



## Short communication

*In vitro* regeneration of adult *Pinus sylvestris* L. trees

N. De Diego, I.A. Montalbán, P. Moncaleán\*

Neiker-Tecnalia, Dpto. Biotecnología, Centro de Arkaute, Apdo. 46, 01080 Vitoria-Gasteiz, Spain

Received 15 December 2008; received in revised form 26 August 2009; accepted 14 September 2009

**Abstract**

The propagation of adult conifer trees by tissue culture has been studied for the last twenty years, but problems related to the juvenile to adult phase change of trees have limited the practical applications of these tissue culture procedures. This paper describes a micropropagation protocol for the *in vitro* propagation of mature Scots pine trees. In this study, dormant shoot buds, which had not started to elongate, were collected from twenty-one adult *Pinus sylvestris* trees (>15 years old) during the winter. The sampled buds were cut transversely into slices of 0.5 to 1 cm in thickness and were cultured on three types of culture media (DCR, WP and LPm) supplemented with four cytokinins (BA, mT, Tdz and Z), at two different concentrations (25 and 50  $\mu\text{M}$ ), except for Tdz, whose concentrations were diluted to 5  $\mu\text{M}$  and 2.5  $\mu\text{M}$ . The evaluated culture media did not show significant differences in the bud organogenesis capacity. In fact, the highest organogenic response was obtained with buds cultured on DCR and WP media and by explants cultured on medium supplemented with 25  $\mu\text{M}$  meta-topolin. This protocol is a successful and efficient biotechnological approach to the micropropagation of adult *P. sylvestris* trees.

© 2009 SAAB. Published by Elsevier B.V. All rights reserved.

**Keywords:** Axillary shoot formation; Conifer; Cytokinin; *In vitro* culture; *Pinus sylvestris*; Scots pine

**1. Introduction**

In Europe, Scots pine (*Pinus sylvestris* L.) is one of the most commercially important conifer species because it induces the development of breeding programs. Unfortunately, the conventional breeding of trees is not as straightforward as that of herbaceous plants (Merkle and Dean, 2000). The economic value of a tree can only be assessed after it reaches maturity, and maturation itself induces changes in meristem behaviour, thus reducing the propagation potential of the tree (Von Aderkas and Bonga, 2000). Vegetative propagation of selected trees offers a rapid way of not only generating clonal planting stocks for reforestation programs, woody biomass production, and

conservation of elite and singular germplasm (Giri et al., 2004), but also effectively captures genetic variation while bypassing long breeding cycles (Ahuja, 1993). Therefore, the application of biotechnology (Campbell et al., 2003) and especially tissue culture provides an important tool to propagate the selected genotypes. The regeneration of plants under aseptic and controlled environmental conditions is referred to as micropropagation because very small pieces of plant tissue or organs are used as starting vegetative tissue (Davis and Becwar, 2007). The most commonly used method of micropropagation of woody plants is shoot-tip culture (Lineberger, 1980).

*In vitro* propagation of selected mature trees could be a powerful tool to improve forestry management and breeding programs. However, few successful micropropagation methods for adult *P. sylvestris* trees have been described (Anderson and Levinsh, 2005). In contrast, numerous studies resulted in efficient micropropagation protocols for other *Pinus* species, using zygotic embryos, young seedlings, or other explants with organogenic capacity such as meristems, axillary shoot and buds (Prehn et al., 2003; Moncaleán et al., 2005; Alonso et al., 2006). During the last years, our research team has focused on the development of *in vitro* regeneration protocols for species of

**Abbreviations:** BA, 6-benzyladenine; mT, meta-topolin; Tdz, thidiazuron; Z, zeatin; B.O.C, bud organogenesis capacity; DCR, Gupta and Durzan, 1985; LPm, modified LP medium, Aitken J., personal communication based in Quorin and Lepoivre, 1977; WP, woody medium, Lloyd and McCown, 1980; EM, elongation medium; IM, induction medium; *P.*, *Pinus*.

\* Corresponding author. Tel.: +34 902540546; fax: +34 902540547.

