International Journal of Botany

ISSN: 1811-9700

science alert

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http://ansinet.com

International Journal of Botany 3 (1): 40-47, 2007
ISSN 1811-9700
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Plant Regeneration via Somatic Embryogenesis Using Secondary Needles of Mature Trees of *Pinus roxburghii* Sarg

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Abstract: This study highlights the positive role of TRIA in inducing the somatic embryogenesis using secondary needles of mature trees of *P. roxburghii*. Very few reports of induction of somatic embryogenesis using secondary needles as explants are available in conifers. Secondary needles of mature pines can be used as the best explant-source-material for the micropropagation of conifers. Explants cold-pretreated at 4°C and incubated for 3 days on 0.3% activated charcoal induced white-mucilaginous embryogenic tissue on DCR basal medium (initiation medium-I) supplemented with 22.62 μM 2, 4-D, 26.85 μM NAA and 5 μM TRIA in all the 3 genotypes (PR17, PR100 and PR321) of *Pinus roxburghii*. Explants without cold pre-treatment induced non-embryogenic tissue on initiation medium supplemented with various concentrations of TRIA. Therefore, both the cold pre-treatment and the presence of TRIA in the initiation medium might have triggered the cells in programming towards the somatic embryogenesis. It was concluded that combination of stress induction by cold pre-treatment and inclusion of TRIA with DCR basal medium has a great potential of inducing embryogenic tissue in all the three genotypes tested. This has created a new possibility of micropropagation of pines for the current demands of commercial forestry.

Key words: Chir pine, micropropagation, secondary needles, stress, triacontanol

INTRODUCTION

Plant regeneration *via* somatic embryogenesis of *Pinus roxburghii* (Chir pine), a species of considerably economic importance in India, plays an important role in the commercial forestry (Malabadi and Nataraja, 2006a, b). *Pinus roxburghii* is distributed throughout in all the parts of India and higher percentage of vegetation is still restricted to Western-Himalayas regions. Its high durability wood is mainly used for the construction of houses, as a household fuel, paper industry and for the production of turpentine oil due to the presence of oleoresin. Turpentine oil is used as a primary in painting industries. However, recent survey by the Forest Department, Government of India revealed and concerned about the depletion of pine forest due to the unplanned urban developmental activities, converting forest area into a agriculture land, extraction of timber for fuel, turpentine oil production and other household purposes. These factors force an immediate attention for the afforestation programmes in the affected regions. In addition, the vegetative propagation of pines is very difficult due to the low viability of seeds, mature hedges do not root easily and large-scale grafting is not economically viable (Malabadi *et al*., 2002, 2003a,b, 2004a,b). These phenomenons hamper the clonal propagation of selected superior genotypes. In *Pinus roxburghii*, somatic embryogenesis has been reported using various explants viz vegetative shoot apices of mature trees (Malabadi and Nataraja, 2006a, b), mature and immature zygotic embryos (Mathur *et al*., 2000; Arya *et al*., 2000). Clonal propagation using tissue culture techniques plays an important role in the commercial forestry. Plant regeneration via somatic embryogenesis using vegetative shoot apices of mature trees has been reported in a few conifers (Bonga and von Aderkas, 1993; Bonga and Pond, 1991; Bonga, 1996, 2004; Westcott, 1994; Smith, 1997; Malabadi *et al*., 2003a, b; Malabadi *et al*., 2004a,b; Malabadi and van Staden, 2003, 2004, 2005a-d, 2006; Malabadi *et al*., 2006; Malabadi and Nataraja, 2006a,b; Malabadi and Nataraja, 2007). However, very few workers have reported regeneration of plants *via* somatic embryogenesis using secondary needles in conifers (Ruaud *et al*., 1992; Ruaud, 1993; Deb and Tandon, 2002, 2004a, b). No reports of somatic embryogenesis using secondary needles in *Pinus roxburghii* are available in the literature.

