

Influence of Triacontanol on Somatic Embryogenesis of *Pinus roxburghii* Sarg.

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Malabadi, R. B. and Nataraja, K. 2007. Influence of Triacontanol on Somatic Embryogenesis of *Pinus roxburghii* Sarg. *Baltic Forestry* 13(1): 39–44.

Abstract

This work highlights the influence of triacontanol on somatic embryogenesis and rooting of somatic seedlings in *Pinus roxburghii*. Mature zygotic embryos produced white-mucilaginous embryogenic callus when cultured on full strength LM (Litvay *et al.*, 1985) basal medium supplemented with 90mM maltose, 2.0g^l⁻¹ Gellan gum, 9.0μM 2,4-D and 7 μg^l⁻¹ triacontanol. On subculture of such embryogenic callus on the maintenance medium (II) containing 2.0μM 2, 4-D and 2.0μg^l⁻¹ triacontanol induced pro-embryos. The highest percentage of somatic embryogenesis (83.5%) was recorded in PR810 genotype. Somatic embryos were successfully germinated on half strength LM basal medium without growth regulators. Therefore, triacontanol can be used as an effective growth regulator for inducing somatic embryogenesis and rooting in *Pinus roxburghii*.

Key words: Chir pine, *Pinus roxburghii*, Somatic embryogenesis, Tissue culture

Abbreviations: ABA-Absciscic acid, 2, 4-D-Dichlorophenoxy acetic acid, I-Initiation medium, II-Maintenance medium, III-Maturation medium, IV-Germination medium, TRIA-Triacontanol

Introduction

Clonal propagation by somatic embryogenesis can capture the benefits of breeding or genetic engineering programs to improve wood quality, quantity and uniformity. Somatic embryogenesis is the most promising technology to multiply high-value forest trees and is expected to play an important role in increasing productivity, sustainability and uniformity of future forests throughout the world (Malabadi *et al.* 2002, 2003a-b, 2004, 2005a). *Pinus roxburghii* Sarg. (Chir pine) is an important Indian pine species of western-Himalays. Some genotypes have tremendous biomass potential and oleoresin prospects (Malabadi and Nataraja 2006). In *Pinus* species somatic embryogenesis has been established in *Pinus patula* (Malabadi and van Staden 2003, 2005a-d; Malabadi *et al.* 2003b, 2006), *Pinus kesiya* (Malabadi *et al.* 2002, 2003a, 2004, 2005a) and *Pinus palustris* (Nagmani *et al.* 1993) using mature, immature and shoot apical domes respectively. A very low percentage (0.5 to 1.0%) of somatic embryogenesis in *Pinus roxburghii* (Sarg.) was also reported by using immature zygotic embryos (Mathur *et al.* 2000; Arya *et al.* 2000).

Triacontanol (TRIA), a long 30-carbon primary alcohol is a naturally occurring plant growth promoter (Ries *et al.* 1977, Ries and Houtz 1983, Ries and Wert 1982, 1988). Its effect on tissue culture was tested on *Pinus kesiya* (Malabadi *et al.* 2005a), *Dendrobium nobile* (Malabadi *et al.* 2005b), *Costus speciosus* (Malabadi *et al.* 2005c), woody plants (Tantos *et al.* 1999, 2001) and *Bupleurum fruticosum* (Fraternali *et al.* 2002, 2003). Reddy *et al.* (2002) reported that TRIA could be used as an effective growth promoter in the micropropagation of *Capsicum frutescens* and *Decalepis hamiltonii*. However, a very few reports are available with respect to the effect of TRIA in the micropropagation and tissue culture. Therefore, the aim of this work is to explore the applicability of TRIA in the micropropagation and improving the rooting of somatic seedlings of *Pinus roxburghii*. Our results demonstrate that TRIA with its multiple functions (shoot/rooting) can be effectively used as a growth promoter in the induction of somatic embryogenesis in conifers, which play an important role in solving the problems of rooting of conifers. This has great potential in commercial forestry.

