu com indução por nove dias em meio ágar–água com 2,68 µM de NAA e 0,44 µM de BAP. A aclimatização de mudas foi bem sucedida (85% de sobrevivência), de modo que foi estabelecido um protocolo de produção de mudas por micropropagação.
Palavras-chave: cultura *in vitro*; espécie florestal; ágar-água.

INTRODUCTION

Pinus taeda L. is one of the main forestry species cultivated worldwide. Its vegetative propagation is often difficult, due to its poor adventitious rooting and response variation with genotype (AMERSON et al., 1985). The two main *in vitro t*echniques used for conifers are somatic embryogenesis and organogenesis. Especially in the genus *Pinus*, shoots can be induced *in vitro* by culturing excised cotyledons in medium containing cytokinins as the sole inducing agent, usually 6-benzylaminopurine (CORTIZO et al., 2009). Conifers are considered difficult to root and major influence on production of a well-branched root system include auxin concentration, shoot quality, donor age and clone (JANG; TAINTER, 1991). Somatic embryogenesis has been described for loblolly pine and could be the most productive propagation method, but it is still unclear how to fully control the embryogenetic process (PULLMAN; JOHNSON, 2002; SILVEIRA et al., 2004), it has low initiation frequencies and sometimes, limited success in the maturation of embryogenic tissue into cotyledonary somatic embryos (MONTALBÁN et al.2011). For−these reasons, the production of clonal plants from seeds via organogenesis has been studied for several conifers in the last 30 years, as *Pinus pinea* (CORTIZO et al., 2009), *Pinus radiata* (MONTALBÁN et al., 2011), *Pinus peuce* (STOJICIC et al., 2012), *Pinus taeda* (OLIVEIRA et al., 2012).

Direct organogenesis does not go through a callus, therefore it could increase the genomic stability of regenerated plants (TANG; GUO, 2001). Direct organogenesis of *Pinus taeda* was already described by Mehra-Palta et al. (1978), Amerson et al. (1985), Sen et al. (1989), Tang and Guo (2001), but an efficient protocol that actually meets commercial production needs has not been established yet. Optimization of factors influencing each step of micropropagation procedure is necessary for successful regeneration through adventitious bud induction (STOJICIC et al., 2012).

The aim of this study was to test the effect of explant length, genotype, culture medium, plant growth regulators and subculture periods to establish an organogenesis protocol for juvenile material of *Pinus taeda*.

MATERIAL AND METHOD

Source of explants

Seeds from selected families were provided by Battistella Florestal Company (Rio Negrinho, Santa Catarina state, Brazil). These families were ranked according to shaft quality. Genotype F27 corresponded to a controlled-pollinated family and B05 to an open-pollinated one, being respectively the 2nd and the 41st best families of the company. Genotype PC corresponded to a mixture of open-pollinated seeds from the best 129 families of company. Seeds were collected in January 2009, stored at 8°C for one month and then soaked for 24 hours. Afterwards, seeds were stratified for 25 days at 8°C, in Petri dishes (100 x 20 mm) with soaked paper filter and placed in new moist Petri dishes, for germination in growth room.

Culture conditions

Growth room with 16h photoperiod, white cool fluorescent lamps (40 $\mu mol.m^{-2}.s^{-1}$), temperature of 27 ± 2ºC (day) and 18 ± 2ºC (night).

Culture media

WV_5 (COKE, 1996a), WV_3 (COKE, 1996b) culture media were used for shoot and root induction. GD (GRESSHOFF; DOY, 1972) was used as modified by Mehra-Palta et al. (1978) and referred as "GDm". Agar-water (AW) was also tested for rooting. The pH was adjusted to 5.8 with NaOH and/or HCl at 0.1 N before autoclaving at 121°C for 20 minutes. 30 $g.L^{-1}$ sucrose was added to shoot induction and multiplication media. Culture media without sucrose or with 20 $g.L^{-1}$ of sucrose were used for rooting experiments. All media were supplemented with 5.6 $g.L^{-1}$ of Himedia® agar. Test tubes (150 x 25 mm) with 10 mL culture medium each were used for shoot induction step. Flasks (125 x 65 mm) containing 40 mL culture medium and five explants each were used for multiplication and rooting steps.