E-mail address: [pmoncalean@neiker.net](mailto:pmoncalean@neiker.net) (P. Moncaleán).

pine (De Diego et al., 2008; Cortizo et al., 2009). Tissue culture of mature Scots pine was reported to be negatively affected by high oxidative stress during culture (Laukkanen et al., 2000), strong wound reactions accompanied by increase in phenolic substances (Hohtola, 1988), and endophytic microbes (Pirttilä et al., 2002). As a result, rapid tissue browning, followed by the deterioration of cellular ultrastructure and necrosis, as well as a high percentage of infections, usually occurs during *P. sylvestris* culture (Anderson and Ievinsh, 2005).

The effects of cytokinins in micropropagation protocols have been well documented in *Pinus* species (Alonso et al., 2006; Moncaleán et al., 2005). Moreover, the culture medium, culture physical conditions and exogenously applied cytokinins influence the process of organogenic induction (Thorpe et al., 1991). Other factors such as the concentration, the application method and the incubation period affect the ability of explants to develop healthy shoots with root-forming ability.

The aim of the present work was to develop a protocol to induce the successful regeneration of adult *P. sylvestris* trees. To this end, we tested the organogenic responses of vegetative buds collected from different genotypes of adult trees, and we focused on determining the most adequate combination of

culture medium and cytokinin treatment to obtain the best micropropagation results.

## 2. Materials and methods

### 2.1. Plant material

Twenty-one *P. sylvestris* L. trees, (>15 years old), were selected in Manzanos (Alava, Spain) between 2004 and 2005. Dormant apical buds were sampled at two independent collection times (November and January) during the winter (Fig. 1A). During sampling, the harvested buds were wrapped at their base with moist paper to prevent dehydration. The material was then stored in polyethylene bags at 4 °C for a maximum of four days until further use.

All explants were disinfected by decontamination in 50% (v/v) commercial bleach (active chloride >5%) diluted with sterile water for 30 min. The explants were then immediately rinsed in sterile water for 5 min; this washing step was repeated three times. The bud scales were subsequently removed (Fig. 1B), and the decontaminated buds were cut transversely with a surgical scalpel blade into slices of 0.5 to 1 cm in thickness, under sterile