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The present study reports the optimized parameters for the initiation, maintenance of embryogenic tissue using secondary needles of mature trees of *Pinus roxburghii* and subsequent regeneration of plantlets. Our results confirmed that cold-pretreatment induced stress conditions and addition of triacontanol in the basal medium has promoted somatic embryogenesis using secondary needles of *P. roxburghii*. This also leads to the conclusion that a non-hormonal factor like cold-pretreatment-induced-stress helps in inducing embryogenic tissue formation in conifers.

MATERIALS AND METHODS

Plant material and initiation of embryogenic culture: Secondary needles from mature trees (10- years old) of *Pinus roxburghii* of 3 genotypes (PR17, PR100 and PR321) were collected from the Western Ghat Forests, India (14°5' to 15° 25' N latitude and 74°45' to 76° E longitude with an average rainfall of 80 cm). Fresh secondary needles were harvested during the month of May where the vegetative growth was found suitable for the collection of needles at least in case of *P. roxburghii*. They were cleansed with 1% Citramide (Sodium hypochlorite 3.5%) for 5 min and rinsed thoroughly with sterilized distilled water. These were surface decontaminated with 70% ethanol for 5 min followed by immersion in 0.5% $HgCl_2$ for 2 min and rinsed 4-times with sterile double distilled water. Longitudinal-sections of approximately 1.0 to 1.5 cm long were cut using sharp sterilized blade or scalpel from secondary needles (basal part was taken and tip of the needles was discarded) for the initiation of embryogenic tissue. These secondary needles sections were cultured individually on full strength (Inorganic salts) DCR (Gupta and Durzan, 1985) basal medium containing 0.2 g L^{-1} polyvinyl pyrollidine (PVP), 2.0 g L^{-1} Gellan gum, 30 g L^{-1} maltose (Analar, Sigma) and 0.2, 0.3 or 0.4% activated charcoal (Sigma) without growth regulators. The cultures were raised in 25 mm×45 mm glass culture tubes (Borosil) containing 15 mL of medium. These cultures were incubated in dark at 4°C and incubated for 3 days. Secondary needle sections after incubation in dark at 4°C and incubated for 3 days were subcultured on full strength DCR basal medium respectively. For the initiation of embryogenic tissue, full strength DCR basal medium was incorporated with different concentrations of NAA (3.76, 5.37, 10.74, 26.85 μM) and 2, 4-D (3.17, 4.52, 9.05, 22.62 μM) with BAP (3.11, 4.44, 8.87, 22.19 μM), TRIA (1, 2, 4, 5, 7, 10, 15, 20, 25, 30 μM), putrescine (0.5, 1, 2, 3, 5, 7, 10, 12 and 15 μM L^{-1}) and 24-epibrassinolide (24-epiBL) concentrations (0.1, 0.5, 1, 2, 5, 10 and 15 μM) singly and in combination was studied. The various combinations of NAA, 2-4, D, BAP, putrescine, TRIA and 24-epiBL were studied for the initiation of embryogenic tissue. On the basis of this preliminary study, the combinations of PGR's were selected for the initiation of embryogenic tissue (full- strength DCR basal medium containing 0.2 g L^{-1} PVP, 2 g L^{-1} Gellan gum, 1 g L^{-1} L-glutamine, 1 g L^{-1} casein hydrosylate, 1 g L^{-1} meso-inositol supplemented with 22.62 μM 2, 4-D, 26.85 μM NAA and 5 μM TRIA) and was used as an initiation medium (I). The growth regulators (BAP, 24-epiBL and putrescine) which induced non-embryogenic tissue were not used for the further experiments since this paper presents only optimum results.

The pH of the medium was adjusted to 5.8 with NaOH or Hcl before Gellan gum was added. The media were then sterilized by autoclaving at 121°C and 1.05 kg cm^{-2} for 15 min. L-glutamine and casein hydrosylate were filter sterilized and added to the media after it had cooled to below 50°C. All the cultures were maintained in the dark at 25±2°C. In all the above experiments medium without BAP TRIA, 24-epiBR and putrescine were treated as a control and all the results were compared against the control. For control, secondary needle sections were cultured directly on the initiation medium without pre-culture with activated charcoal and incubated at 4° for 3 days.