Materials and methods

Plant material

Pinus roxburghii (Sarg.) seeds of 3 genotypes (PR481, PR35 and PR810) belonging to different families of open pollinated trees were procured from Forest Department, Solan, Himachal Pradesh state, India. The seeds were surface washed with 1% Citramide for 2 min, and washed thoroughly with sterilized –distilled water for three times. Seeds were further treated with sodium hypochlorite solution (4-5% available chlorine) for 2 min, rinsed five times with sterile double distilled water and stratified with 6% hydrogen peroxide for 24hr. Prior to dissection of embryos, seeds were surface decontaminated sequentially with 0.1% HgCl₂ for 2 min, immersed in 70% ethanol for 3 min and finally rinsed thoroughly five times with sterile-double distilled water. Triacontanol (CH₃(CH₂)₂₈CH₂OH) was obtained from Sigma (St Louis, USA). Stock solution of TRIA was prepared by dissolving 1mg of TRIA in 0.75ml of CHCl₃ containing 1 drop of Tween 20 and this stock solution was gradually diluted with distilled water to the final volume of 200ml (Tantos *et al.* 1999).

I) Culture medium and initiation of embryogenic cultures

Mature zygotic embryos of 3 genotypes (PR481, PR35 and PR810) of *Pinus roxburghii* Sarg. were cultured individually on both half and full strength inorganic salts Litvay's basal medium (Litvay *et al.* 1985) containing 2.0 g l⁻¹ Gellan gum (Sigma), 90mM maltose (Hi-media, Mumbai), 1g l⁻¹ L-glutamine, 1g l⁻¹ casein hydrosylate, 0.5g l⁻¹ meso-inositol, 0.2g l⁻¹ p-aminobenzoic acid and 0.1g l⁻¹ folic acid. The medium was supplemented with 9.0μM 2,4-D and various concentrations of TRIA (1, 2, 4, 5, 7, 10, 15, 20, 25, 30μg l⁻¹) singly and in combination without any other growth hormones. The cultures were raised in 25mmX145mm glass culture tubes (Borosil) containing 15ml of the medium and maintained in dark for 4-6 weeks at 25± 3°C with a relative humidity of 55-60%. Nutrient medium without TRIA served as the control. 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 1.08 Kg/cm² for 15min. L-glutamine, p-aminobenzoic acid and TRIA were filter sterilized and added to the media after it had cooled to below 50°C.

All the cultures were examined for the presence of embryonal suspensor masses by morphological and cytological observations of callus. The cultures showing white mucilaginous embryogenic callus were identified and subcultured on the initiation medium for further two weeks for the better development of embryonal suspensor masses. The full strength inorganic salts LM basal medium (Litvay *et al.* 1985) supple-

mented with 9.0μM 2, 4-D and 7 μg l⁻¹ TRIA (initiation medium I) was used for this purpose.

II) Maintenance of embryogenic cultures

The white-mucilaginous embryogenic callus developed on the above initiation medium (I) was subcultured on maintenance medium (II). The full strength (inorganic salts) LM basal medium containing 130mM maltose, 4g l⁻¹ Gellan gum and supplemented with 2μM 2,4-D and 2g l⁻¹ TRIA (maintenance medium II) was used for this purpose. On maintenance medium, the white mucilaginous embryogenic callus with embryonal suspensor masses was maintained for 3 to 4 weeks with two subcultures. All the cultures were maintained in dark and microscopic observation of cultures was conducted to ensure the development of proembryo.

III) Maturation of somatic embryos

The white-mucilaginous embryogenic callus showing the proembryo development was transferred to maturation medium to induce cotyledonary embryo development. The full strength (inorganic salts) LM basal medium supplemented with 180mM maltose, 60μM ABA and 8 g l⁻¹ Gellan gum (maturation medium III) was tested for this purpose. All the cultures were again maintained in the dark for 8 to 12 weeks. Microscopic observation was conducted to ensure the development of somatic embryos.

IV) Germination and recovery of plantlets

After 8 to 12 weeks of maturation in the presence of ABA, higher concentration of maltose and Gellan gum, advanced cotyledonary somatic embryos were taken from the cultures for germination. The germination medium (IV) used was half strength (inorganic salts) LM basal medium with 2 g l⁻¹ Gellan gum. Somatic embryos were considered germinated as soon as radicle elongation occurred and conversion to plantlet was based on the presence of an epicotyl. After 6 weeks on germination medium, the plantlets were directly transferred to vermiculite. Plantlets were placed in a growth room under a 16hr photoperiod (50μ mol m⁻² s⁻¹) for hardening.