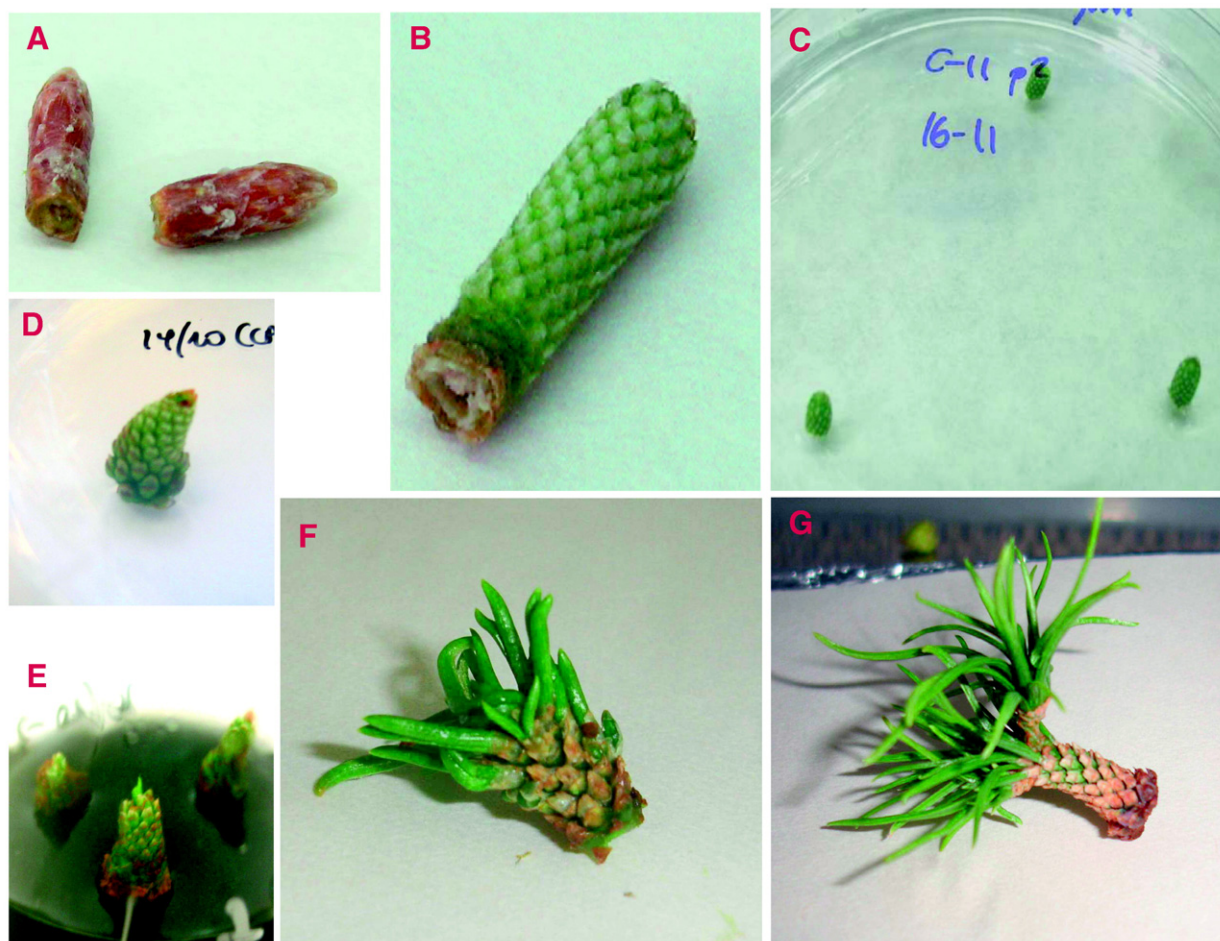


Fig. 1. *In vitro* regeneration from shoot buds of 15-year-old *Pinus sylvestris* trees: (A) Dormant buds from >15 year-old trees; (B) Shoot bud without bud sheath; (C) Shoot bud slices in culture; (D) Development of needle fascicles on induction medium (WP) supplemented with cytokinins; (E) Bud cultured on elongation medium (WP with AC) without cytokinins for promoting needle elongation; (F) Well-developed needles; (G) Axillary shoot formation.

conditions. Three slices were cultured per Petri dish (Ø 96X21 mm) in bud induction medium (Fig. 1C).

### 2.2. Bud induction and shoot formation

Dormant buds of *P. sylvestris* from selected trees were cultured in three different culture media: DCR (Gupta and Durzan, 1985), modified LP (LPm) (Aitken, personal communication based on Quorin and Lepoivre, 1977) and WP medium (Lloyd and McCown, 1980). In order to induce the organogenic response, each basal medium was supplemented individually with four cytokinins [benzyladenine (BA), meta-Topolin (mT), thidiazuron (TdZ) and zeatin (Z)]. Medium supplemented with some of the tested cytokinins was called induction medium (IM). Cytokinins were applied at concentrations of 25 µM and 50 µM, except for TdZ whose concentrations were diluted to 5 µM and 2.5 µM according to previously published reports (Goldfarb et al., 1991). Culture media were supplemented with sucrose 3% (w/v) and 10 g/L of Difco Agar<sup>®</sup> granulated (Becton, Dickinson and Company, France) was added. The pH was adjusted to 5.8 before autoclaving at 121 °C for 20 min. Exactly 15 ml of culture media were poured in each 90 mm Petri dish. All cultures were maintained at 22 °C under a 16 h photoperiod of 120 µmol m<sup>-2</sup> s<sup>-1</sup> (Fig. 1D) provided by cool white fluorescent tubes (TLD 58 W/33; Philips, France).

Upon bud sprouting, the explants were placed on elongation media (EM) (Fig. 1E). The composition of EM was identical to that of IM but lacked cytokinins, and was supplemented with 0.2% (w/v) activated charcoal (AC), 3% (w/v) sucrose and 10 g/L Difco Agar<sup>®</sup>. When elongating needle fascicles were more than 2 cm length, they were cut transversally and transferred to the IM to stimulate the development of the shoots in the brachyblast primordia, thereby inducing axillary shoot formation. One month later, explants that showed new axillary shoot formation were subcultured onto fresh EM (Fig. 1G). The remaining explants were cultured again on the IM to promote axillary bud development. Shoots reaching 1.5 cm in length were separated individually and subcultured on EM.