***In vitro* establishment and shoot induction**

Five-day germinated seeds had their teguments off and were immersed for 20 min in 30% hydrogen peroxide followed by immersion in a 6% sodium hypochlorite plus 0.1% Tween® 20 solution, for 10 min. Three rinses with sterilized distilled water were realized. Germinated seeds were then immersed in 0.05% mercuric chloride plus 0.1% Tween® 20 solution during five minutes and finally rinsed six times in sterile distilled water. The excised explants (apical shoots of seedlings) were vertically inoculated in WV_5 culture medium (COKE, 1996a) with 44 µM 6-benzylaminopurine (BAP) and 0.05 µM α- naphtaleneacetic acid (NAA) (MEHRA-PALTA et al. 1978). Shoot induction lasted 14 days and was followed by four subcultures on WV_5 growth regulator free, each subculture during four weeks (Figure 1a). These initial cultures produced elongated adventitious and axillary shoots which were sectioned into nodal segments and apical shoots.

Nodal segment multiplication

Experiment I

Nodal segments (1.0 - 1.9 cm, 2.0 - 2.9 cm or 3.0 - 4.0 cm long) of F27 genotype were cultivated on WV_5 medium with 0.1 µM BAP during four weeks and then subcultivated on WV_5 medium without regulators, being evaluated after four and eight weeks of subculture. The experimental design was completely randomized in a 3 x 2 factorial arrangement (length x subculture time) with three repetitions and experimental unit of 15 explants.

Experiment II

Nodal segments (1.0 cm long) of F27, B05 and PC genotypes were cultivated on WV_5 medium supplemented with 0.25, 0.5 or 2.5 µM BAP. The experimental design was completely randomized in a 3 x 3 factorial arrangement (genotype x BAP concentration) with four repetitions and experimental unit of 10 explants.

Apical shoots multiplication

Apical shoots (0.5,1.0 or 2.0 cm long) of F27 genotype were cultivated on WV_5 or GDm without growth regulators for eight weeks. The experimental design was completely randomized in a 3 x 2 factorial arrangement (length x culture medium) with four repetitions and experimental unit of 15 explants.

Rooting

Experiment I

Apical shoots, 1.5 cm long, elongated on WV_5 medium without growth regulators for eight weeks were used as explants. Shoots of F27 genotype were inoculated on WV_5, WV_3 (COKE, 1996b), GDm or AW, supplemented with 2.68 µM NAA and 0.44 µM BAP (MEHRA-PALTA *et al*., 1978) for nine days. Culture media WV_5, WV_3 e GDm had their salts reduced by half and contained 20 $g.L^{-1}$ sucrose. Agar-water medium did not contain sucrose. After rooting induction period, explants were transferred to the flasks with culture medium of the same composition, without growth regulators, except explants on agar-water formulation, which were subcultured on GDm/2 with 20 $g.L^{-1}$ sucrose.

Experimental design was completely randomized, with four repetitions and experimental unit of ten explants.

Experiment II

Shoots of genotypes F27, B05 and PC were cultured for nine days on GDm/2 or agar-water, combined with 0 or 20 g $.L^{-1}$ of sucrose, supplemented with 2.68 µM NAA and 0.44 µM BAP and then transferred to GDm/2 without plant growth regulators. The experimental design was completely randomized in a 2 x 2 factorial arrangement (culture medium x sucrose concentration) with four repetitions and experimental unit of 12 explants.

Evaluations

Percentage of explants with shoots and average number of shoots per explant were evaluated after four and eight weeks for nodal segments multiplication. Percentage of elongation [(final length – initial length/ initial length) x 100] after eight weeks was evaluated for apical shoots. Percentage of rooted shoots and average number of roots per shoot were evaluated after six weeks.

Statistical analysis

The data were submitted to Bartlett's Test and analysis of variance (ANOVA) and means were compared by Tukey's test at $P \leq 0.05$. When necessary variables were transformed by log (x + 10) or $[\log(x+ 10)]^{1/2}$.

RESULTS AND DISCUSSION

Nodal segments and apical shoot multiplication

There were no differences of shoot formation percentage or the average number of shoots per explant among the three groups of nodal segments with different initial length (Table 1; Figure 1b), differing from the results observed for other *Pinus* species, like *Pinus tecunumanii* and *Pinus oocarpa* which showed increased multiplication with longer segments (BAXTER et al., 1989). Therefore, the use of shorter nodal segments for *Pinus taeda* (1.0-1.9 cm) can be more advantageous, since it is possible to obtain more short segments than longer segments from the same initial stock material.