Statistical analysis

In all the above experiments each culture tube received a single embryo. Each replicate contained 50 cultures and one set of experiment is made up of 3 replicates (150 cultures). All the experiments were repeated three times (total 3x150=450 cultures). The percentage data were arcsine transformed before being statistically analyzed by using ANOVA. Data presented in the tables were analyzed for significance using ANOVA and the differences contrasted using a Duncan's multiple range test. All statistical analysis was performed at the 5% level using SPSS statistical software package.

Results

It was found that TRIA has a high potential for inducing embryogenic tissue using mature zygotic embryos in three genotypes of *Pinus roxburghii*. In the present study mature zygotic embryos cultured on full strength LM basal medium supplemented with 9.0µM 2,4-D and lower concentrations of TRIA (4, 5 and 7µg l⁻¹) induced white mucilaginous embryogenic callus in all the three genotypes (Table 1) (Fig. 1 A, B, C, D). Microscopic observation of callus revealed actively dividing elongated cells with few undergoing cleavage polyembryony. The presence of actively di-

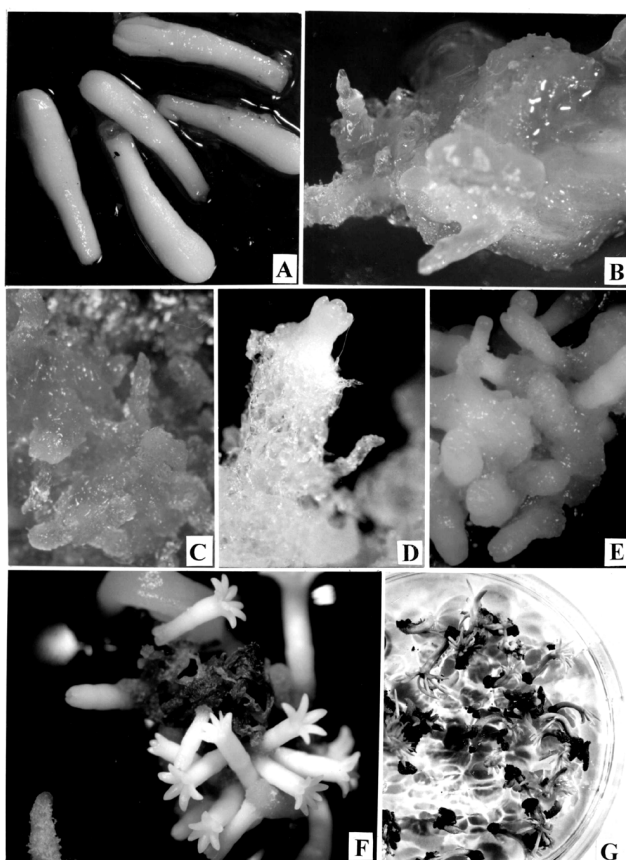


Figure 1. Plant regeneration via somatic embryogenesis using TRIA in *Pinus roxburghii*
 A-Mature zygotic embryos dissected from seed (10mm=0.97mm). B-White mucilaginous embryogenic callus on initiation medium (I) (10mm=8mm). C-Luxuriant growth of embryogenic callus on maintenance medium (II) (10mm=4mm). D-Embryogenic callus showing the development of somatic embryos on maturation medium (III) (10mm=3.5mm). E-Variou developmental stages of somatic embryos seen under the microscope (10mm=10.97mm). F-Development of advanced cotyledonary somatic embryos on maturation medium (III) (10mm=9.3mm). G-Somatic seedlings on germination medium (IV)

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 LM basal medium containing 9.0µM 2,4-D

TRIA (µg l ⁻¹)	Embryogenic tissue in 3 genotypes (%)		
	PR481	PR35	PR810
1	0	0	0
2	0	0	0
4	8.0±0.5a	2.0±0.01 b	17.3±1.27 c
5	31.5±3.89e	16.2±1.2c	55.0±5.9e
7	67.4±8.3d	40.8±5.02 e	87.5±10.08d
10	3.0±0.06b	1.0±0.0	3.2±0.08b
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 letters are significantly different (P<0. 5).