### 2.3. Statistical analysis

Statistical analysis was applied to the explant collection data. A total of 72 bud slices per sampling point and per genotype (3 bud slices X 3 culture media X 4 cytokinins X 2 cytokinin concentrations) were used to carry out the present work. Bud organogenesis capacity (B.O.C) was evaluated as the number of buds that showed axillary shoot development. The data followed a normal distribution and were used directly for analysis of variance (ANOVA) according to the following model:

$$Y_{ijr} = \mu + Me_i + H_j + MeH_{ij} + e_{ijr}$$

where  $Y_{ijr}$  is the  $r$ th number of collected buds that were introduced into the  $i$ th medium with the  $j$ th cytokinin;  $\mu$  is the experimental mean;  $Me_i$  is the effect of the  $i$ th medium;  $H_j$  is the effect of the  $j$ th cytokinin;  $MeH_{ij}$  is the effect of the interaction between the  $i$ th medium and the  $j$ th cytokinin and  $e_{ijr}$  is the

random error component. Residual normality was evaluated by the Shapiro–Wilk normality test (Shapiro and Wilk, 1965) without transformation. However, homocedasticity was analyzed by the Levene's test (Brown and Forsythe, 1974). Multiple comparisons were obtained by significant Tukey's HSD test after ANOVA at a probability of  $\alpha=0.05$ . The model was subjected to analysis by the R software (version 2.6.1<sup>®</sup>, R Foundation for Statistical Computing, Vienna).

## 3. Results and discussion

Explants cultured on DCR, LPm and WP media did not show significant differences in B.O.C; they were 17.09, 16.04 and 17.71% respectively (Table 1). Shoot buds cultured on LPm medium presented the lowest organogenic response perhaps as a result of the relatively high nitrate concentration when compared to the DCR or WP media. Tuskan et al. (1990) showed that the excess nitrate could have a negative impact on the organogenic response during micropropagation. Others have shown a higher organogenic response when pine explants were cultured on DCR and WP (Mathur and Nadgouda, 1999; Anderson and Ievinsh, 2002). However, more recently, new successful micropropagation technologies using DCR medium have been developed for cloning adult trees of *Pinus patula* (Malabadi and Van Staden, 2005) and *Pinus kesiya* (Malabadi et al., 2004).

*In vitro* regeneration is less successful from explants of mature woody plants than from their juvenile counterparts because numerous physiological and biochemical changes are implicated in the juvenile to adult phase transition (Bonga and Von Aderkas, 1993). Mature Scots pine tissue culture presents peculiarities that have been evaluated in previous works (Laukkanen et al., 2000; Pirttilä et al., 2002). For instance, Anderson and Ievinsh (2005) induced callus formation in order to regenerate new needles. In our experiment, the callus phase was circumvented and new needle formation was stimulated directly from buds cultured in the different media supplemented with cytokinins.

Table 1

Organogenic response (%) of shoot bud explants from 15-year-old *Pinus sylvestris* trees cultured in three different media (DCR, LPm and WP) supplemented with different cytokinins (BA, mT, TdZ or Z) at two lower and upper confidence limits (95%).

Factor	Treatment	B.O.C	95% confidence limits	
			Low	High
Medium	DCR	17.09 a	14.07	20.93
	LP	16.04 a	12.21	19.87
	WPM	17.71 a	13.88	21.54
Cytokinin	BA-50	17.46 ab	23.71	11.21
	BA-25	24.58 ab	30.83	18.33
	mT-50	14.12 b	21.29	6.95
	mT-25	24.65 a	30.95	18.35
	TdZ-5	11.67 b	17.92	5.42
	TdZ-2.5	16.83 ab	23.13	10.53
	Z-50	16.13 ab	22.38	9.88
	Z-25	13.08 b	19.75	6.41

The different letters refer to statistically significant values obtained at  $p<0.05$  by the Tukey's HSD test to separate means after ANOVA.

Phytohormones and their concentration play an important role in the explant organogenic response and in maturation changes during the micropropagation process. In fact, Zhang et al. (2003) revealed that juvenile buds exhibited different levels of free base and riboside cytokinin than adult buds. For this reason, exogenously applied cytokinins may provoke the reinvigoration of adult buds and the acquisition of juvenile characteristics. In order to regenerate plants from dormant buds of adult *Pinus brutia* (Abdullah et al., 1987) and *Pinus pinaster* (De Diego et al., 2008) trees, the primary explants were cultured on media supplemented with different cytokinins at a 25  $\mu\text{M}$  concentration. Moreover, the IM were only individually supplemented with different cytokinins, because auxin application sometimes caused a marked decrease in the explant organogenic response (García-Férriz et al., 1994).