A higher percentage of explants with shoots was observed after eight weeks of subculture than after four weeks (Tables 1 and 2) and the shoots were vigorous. This result contrasts with the subculture period of four weeks without losses of vigor suggested by Amerson et al. (1985) for *Pinus taeda* organogenic cultures. It also suggests that WV_5 formulation probably allowed a longer subculture period than the described for other culture media used for *Pinus taeda*, like GD Gresshoff and Doy's (1972) medium (AMERSON et al., 1985; JANG; TAINTER, 1991) or Litvay's (LITVAY et al., 1981) medium (JANG; TAINTER, 1991).

The simple sectioning and decapitation of shoots allow the production of new axillary shoots and can increase multiplication rate (BAXTER et al., 1989; GEORGE et al, 2008), hence nodal segments can be multiplied without using BAP. However, the

TABLE 1: Shoot formation on *Pinus taeda* nodal segments from F27 family with different lengths, after four weeks on WV_5 medium supplemented with 0.1 µM BAP, after subculture on WV_5 medium without growth regulators.

TABELA 1: Formação de brotações em segmentos nodais com diferentes comprimentos da família F27 de *Pinus taeda,* após quatro semanas de cultivo em meio WV5, suplementado com 0,1 µM BAP, seguido de subcultivo em meio WV5, sem reguladores de crescimento.

Explant length (cm)	Explants with shoots (%)			Average number of shoots/explants
	4 weeks	8 weeks	Average	
1.0-1.9	77.8	85.0	81.4 A	1.9 A
2.0 - 2.9	76.2	97.6	86.9 A	2.0 A
3.0 - 4.0	63.6	98.1	80.8 A	2.5 A
Average	72.7 B	93.6 A		2.1
CV(%)	11.5			26.3

Where in: Means followed by same letter do not differ significantly by F or Tukey's test at 5% probability.

use of this cytokinin in increasing concentrations raised the average number of shoots per explant after eight weeks (Table 2). Similar results were obtained by Lambardi et al. (1993) and Nandwani et al. (2001) who verified that the addition of cytokinin to culture medium increased proliferation of axillary shoots of *Pinus halepensis* and *Pinus kesiya*, respectively. Since long term maintenance on a cytokinin supplemented medium may cause elongation inhibition in subsequent subcultures or rooting difficulties (KAUL, 1990; ZEL, 1993), it is suggested that, after an eight week subculture period with BAP, a subculture on BAP–free medium should be done for *Pinus taeda*. Similar procedure was suggested by Álvarez et al. (2009) for *Pinus pinaster*.

F27 genotype explants presented a significantly higher number and percentage of explants with shoots than PC explants after four weeks of culture (Table 2). Since the F27 family was the genotype with best shaft quality a relationship between field and *in vitro* performances may be inferred and further tests being necessary to prove this hypothesis. In the first studies made by Westvaco, genetically superior families of *Pinus taeda* showed positive *in vitro* responses (HANDLEY et al., 1995), indicating that family influenced shoot production, though it was not confirmed whether or not the propagation capacity was related to field characteristics, like growth in height or volume.

Percentage of elongation was higher for the shorter initial length explants and for the explants grown on WV_5 culture medium (Table 3; Figure 1c), indicating that this medium could be more adequate for this species. Shoots cultivated in GDm culture medium presented shorter internodes, darker green leaves and more lignified explant bases than the ones cultivated in WV_5 medium, which contains higher concentrations of N, B, Ca, Mg, S, K, P, Mn, I, Zn, Cl and myo-inositol than the GDm formulation. Comparing the composition of these two culture media with the nutritional requirements of cellular suspension cultures of *P. taeda* (TEASDALE et al., 1986), it was verified that GDm medium has lower B and Mg quantities than the minimum required, what does not occur with WV_5 salt formulation. Moreover, WV_5 culture medium was specifically developed for *P. taeda* cultures (COKE, 1996a), considering the nutritional requirements of the species and it is richer in mineral salts than GD or Litvay's media.