viding elongated cells with a cleavage polyembryony is a prerequisite for somatic embryogenesis in conifers. Mature zygotic embryos induced higher percentage of white glossy non-embryogenic callus with lowest (1, 2 µg l⁻¹) and higher concentrations of TRIA (15, 20, 25, 30 µg l⁻¹) on full strength LM basal medium in all three genotypes (Table 1). Microscopic observation of callus showed round, oval, globular with few elongated cells. The subculture of callus showed only proliferation and failed to produce embryonal suspensor masses even after 6 to 8 weeks on full strength LM basal medium. The most effective range of TRIA, which induced white-mucilaginous embryogenic callus on full strength LM basal medium containing 9.0µM 2,4-D was 4-7 µg l⁻¹ in all three genotypes (Table 1). The percentage of initiation of embryogenic tissue was not similar in all the three genotypes of *Pinus roxburghii*. In the PR481 genotype, 67.4% of embryogenic tissue was recorded (Table 1). The highest percentage of embryogenic tissue (87.5%) was initiated in PR810. Lowest percentages of embryogenic tissue (40.8%) were initiated in case of PR35 genotype (Table 1). Therefore, 9.0µM 2, 4-D and 7 µg l⁻¹ TRIA supplemented full strength LM basal medium is the optimum for the initiation of embryogenic tissue in *Pinus roxburghii* (Table 1). On the other hand mature zygotic embryos induced white glossy non embryogenic callus on half strength LM basal medium supplemented with 9.0µM 2,4-D with different concentrations of TRIA in all the 3 genotypes. The subculture of callus showed maximum proliferation but failed to produce embryonal suspensor masses on initiation medium (I).

Mature zygotic embryos induced white mucilaginous embryogenic callus on initiation medium (I) within 5 weeks of culture. The callus was subcultured on the same medium for every week for the further proliferation and inducing cleavage polyembryony.

The white mucilaginous embryogenic callus was subcultured on maintenance medium (II) for further development of embryonal suspensor masses. The full strength LM basal medium supplemented with 130 mM maltose, 4 g l⁻¹ Gellan gum with reduced concentrations of 2 µM 2,4-D and 2 g l⁻¹ TRIA (maintenance medium II) was used for this purpose. On the maintenance medium (II) embryonic suspensor masses showed the development of proembryos within three weeks of culture. The cultures were maintained for 4 weeks with two subcultures. The proembryos developed on the maintenance medium (II) could not grow further, until they were transferred on a medium with enhanced maltose, ABA and Gellan gum respectively. The full strength LM basal medium supplemented with 180 mM maltose, 60 µM ABA and 8 g l⁻¹ Gellan gum was used as a maturation medium (III). The callus developed somatic embryos on maturation medium after a period of 9 to 12 weeks (Fig. 1 C, D and E). The percentage of somatic embryogenesis was not similar in all the three genotypes of *Pinus roxburghii* (Table 2). The highest percentage of somatic embryogenesis (83.5%) was recorded in case of PR810 genotype, with a total of 42 somatic seedlings recovered per gram fresh weight of embryogenic tissue (Table 2). The genotype PR481, 62.8% of somatic embryogenesis produced a total of 29 somatic seedlings per gram fresh weight of embryogenic tissue (Table 2). Lowest recovery of somatic seedlings (16) was recorded with 35.7% of somatic embryogenesis in a genotype PR35 (Table 2).

After 9 to 12 weeks of maturation, the advanced cotyledonary somatic embryos were picked from the cultures for germination (Fig. 1 D, E, and F). The half strength LM basal medium with 2 g l⁻¹ Gellan gum with

out growth regulators was used as a maturation medium (IV). No post maturation treatment was applied to mature somatic embryos for germination. After 6 weeks on germination medium, the plantlets were recovered and hardened in all the three genotypes of *Pinus roxburghii*.

Discussion and conclusions

The plant growth stimulating property of TRIA in plants was already reported by Tantos *et al.* (2001). However, there is only one report of induction of somatic embryogenesis in conifers using triacontanol (Malabadi *et al.* 2005a). This is the second report of TRIA induced somatic embryogenesis in *Pinus roxburghii*, an important conifer of India. The positive effect of TRIA as a growth regulator was studied by various workers on herbaceous and woody plant micropropagation (Tantos *et al.* 2001; Fraternali *et al.* 2002). On average 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 (Bierbaum *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 1988) and growth processes (Ries *et al.* 1977). In the present study the lowest concentration of TRIA (1, 2 µg l⁻¹) also stimulated the formation of white non-embryogenic callus. The increase in the concentration of TRIA from 4-7 µg l⁻¹ resulted in the formation of white mucilaginous embryogenic callus and formed elongated cells. These elongated cells might have programmed towards embryogenesis leading to successful cleavage polyembryogenesis. This also confirms the results of our previous studies where 7-10 µg l⁻¹ of triacontanol induced somatic embryogenesis of *Pinus kesiya* (Malabadi *et al.* 2005a).