In this study, explants cultured on media with the lowest cytokinin concentration presented the highest organogenic capacity with a B.O.C of 24.58% for BA, 24.65% for mT, and 16.89% for Tdz. On the other hand, the B.O.C values for buds cultured on media supplemented with 50  $\mu\text{M}$  BA and Z (17.46% and 16.13%, respectively) were not statistically different (Table 1). Analysis of variance (ANOVA) did not show significant differences ( $p < 0.05$ ) in the organogenic response of shoot buds cultured on media supplemented with 50  $\mu\text{M}$  mT, 5  $\mu\text{M}$  Tdz, and 25  $\mu\text{M}$  Z and for the following treatments: 50  $\mu\text{M}$  and 25  $\mu\text{M}$  BA, 25  $\mu\text{M}$  mT, 2.5  $\mu\text{M}$  Tdz, and 50  $\mu\text{M}$  Z (Table 1). These differences among averages could be due to the accumulation of high cytokinin levels inside the explants, resulting from application to the culture media. When BA was used, this phenomenon seemed less severe as we previously observed in other pine species (De Diego et al., 2008). When the explants were subcultured on EM, the residual cytokinin accumulated in the explants might have not been rapidly adsorbed by the AC (Pan and Van Staden, 1998), causing the buds to develop abnormally and subsequently die.

When the shoot buds were cultured on media supplemented with the lowest concentration of mT (25  $\mu\text{M}$ ), the B.O.C was highest. The application of mT has been used in several micropropagation protocols and gave satisfactory results (Strnad et al., 1997; Palavan-Unsal et al., 2002; Escalona et al., 2003). For instance, Bairu et al. (2007) optimized the micropropagation protocol for the endangered Spiral Aloe, *Aloe polyphylla*, and showed that mT was the preferred cytokinin both in terms of multiplication rate and rooting. For this reason, and in concordance with our results, mT could serve as an alternative for other cytokinins, such as BA, in plant micropropagation.

Supplementation of AC to the culture medium of *in vitro* plant protocols has been frequently described (Stojicic and Budimir, 2004; Lelu-Walter et al., 2008). The AC was beneficial in the micropropagation process of adult pine trees as shown by Dumas and Monteuis (1995) and De Diego et al. (2008) in maritime pine. Pan and Van Staden (1998) reviewed the use of AC in micropropagation, highlighting the absorption of elements such as phenolic metabolites and residual plant growth regulators by AC which often hinder organogenesis and plantlet development. These are some of the important beneficial functions of using activated charcoal (Ebert et al.,

1993). Also, culture media supplemented with AC have allowed the improvement of *Pinus* organogenesis protocols (Calixto and Pais, 1997). Hence, we considered that the presence of AC in EM was essential.

In conclusion, an effective protocol for regenerating adult Scots pine trees was developed. This protocol provides an advantage in breeding programs because it may be used for germplasm conservation of elite trees and for establishing seed orchards or plantations. The method we described can be applied to all of the assayed genotypes and requires less starting material than grafting. Future research will focus on the improvement of rooting rates.

### Acknowledgements

This work was supported by the Spanish Ministry of Science and Technology (MEC-AGL-2005-08214) and the Department of Agriculture and Fisheries of the Basque Government (VED2007014). Nuria De Diego was supported by a full-time grant from the Basque Government.