Triacontanol ($CH_3 (CH_2)_{28} CH_2OH$) was obtained from Sigma (St Louis, USA). Stock solution of TRIA was prepared by dissolving 1 mg of TRIA in 0.75 mL of $CHCl_3$ containing 1 drop of Tween 20 and this stock solution was gradually diluted with distilled water to the final volume of 200 mL (Tantos *et al*., 1999). 24-epibrassinolide was purchased from CID Tech. Research Inc., Mississauga, Ontario, Canada (www.cidtech-research.com/brass.html). The stock solutions of 24-epiBrassinolide were prepared in absolute ethanol whereas putrescine was obtained from Sigma Co. St Louis, USA.

Maintenance of embryogenic cultures: The embryogenic tissue showing proembryonal masses was again subcultured onto maintenance medium. The full-strength DCR basal medium containing 30 g L^{-1} maltose, 1.8 g L^{-1} Gellan gum supplemented with 2.26 μM 2,4-D, 2.68 μM NAA and 2 μM TRIA (Maintenance medium) (II) was used for this purpose. On the maintenance medium, embryonal suspensor masses were cultured for 30 days with 2 subcultures. The presence of embryonal masses was determined by morphological and cytological

observations (Malabadi *et al*., 2003a, b; Malabadi *et al*., 2004a, b; Malabadi and van Staden, 2003, 2004, 2005a-d, 2006; Malabadi and Nataraja, 2006a, b).

Partial desiccation: For the partial desiccation, one gram fresh weight of embryogenic tissue (after 15 days on the maintenance medium) of all the three genotypes was transferred to sterile empty Petri dishes (65 mm) containing two sterile Whatman filter paper disks (60 mm). The Petri dishes were sealed with parafilm and kept at 25±2°C and incubated in dark for 24 h to obtain the desired extent of desiccation (Malabadi *et al*., 2003a, b; Malabadi *et al*., 2004a, b; Malabadi and van Staden, 2003, 2004, 2005a-d, 2006; Malabadi and Nataraja, 2006a,b). The relative water content of tissue was calculated as;

$$\frac{\text{Initial weight of tissue-Final weight of tissue after desication}}{\text{Initial weight of tissue}} \times 100$$

Maturation of somatic embryos: After desiccation, the embryogenic tissue was transferred to maturation medium to induce cotyledonary embryo development. The full strength DCR basal medium with 50 g L^{-1} maltose, 37.84 μM ABA and 7 g L^{-1} Gellan gum (III) (Maturation medium) was tested for this purpose (Malabadi *et al*., 2003a, b; Malabadi *et al*., 2004a, b; Malabadi and van Staden, 2003, 2004, 2005a-d, 2006; Malabadi and Nataraja, 2006a,b). All the cultures were placed in the dark at 25±2°C and were maintained for 8 to 12 weeks.

Germination and plantlet recovery: After 12 to 14 weeks of maturation in presence of ABA and higher concentrations of maltose, advanced cotyledonary somatic embryos were picked from the cultures for germination. The germination medium (IV) used was half DCR basal medium with 2 g L^{-1} Gellan gum (Malabadi *et al*., 2003a, b; Malabadi *et al*., 2004a, b; Malabadi and van Staden, 2003, 2004, 2005a-d, 2006; Malabadi and Nataraja, 2006a,b). Somatic embryos were considered germinated as soon as radical elongation occurred and conversion to plantlet was based on the presence of epicotyls. After 4 to 6 weeks on germination medium, plantlets were transferred to vermiculite. Plantlets were placed in growth room under a 16 h photoperiod (50 μmol m^{-2} s^{-1}) for hardening.

Statistical analysis: In all the above experiments each culture tube received a single explant. Each replicate contained 50 cultures and one set of experiment is made up of 2 replicates (100 thin longitudinal secondary needle sections were cultured for each genotype for one set of experiment). All the experiments were repeated 3 times (total 900 cultures for 3 independent experiments of three genotypes). Data presented in the tables were arcsine transformed before being analyzed for significance using ANOVA (analysis of variance, $p<0.05$) or evaluated for independence using Chi-square test. Further, the differences contrasted using a Duncan's multiple range test (a = 0.05) following ANOVA. All statistical analysis was performed using the SPSS statistical software package.