In this work, the maintenance of vigorous organogenic cultures of *Pinus taeda* occurred mostly on WV_5 culture medium, for 24 months, with four or eight-week subcultures, with and without BAP, without major symptoms of nutritional deficiency. That is longer than the observed for the same species by Handley et al. (1995) and by Jang and Tainter (1991), who succeeded in maintaining cultures

TABLE 2: Shoots formation on 1.0 cm long nodal segments of different genotypes of *Pinus taeda*, after four and eight weeks on WV_5 medium supplemented with 0.25, 0.5 or 2.5 µM BAP.

TABELA 2: Formação de brotações em segmentos nodais de 1,0 cm de comprimento em diferentes genótipos de *Pinus taeda*, após quatro e oito semanas de cultivo em meio WV5 suplementado com 0,25; 0,5 ou 2,5 µM de BAP.

		Explant with shoots (%)				Average number of shoots/explant			
	Genotype	BAP (µM)			Average	BAP (µM)			Average
		0.25	0.5	2.5		0.25 µM	0.5 µM	2.5 µM	
4 weeks	F27	97.5	95.0	100.0	97.5 A	2.7	2.9	2.8	2.8 A
	B05	70.7	72.8	77.7	73.7 B	1.8	1.9	1.9	1.9 B
	PC	63.9	65.0	52.5	60.5 B	1,7	1.6	1.7	1.7 B
	Average	77.4 A	77.6 A	76.7 A	77.2	2.2A	2.2A	2.2A	2.2
8 weeks	F27	97.6	100.0	100.0	99.2 A	3.0	3.4	4.6	3.6 A
	B05	92.8	91.8	90.5	91.7 AB	2.3	2.4	4.3	3.0 A
	PC	94.4	100.0	77.8	90.7 B	3.1	2.8	3.2	3.0 A
	Average	95.0 A	97.3 A	89.4 A	93.9	2.7 B	2.8 B	4.0 A	3.2
	CV(%)		15.5				17.5		

Where in: Means followed by same letter do not differ significantly by F or Tukey test at 5% probability. PC= Commercial orchard.

TABLE 3: Percentage of elongation of *Pinus taeda* apical shoots (0.5, 1.0 or 2.0 cm long), from F27 family, after eight weeks of culture on WV_5 or GDm media without growth regulators.
TABELA 3: Percentagem de alongamento de brotações apicais da família F27 de *Pinus taeda* (0,5, 1,0 ou 2,0 cm de comprimento), após oito semanas de cultivo nos meios WV5 ou GDm, sem reguladores de crescimento.

Initial length (cm)	Elongation (%)		
	WV_5	GDm	Average
0.5	286.5	41.0	163.8 A
1.0	206.3	15.0	110.7 B
2.0	162.0	10.8	86.4 B
Average	218.3 A	22.3 B	120.3
CV(%)	5.1		

Where in: Means followed by same letter do not differ significantly by Tukey's test at 5% probability. Data were subjected to log (x+10) transformation.

for six and ten months, respectively, on GDm or Litvay's culture media.

Rooting

Spontaneous rooting occurred at low frequency (less than 1%). According to Diaz-Sala et al. (1996), *Pinus taeda* rooting depends on the application of exogenous auxin. In the present work, we used growth regulator concentrations like proposed by Mehra-Palta et al. (1978) *i.e*., a combination of NAA and BAP for root induction. Mehra-Palta et al. (1978) and Tang and Ouyang (1999) observed that addition of other regulators (cytokinin or gibberellin), together with an auxin, was necessary to increase rooting rates of shoots of *Pinus taeda*, rather than auxin alone.

In this work, the best rooting percentages were similar to those obtained by Mehra-Palta et al. (1978) who related 50% of *in vitro* rooting for *Pinus taeda*. However, in that work, the first roots appeared after five months in medium with regulators, while in the present study first roots were observed after three weeks, considering the nine-day period of rooting induction with regulators plus two weeks of subculture without regulators. De Klerk et al. (1997) verified that the hormonal stimulation which induces an *in vitro* physiological reaction could be inhibitory when the new organ is developing. The necessary time for root formation was reduced when compared to the results of Tang and Ouyang (1999), who observed the first responses of *in vitro* adventitious rooting of *Pinus taeda* after six weeks of culture.

Roots formed after two weeks on culture media without regulators were white-colored without secondary roots. They acquired a darker color and presented secondary ramifications after six weeks of culture (Figure 1d). Average length of roots was about 0.2-0.5 cm after two weeks of subculture (Figure 1d) and about 1.0-1.7 cm after six weeks of subculture, without differences among the treatments, in both rooting experiments.