### References

- Abdullah, A.A., Yeoman, M.M., Grace, J., 1987. Micropropagation of mature Calabrian pine (*Pinus brutia* Ten.) from fascicular buds. *Tree Physiology* 3, 123–136.
- Ahuja, M.R., 1993. Biotechnology and clonal forestry. In: Ahuja, M.R., Libby, W.J. (Eds.), *Clonal Forestry I, Genetics and Biotechnology*. Springer-Verlag, Berlin, pp. 135–144.
- Alonso, P., Moncaleán, P., Fernández, B., Rodríguez, A., Centeno, M.L., Ordás, R., 2006. An improved micropropagation protocol for stone pine (*Pinus pinea* L.). *Annals of Forest Science* 63, 879–885.
- Andersone, U., Ievinsh, G., 2002. Changes of morphogenic competence in mature *Pinus sylvestris* L. buds *in vitro*. *Annals of Botany* 90, 293–298.
- Andersone, U., Ievinsh, G., 2005. *In vitro* regeneration of mature *Pinus sylvestris* buds stored at freezing temperatures. *Biologia Plantarum* 49, 281–284.
- Bairu, M.W., Stirk, W.A., Dolezal, K., Van Staden, J., 2007. Optimizing the micropropagation protocol for the endangered *Aloe polyphylla*: can metatoplin and its derivatives serve as replacement for benzyladenine and zeatin? *Plant Cell, Tissue and Organ Culture* 90, 15–23.
- Bonga, J.M., Von Aderkas, P., 1993. Rejuvenation of tissues from mature conifers and its implications for propagation *in vitro*. In: Ahuja, M.R., Libby, W.J. (Eds.), *Clonal Forestry: Genetics and Biotechnology*. Springer-Verlag, Berlin, pp. 182–199.
- Brown, M.B., Forsythe, A.B., 1974. Robust test for the equality of variance. *Journal of the American Statistical Association* 69, 364–367.
- Calixto, F., Pais, S., 1997. Adventitious shoot formation and plant regeneration from *Pinus pinaster* Sol. ex Aiton. *In Vitro Cellular and Development Biology — Plant* 33, 119–124.
- Campbell, M.M., Brunner, A.M., Jones, H.M., Strauss, S.H., 2003. Forestry's fertile crescent: the application of biotechnology to forest trees. *Plant Biotechnology Journal* 1, 141–154.
- Cortizo, M., De Diego, N., Ordás, R., Moncaleán, P., 2009. Micropropagation of adult Stone Pine (*Pinus pinea* L.). *Trees — Structure and Function* 23, 835–842.
- Davis, J.M., Becwar, M.R., 2007. Examples of current implementation of cloning technology worldwide. In: Page, A. (Ed.), *Developments in tree cloning*. Pira International Ltd, Leatherhead, UK, pp. 32–33.
- De Diego, N., Montalbán, I.A., Fernández, E., Moncaleán, P., 2008. *In vitro* regeneration of *Pinus pinaster* adult trees. *Canadian Journal of Forest Research* 38, 2607–2615.