RESULTS AND DISCUSSION

This study demonstrated that combination of stress induction by cold pre-treatment and inclusion of TRIA with basal medium has a great potential of inducing embryogenic tissue in all the three genotypes tested using secondary needles as the explants. Explants cold-pre treated at 4°C and incubated for 3 days on 0.3% activated charcoal induced white-mucilaginous embryogenic tissue on initiation medium (I) supplemented with 22.62 μM 2, 4-D, 26.85 μM NAA and 5 μM TRIA in all the 3 genotypes (PR17, PR100 and PR321) of *Pinus roxburghii* (Table 1) (Fig. 1A). This clearly indicates a positive role of TRIA in inducing the somatic embryogenesis of *P. roxburghii.* On the other hand secondary needle sections even after cold pre-treatment at 4°C and incubated for 3 days on 0.3% activated charcoal failed to produce embryogenic tissue on the initiation medium lacking TRIA. Explants without cold pre-treatment induced non- embryogenic tissue on initiation medium supplemented with various concentrations of TRIA. Therefore, both the cold pre-treatment and the presence of TRIA in the initiation medium triggered the cells in programming towards the somatic embryogenesis. It was also concluded that both cold pre-treatment and the presence of TRIA are dependent processes in influencing the embryogenic potential of secondary needles. The most effective range of TRIA which induced white-mucilaginous embryogenic tissue on initiation medium in all three genotypes was found to be 4-7 μM (Table 1). The highest percentage of explants produced embryogenic tissue in all the three genotypes was 5 μM TRIA (Table 1). Lower or higher concentrations of TRIA supplemented initiation medium have inhibited the somatic embryogenesis. In our separate experiments, cold pre-treated explants failed to produce embryogenic tissue on the initiation medium supplemented with various concentrations of TRIA lacking 2, 4-D and NAA (data not shown). This has led to the conclusion that TRIA alone has only induced cell division but not the somatic embryogenesis. Therefore, the combination TRIA with other growth regulators like 2, 4-D and NAA is very much

Table 1: The effect of various concentrations of TRIA on the initiation of embryogenic tissue in three genotypes of *Pinus roxburghii* cultured on full strength DCR basal medium containing 22.62 µM 2, 4-D and 26.85 µM NAA

	Embryogenic tissue in 3 genotypes** (%)		
TRIA (µM)	PR17	PR100	PR321
1	0	0	0
2	0	0	0
4	2.0±0.1c	2.0±0.01 c	1.0±0.0c
5	51.5±4.09a	46.2±3.2a	65.0±1.9a
7	27.4±8.3b	10.8±5.02b	17.5±10.08b
10	1.0±0.0c	1.0±0.0c	3.2±0.02c
15	0	0	0
20	0	0	0
25	0	0	0
30	0	0	0

Data scored after 6 weeks and represents the mean±SE of at least 3 different experiments. In each column the values with different letter(s) are significantly different ($p<0.5$). **Secondary needles (explants) were incubated at 4°C for 3 days before transferring on the DCR basal medium containing 22.62 µM 2, 4-D and 26.85 µM NAA with different concentrations of TRIA