The other factors, which influence the initiation of embryogenic cultures were maltose and glutamine. Maltose is primarily linked to more effective white callus formation and subsequently formed embryogenic cells (Norgaard 1997, Li *et al.* 1998). The beneficial effect of maltose derives from its slow hydrolysis in the nutrient media. This could be a factor that limits callus carbon nutrition. Glutamine is a common organic nitrogen source and provides reduced nitrogen source in a form that is energetically less costly to assimilate than nitrate or ammonium (Leustek and Kirby 1988).

Table 2. Somatic embryogenesis and seedling recovery in three genotypes of *Pinus roxburghii*

Genotype	Somatic embryogenesis (%)	Somatic embryos recovered per gram fresh wt of embryogenic tissue	Seedlings recovered per gram fresh wt of embryogenic tissue
PR481	62.8±7.95 a ^z	35.0±3.85 b	29.0±2.63 a
PR35	35.7±4.95 b	20.0±1.85 c	16.0±1.12 d
PR810	83.5±9.86 e	54.6±5.60 a	42.0±5.39a

^zData scored after 6 weeks and represents the mean ±SE of at least 3 different experiments.

In each column the values with different letters are significantly different (P<0.05).

The higher concentrations of maltose, Gellan gum and reduced growth regulators improved the development of proembryos on the maintenance medium (II) due to reduced water availability of the medium (Klimaszewska and Smith 1997; Klimaszewska *et al.* 2000a). In conifer somatic embryogenesis it is a common practice to restrict water availability by exposing embryogenic tissue to maturation media of high osmolality (low water potential). The somatic embryos of *Pinus strobus* mature in large numbers on media with a high concentration of Gellan gum. Somatic embryos maturing on such medium were characterized by lower water potential and could germinate at a higher rate without any post maturation treatment being applied (Klimaszewska *et al.* 2000b).

The embryogenic tissue was initiated and established using mature zygotic embryos and TRIA in three genotypes of *Pinus roxburghii*. TRIA induced embryogenesis improved both initiation of embryogenic tissue and the rooting of recovered plantlets. Therefore, TRIA can be effectively used as a growth promoter in conifer somatic embryogenesis for the improvement of existing protocols to induce higher percentage of embryogenic tissue. This protocol is very simple, reproducible and suitable for *Agrobacterium*-mediated genetic transformation.

Acknowledgements

We are grateful to the Head, Department of Botany, Karnatak University, Dharwad, India for providing all the necessary facilities for this work. Rinu Thomas, Nancy Miranda and Shahnaz were warmly acknowledged for every help in this investigation.

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Received 21 April 2006

Accepted 17 May 2007

ВЛИЯНИЕ ТРИАКОНТАНОЛА НА СОМАТИЧЕСКИЙ ЭМБРИОГЕНЕЗ *PINUS ROXBURGHII* SARG.

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Резюме

Представленная работа выявляет влияние триаконоланола на соматический эмбриогенез и укоренение соматических семян *Pinus roxburghii*. Зрелые зиготные эмбрионы продуцировали белые слизистые эмбриогенные калусы, выращиваемые на ЛМ основной среде, дополненной 90mM мальтозой, 2.0g^l⁻¹ Гелановой камедью, 9.0μM 2,4-D и 7 μg^l⁻¹ триаконоланола. На субкультуре эмбриогенного калуса в питательной среде (II), содержащей 2.0μM 2, 4-D и 2.0μg^l⁻¹ триаконоланола, выявлялись про-эмбрионы. Наиболее высокий процент соматического эмбриогенеза (83.5%) определен у PR810 генотипа. Соматические эмбрионы были успешно пророщены на полуконцентрированной ЛМ основной среде без регуляторов роста. Таким образом, триаконоланола может использоваться в качестве эффективного регулятора роста индуцирующего соматический эмбриогенез и укоренение *Pinus roxburghii*.

Ключевые слова: *Pinus roxburghii*, соматический эмбриогенез, тканевая культура