- Dumas, E., Monteuis, O., 1995. *In vitro* rooting of micropropagated shoots from juvenile and mature *Pinus pinaster* explants: influence of activated charcoal. *Plant Cell, Tissue and Organ Culture* 40, 231–235.
- Ebert, A., Taylor, F., Blake, J., 1993. Changes of 6-benzylaminopurine and 2, 4 dichlorophenoxyacetic acid concentrations in plant tissue culture media in the presence of activated charcoal. *Plant Cell, Tissue and Organ Culture* 33, 157–162.
- Escalona, M., Cejas, I., González-Olmedo, J., Capote, I., Roels, S., Cañal, M.J., Rodríguez, R., Sandoval, J., Debergh, P., 2003. Efecto de meta-topolina sobre la propagación del plátano utilizando un biorreactor de inmersión temporal. *InfoMusa* 12, 30–32.
- García-Férriz, L., Serrano, L., Pardos, A., 1994. *In vitro* shoot organogenesis from excised immature cotyledons and microcuttings production in Stone Pine. *Plant Cell, Tissue and Organ Culture* 36, 135–140.
- Giri, C.C., Shyamkumar, B., Anjaneyulu, C., 2004. Progress in tissue culture, genetic transformation and applications of biotechnology to trees: an overview. *Trees* 18, 115–135.
- Goldfarb, B., Howe, G.T., Bailey, L.M., Strauss, S.H., Zaerr, J.B., 1991. A liquid cytokinin pulse induces adventitious shoot formation from Douglas-fir cotyledons. *Plant Cell Reports* 10, 156–160.
- Gupta, P.K., Durzan, D.J., 1985. Shoot multiplication from mature trees of Douglas fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). *Plant Cell Reports* 4, 177–179.
- Hohtola, A., 1988. Seasonal changes in explant viability and contamination of tissue culture from mature Scots pine. *Plant Cell, Tissue and Organ Culture* 15, 211–222.
- Laukkanen, H., Rautiainen, L., Taulavuori, E., Hohtola, A., 2000. Changes of cellular structures and enzymatic activities during browning of Scots pine callus derived from mature buds. *Tree Physiology* 20, 467–475.
- Lelu-Walter, M.A., Bernier-Cardou, M.B., Klimaszewska, K., 2008. Clonal plant production from self- and cross-pollinated seed families of *Pinus sylvestris* (L.) through somatic embryogenesis. *Plant Cell, Tissue and Organ Culture* 92, 31–45.
- Lineberger, R.D., 1980. Tissue culture of woody plants. The Buckeye Nurseryman (Columbus, Ohio). <http://aggie-horticulture.tamu.edu/tisscult/microprop/woodypl.html> (Access date: 20-05-2009).
- Lloyd, G., McCown, B., 1980. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. *Proceedings of the International Plant Propagation Society* 30, 421–427.
- Malabadi, R.B., Choudhury, H., Tandon, P., 2004. Initiation, maintenance and maturation of somatic embryos from thin apical dome sections in *Pinus kesiya* (Royle ex. Gord) promoted by partial desiccation and Gellan gum. *Scientia Horticulturae* 102, 449–459.
- Malabadi, R.B., Van Staden, J., 2005. Somatic embryogenesis from vegetative shoots apices of mature trees of *Pinus patula*. *Tree Physiology* 25, 11–16.
- Mathur, G., Nadgauda, R., 1999. *In vitro* plantlet regeneration from mature zygotic embryos of *Pinus wallichiana* A.B. Jacks. *Plant Cell Reporter* 19, 74–80.
- Merkle, S.A., Dean, J.F., 2000. Forest tree biotechnology. *Current Opinion in Biotechnology* 11, 298–302.
- Moncaleán, P., Alonso, P., Centeno, M.L., Cortizo, M., Rodríguez, A., Fernández, B., Ordás, R., 2005. Organogenic response of *Pinus pinea* cotyledons to hormonal treatments: BA metabolism and cytokinin content. *Tree Physiology* 25, 1–9.
- Palavan-Unsal, N., Cag, S., Cetin, E., 2002. Growth responses of excised radish cotyledons to meta-topolin. *Canadian Journal of Plant Science* 82, 191–194.
- Pan, M.J., Van Staden, J., 1998. The use of charcoal in *in vitro* culture — a review. *Plant Growth Regulation* 26, 155–163.
- Prehn, D., Serrano, C., Mercado, A., Stange, C., Barrales, L., Arce-Johnson, P., 2003. Regeneration of whole plants from apical meristems of *Pinus radiata*. *Plant Cell, Tissue and Organ Culture* 73, 91–94.
- Pirttilä, A.M., Laukkanen, H., Hohtola, A., 2002. Chitinase production in pine callus (*Pinus sylvestris* L.): a defense reaction against endophytes? *Planta* 214, 848–852.
- Quorin, M., Lepoivre, P., 1977. Étude de milieux adaptés aux cultures *in vitro* de *Prunus*. *Acta Horticulturae* 78, 437–442.
- Shapiro, S.S., Wilk, M.B., 1965. An analysis of variance test for normality. *Biometrika* 52, 591–611.
- Stojicic, D., Budimir, S., 2004. Cytokinin-mediated axillary shoot formation in *Pinus heldreichii*. *Biologia Plantarum* 48, 477–479.
- Strnad, M., Hanus, J., Vanek, T., Kaminek, M., Ballantine, J., Fusell, B., Hanke, D., 1997. Meta-topolin, a highly active aromatic cytokinin from Poplar leaves (*Populus x Canadensis* Moench., cv. Robusta). *Phytochemistry* 45, 213–218.
- Thorpe, T.A., Harry, I.S., Kumar, P.P., 1991. Application of micropropagation to forestry. In: Debergh, P.C., Zimmerman, R.H. (Eds.), *Micropropagation: Technology and Application*. Kluwer Academic, Dordrecht, pp. 311–316.
- Tuskan, G.A., Sargent, W.A., Rensema, T., Walla, J.A., 1990. Influence of plant growth regulators, basal media and carbohydrate levels on the *in vitro* development of *Pinus ponderosa* (Dougl. ex Law.) cotyledon explants. *Plant Cell, Tissue and Organ Culture* 20, 47–52.
- Von Aderkas, P., Bonga, J.M., 2000. Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment. *Tree Physiology* 20, 921–928.
- Zhang, H., Horgan, K.J., Reynolds, P.H.S., Jameson, P.E., 2003. Cytokinins and bud morphology in *Pinus radiata*. *Physiologia Plantarum* 117, 264–269.