Similarly to what occurred in multiplication experiments, the saline formulation had an effect on rooting induction of *Pinus taeda* shoots. However, unlike the multiplication experiments, the more concentrated saline formulations, $WV_3/2$ and $WV_5/2$, presented low or no positive results, while the higher rooting percentages were seen in AW and GDm/2 media (Table 4). Webb et al. (1988) obtained greater success for *Pinus ponderosa in vitro* rooting using agar-water culture medium than using other formulations, similarly to what was observed in this work for *Pinus taeda*. Oliveira et al. (2012) also observed that *P. taeda* shoots did not need salts to acquire rooting competence; however they pointed out that absence of salts reduces significantly further root development. Mean number of roots per explants was higher for the explants induced in GDm/2, when compared to AW medium (Table 5). The presence or absence of sucrose in the induction culture medium did not influence adventitious rooting (Table 5).

Genetic factors could influence rooting results, once similar treatments with GDm and agar-water formulations from the two experiments presented different rooting percentages (Tables 4 and 5). This is in accordance with the results of Tang and Ouyang (1999) who observed variation of rooting

TABLE 4: *Pinus taeda* rooting from apical shoots of F27 family, induced for nine days on GDm/2, $WV_5/2$, $WV_3/2$ culture media with 20 $g.L^{-1}$ sucrose or AW medium without sucrose, supplemented with 2.68 µM NAA and 0.44 µM BAP, after six weeks of subculture on culture media without growth regulators.

TABELA 4: Enraizamento de brotações apicais da família F27 de *Pinus taeda,* induzido nove dias em meios de cultura GDm/2, $WV_5/2$, $WV_3/2$, com 20 $g.L^{-1}$ de sacarose ou meio ágar-água sem sacarose, suplementado com 2,68 µM de NAA e 0,44 µM de BAP, após seis semanas de subcultivo em meios de cultura sem reguladores de crescimento.

Rooting induction medium	Subculture medium	Rooted explants (%)	Average number of roots/ explants
GDm/2	GDm/2	17.9 AB	2.7 A
WV5/2	WV5/2	5.0 B	1.0 B
WV3/2	WV3/2	0.0 B	-
AW	GDm/2	35.0 A	2.3 B
CV(%)		16.2	3.4

Where in: Means followed by same letter do not differ significantly by Tukey's test at 5% probability. Data for rooted explants and average number of roots/explants were subjected to log (x+10) transformation. AW = agar-water.

TABLE 5: *Pinus taeda* rooting from apical shoots of F27, B05 and PC genotypes, induced for nine days on GDm/2 or AW culture medium, supplemented or not with 20 $g.L^{-1}$ sucrose, 2.68 µM NAA and 0.44 µM BAP, after six weeks of subculture on GDm/2 medium with 20 g. L^{-1} sucrose and without growth regulators.

TABELA 5: Enraizamento de brotações apicais dos genótipos F27, B05 e PC de *Pinus taeda,* induzidas por nove dias em meios de cultura GDM/2 e ágar-água, acrescido ou não com 20 g. L^{-1} de sacarose, 2,68 µM de NAA e 0,44 µM de BAP, após seis semanas de subcultivo em meio GDm/2, sem 20 g. L^{-1} de sacarose e reguladores de crescimento.

	Culture medium of root induction	Sucrose ($g.L^{-1}$)		Average
		0	20	
Rooted explants (%)	AW			49.8 A
	GDm/2			33.4 A
	Average	40.9 A	42.2 A	41.6
	CV(%)	66.1		
Average number of roots/explant	AW	1.7	1.4	1.5 B
	GDm/2	3.0	2.2	2.6 A
	Average	2.0 A	2.2 A	
	CV(%)	22.9		

Where in: Means followed by same letter do not differ significantly by F test. AW = agar-water

percentage (8.7% to 46.7%) among six families of *Pinus taeda*. The influence of genotype on *in vitro* responses of *Pinus taeda* is often reported in the literature (JANG; TAINTER, 1991; HANDLEY et al., 1994; TANG et al., 1998; TANG; GUO, 2001), therefore being a challenge to establish a reliable protocol for the species, not restricted to only few genotypes.