essential for programming the somatic embryogenesis process at least in case of *Pinus roxburghii* genotypes. Hence TRIA regulates the entire process of somatic embryogenesis in combination with other growth regulators. Explants failed to produce embryogenic tissue on the initiation medium supplemented with various concentrations of BAP, putrescine and 24-epiBL even after the cold pre-treatment at 4°C and incubated for 3 days on 0.3% activated charcoal (data not shown). In all the rest of the combinations of NAA, 2, 4-D and BA, the explants produced white glossy non-embryogenic callus and hence cultures discarded (Data not shown). During May, the secondary needles started sprouting and showed significant growth against the rest of collections. Secondary needles of mature trees serves as the excellent source plant material for somatic embryogenesis since they represent the superior characteristics of mother plants, which is very much needed for the clonal forestry. Longitudinal sections of secondary needles from the basal part have resulted in the formation of embryogenic tissue whereas the tip or upper part of secondary needles resulted in the non-embryogenic tissue. Therefore, it was also concluded that the basal part of secondary needles might be having actively dividing meristematic cells, which are absent in the tip portion of needles. Actively dividing meristematic cells plays an important role in the induction of somatic embryogenesis, which has been demonstrated in a number of plant species including conifers. Furthermore, in a separate experiment, the transverse thin sections of the explants were not that much effective for the initiation of embryogenic tissue and therefore, we preferred to take longitudinal sections. This has resulted in the formation of embryogenic tissue and responsive explant was high enough against transverse sections of explants (data not shown). This might be due the more exposure of explants surface area to the nutrient medium under *in vitro* culture conditions than transverse sections. In case of thin transverse sections of secondary needles having very less diameter, there might be less surface area touching nutrient medium under *in vitro* conditions has influenced the percentage of embryogenesis. Therefore, surface area of explants has been an important factor which influences somatic embryogenesis at least in case of *P. roxburghii* (data not shown).

Culture system for inducing embryogenesis has been developed for many angiosperm and gymnosperm species. To date, the only conclusive evidence on this subject is that a cell culture has to be grown in a medium containing a high concentration of auxin in order to become embryogenic. Young embryos develop more mature stages following a reduction in the concentration of the auxin or its removal from the medium (Thorpe and Stasolla, 2001). Stress induced by cold pre-treatment significantly played an important role in inducing somatic embryos from mature trees has been reported in many plant species including conifers (Malabadi and Nataraja, 2006a, b; Malabadi *et al*., 2004a, b; Malabadi and Nataraja, 2007; Malabadi and van Staden, 2005a-d). Stress induced by cold pre-treatment of secondary needle explants resulted in the formation of embryogenic tissue on the initiation medium in *Pinus kesiya* (Deb and Tandon, 2002, 2004a, b). Therefore, stress induced by environmental stimulus is a common phenomenon for inducing somatic embryogenesis in plant species including conifers (Malabadi and Nataraja, 2007). Somatic embryogenesis can also be triggered by non-hormonal factors for example, various stress conditions such as an electric field (Dijak *et al*., 1986), non-optimal salt concentration (Kiyosue *et al*., 1989), heavy metals (Kamada *et al*., 1989; Kiyosue *et al*., 1990), ethanol or gamma irradiation (Pechan and Keller, 1989), antibiotics (Nakano and Mii, 1993) and many more. Inorganic nitrogen forms such as NO_3^- and NH_4^+ also promotes embryo development (Halperin and Wetherell, 1965; Reinert *et al*., 1967; Smith and Krikorian, 1990; Burza and Malepszy, 1995; Leifert *et al*., 1995; Preece, 1995; Joy *et al*., 1996; Poddar *et al*., 1997). Cold pre-treatment has been used as an environmental stimulus to promote somatic-embryogenesis in many plant species including *Pinus roxburghii* (Malabadi and Nataraja, 2006a, b; Krul, 1993; Tamaszewski *et al*., 1994; Janeiro *et al*., 1995; Malabadi and van Staden, 2006). It was suggested that cold pre-treatment can stimulate the formation of pro-embryogenic masses in cell cultures, which results in an enhancement of somatic embryogenesis (Krul, 1993). Recently the possible inter-relationship between