In the present study, axillary shoots obtained within 12 and 24 months of culture could be induced to root, in contrast to the results of Jang and Tainter (1991), who observed loss of adventitious rooting capacity of *Pinus taeda* cultures after eight months of culture on GD or Litvay's media.

Plants were acclimatized by transfer to vermiculite: plantmax (1:1) substrate, in open glass flasks, in growth room conditions, for 20 days, followed by transfer to greenhouse under uncontrolled conditions, with survival rate of 85% after 40 days of acclimatization.

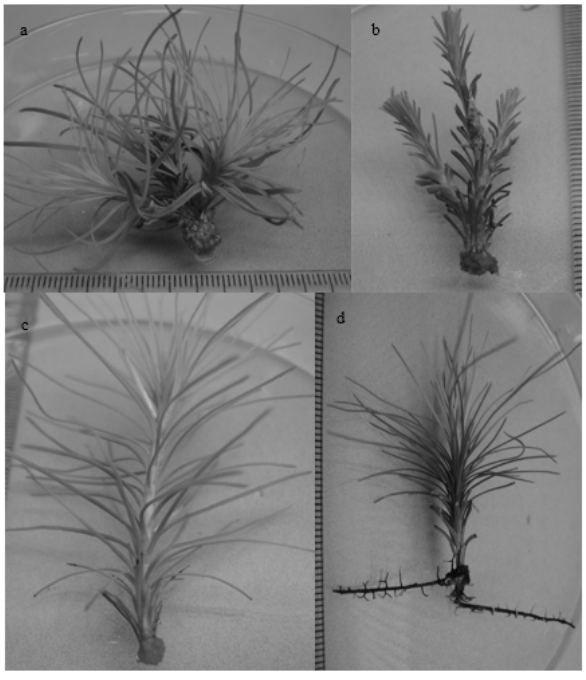

FIGURE 1: Micropropagation of *Pinus taeda.* a-Apical shoot induced on WV5 culture medium with 44μM BAP and 0.05 μM NAA for 14 days, followed by two subcultures on growth regulator-free medium; b- Axillary shoots developed from nodal segment 2.0 to 2.9 cm long, after four weeks on WV_5 culture medium with 0.1 μM BA and eight weeks in WV_5 medium without plant growth regulators. c- Elongation of apical shoots with initial length of 1.0 cm, after eight weeks on growth regulator free GDm or WV_5, respectively. d- Secondary root formation after nine days of induction on GDm/2 medium with 20 $g.L^{-1}$ of sucrose, 2.68 μM NAA and 0.44 μM of BA, followed by six weeks of subculture on the culture medium of same composition, without growth regulators.

FIGURA1: Micropropagação de *Pinus taeda.* a- Brotação apical induzida em meio de cultura WV5 com 44 μM BAP e 0.5 μM NAA por 14 dias, seguida de dois subcultivos em meio sem regulador de crescimento; b- Brotações axilares desenvolvidas a partir de segmentos nodais de 2,0 a 2,9 cm de comprimento, após quatro semanas de cultivo em meio de cultura WV_5, contendo 0,1 μM de BA, seguido de oito semanas em meio WV5, sem reguladores de crescimento; c – Alongamento de brotações apicais com comprimento inicial de 1,0 cm, após oito semanas em meio GDm ou WV_5, respectivamente; d- Formação de raízes secundárias após nove dias de indução em meio GDm/2, com 20 g. L^{-1} de sacarose, 2,68 μM de NAA e 0,44 μM de BA, seguida de seis semanas de subcultivo em meio de mesma composição, sem reguladores de crescimento.

CONCLUSIONS

It was concluded that genotype had influenced shoot formation and rooting. It is recommended the length of 1.0 cm for nodal segment explants to obtain a higher number of axillary shoots. For apical shoots, the 0.5 cm explant length and WV_5 medium formulation allowed their bigger elongation, this way they can be used as stocks for new nodal segments sections, increasing multiplication rates. The best period of subculture was eight weeks, both for nodal segments and for apical shoots.

With the use of WV_5 culture media, it was possible to maintain cultures of vigorous shoots during two years, allowing the maintenance of an in vitro clonal micro garden.

In vitro, rooting of *P. taeda* does not need salts during the nine days induction period with 2.68 μM NAA and 0.44 μM BAP and the presence of sucrose in the induction culture medium does not influence adventitious rooting. Plantlets were acclimatized successfully, so a direct organogenesis protocol was established.

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