exogenous calcium during cold-pretreatment-induced somatic embryogenesis was investigated using vegetative shoot apices of mature trees of *Pinus patula*. These results suggest that embryogenic cells require minimal concentrations of calcium during pre-treatment for the expression of the cold-enhancement of embryogenesis in *Pinus patula* (Malabadi and van Staden, 2006). The cold pre-treatment of shoot apical domes at 2°C and incubated for 3 days on 0.3% activated charcoal induced white-mucilaginous embryogenic callus when subcultured on the DCR basal medium supplemented with 23 μM 2,4-D, 27 μM NAA and 9 μM BA in 3 different genotypes of *Pinus patula* (Malabadi and van Staden, 2003, 2004, 2005a-d; Malabadi *et al*., 2006). Other positive effects of cold pre-treatment on the initiation of embryogenic tissue could be the release of substances necessary for embryogenesis, mainly amino acids and shock thermic proteins (Xie *et al*., 1997). However, the role of cold pre-treatment in regulating the enhancement of somatic embryogenesis is not well understood (Malabadi *et al*., 2004a, b) and warrants further investigation of gene expression studies in regulating the process. This will certainly highlights the mechanism in understanding the role of cold pre-treatment induced proteins.

The positive effect of TRIA as a growth regulator was studied by various workers on both herbaceous and woody plant micropropagation system (Tantos *et al*., 2001; Fraternale *et al*., 2002). However, there is only one report of induction of somatic embryogenesis in conifers using triacontanol (Malabadi *et al*., 2005). This is the second report of TRIA induced somatic embryogenesis in *Pinus roxburghii*. The plant growth stimulating property of TRIA in plants was already reported by Tantos *et al*. (2001). An average of 2-3 shoots with an increase in the number of nodes and leaves were noticed in *Capsicum frutescens* and *Decalepis hamiltonii* after 5 μg L^{-1} TRIA treatment (Reddy *et al*., 2002). TRIA was also found to promote shoot multiplication in *Melissa officinalis* (Tantos *et al*., 1999). Lower concentrations of TRIA may be biologically effective because of the sensitivity of whole explants to extremely low doses of TRIA (Biernbaum *et al*., 1998). TRIA, a metabolite of TRIA or a secondary messenger moves rapidly in plants after initial application and influences enzymes related to carbohydrates metabolism in plants (Ries and Wert, 1982, 1988) and growth processes (Ries *et al*., 1977). In the present study, the lower concentration of TRIA does not have any significant effect on somatic embryogenesis. The increase in the concentration of TRIA from 4-7 μM resulted in the formation of white mucilaginous embryogenic tissue might be due to the meiotic division of actively dividing meristematic cells. This also confirms the results of our previous studies where 7-10 μM of triacontanol induced somatic embryogenesis in *Pinus kesiya* (Malabadi *et al*., 2005).

After 4-6 weeks of culture on the initiation medium (I), the white mucilaginous embryogenic tissue was subcultured on to the maintenance medium for further proliferation of embryonal suspensor masses (Fig. 1B). Full- strength DCR basal medium supplemented with 30 g L^{-1} maltose, 1.8 g L^{-1} Gellan gum with reduced growth regulators of 2.26 μM 2,4-D, 2.68 μM NAA and 2.0 μM TRIA was used for this purpose. This improved the development of proembryos on the maintenance medium (II) (Fig. 1B). Embryogenic cultures were maintained for 2-4 weeks on the maintenance medium (II) and used for the maturation experiments. The proemryos which developed on the maintenance medium (II) did not grow until they were transferred on a maturation medium containing higher concentrations of maltose, ABA and Gellan gum respectively. Before transfer to the maturation medium, the white mucilaginous embryogenic tissue showing proembryos was partially desiccated for 24 h (Malabadi and Nataraja, 2006a, b). The partial desiccation has improved the maturation of somatic embryos and newly developed cotyledonary somatic embryos were obtained after 8 to 12 weeks. Advanced cotyledonary somatic embryos were picked from the cultures for germination (Fig. 1C). The percentage of somatic embryogenesis was not similar in all the three genotypes of *Pinus roxburghii* (Table 2). The highest percentage of somatic embryogenesis (65.0%) was recorded in case of PR321 genotype, with a total of 3 somatic seedlings recovered per gram fresh weight of embryogenic tissue (Table 2). The genotype PR17, 51.5% of somatic embryogenesis produced a total of 9 somatic seedlings per gram fresh weight of embryogenic tissue (Table 2). The lowest percentage of somatic embryogenesis (46.2%) was recorded with 4 of somatic embryos per gram fresh wt of tissue was recorded in a genotype PR100 (Table 2). The germination medium (IV) used was half DCR basal medium with 2 g L^{-1} Gellan gum (Malabadi *et al*., 2003a, b; Malabadi *et al*., 2004a, b;

Table 2: Somatic embryogenesis and seedling recovery in three genotypes of *Pinus roxburghii* using secondary needles of mature trees as the explants for the initiation of embryogenic tissue

Genotype	Somatic embryogenesis (%)	Somatic embryos recovered per gram fresh wt of embryogenic tissue	Seedlings recovered per gram fresh wt of embryogenic tissue
PR17	51.5±4.0a	15.0±1.8b	9.0±0.3b
PR100	46.2±3.2a	10.0±1.0b	4.0±0.1c
PR321	65.0±1.9a	11.0±1.6b	3.0±0.1c

Data scored after 14 weeks and represents the mean±SE of at least 3 different experiments. In each column the values with different letter(s) are significantly different ($p<0.05$)

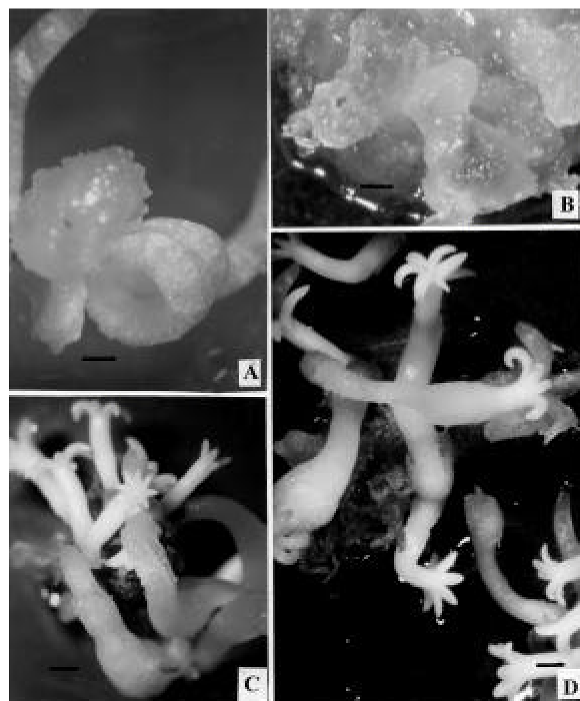

Fig. 1: Somatic embryogenesis using secondary needles as explants in *Pinus roxburghii.* (a) Longitudinal thin section of secondary needle showing embryogenic tissue on initiation medium (10 mm = 0.97 mm). (b) Luxuriant growth of embryogenic tissue on maintenance medium (II) (10 mm = 4 mm). (c) Advanced cotyledonary stages of somatic embryos on maturation medium seen under the microscope (10 mm = 10.97 mm) and (d) Somatic seedlings on germination medium

Malabadi and van Staden, 2003, 2004, 2005a-d, 2006; Malabadi and Nataraja, 2006a,b). Somatic embryos were considered germinated as soon as radical elongation occurred and conversion to plantlet was based on the presence of epicotyls (Fig 1-D). After 4 to 6 weeks on germination medium, plantlets were transferred to vermiculite. Plantlets were placed in growth room under a 16 h photoperiod (50 µmol m^{-2} sec^{-1}) for hardening.

In conclusion, the embryogenic system was established by the influence of TRIA and stress induced-cold-pretreatment of vegetative explants like secondary needles of *Pinus roxburghii*. The use of TRIA in the initiation medium has played an important role in programming the cells towards effective embryogenesis. This study clearly indicates the positive role of TRIA as a best growth regulator influencing somatic embryogenesis in many plant species including conifers.

ACKNOWLEDGMENTS

We are grateful to the Head, Department of Botany, Karnatak University, Dharwad, India for providing all the necessary facilities for this research. We also acknowledge the help during the collection of plant material from Western Ghat during our experiments.

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