The Americas Journal of Plant Science and Biotechnology ©2008 Global Science Books

A New Approach Involving Salicyclic Acid and Thin Cell Layers for Cloning Mature Trees of *Pinus roxburghii* (Chir Pine)

Ravindra B. Malabadi[1,3*] • Jaime A. Teixeira da Silva[2] • K. Nataraja[1]

[1] Division of Plant Biotechnology, Department of Botany, Karnatak University, Pavate nagar, Dharwad-580003, Karnataka state, India
[2] Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Ikenobe 2393, Kagawa-ken, 761-0795, Japan
[3] Present address: Department of Wood Sciences, 4th floor, Forest Sciences Centre # 4030-2424 Main Mall, University of British Columbia, Vancouver, V6T 1Z4, BC, Canada
Corresponding author: * **Ravindra.Malabadi@forestry.ubc.ca**

ABSTRACT

The cloning of mature conifers by somatic embryogenesis is potentially a low-cost method and is capable of producing unlimited numbers of normal individuals. This paper highlights for the first time the role of salicyclic acid (SA) on induction of embryogenic cultures using thin cell layers of vegetative shoot apices of mature trees of *P. roxburghii*. Embryogenic cultures were successfully initiated at a high percentage (12 to 31%) in 10 different genotypes following the addition of 1.0 mg l^{-1} SA to DCR basal medium. On the other hand, DCR basal medium lacking SA (control) showed a lower percentage (0 to 13%) of somatic embryogenesis in these 10 genotypes. SA improved the initiation of embryogenic cultures and also resulted in the formation and development of more pro-embryos on maintenance medium than in our previous protocols of somatic embryogenesis which lacked SA. Higher concentrations (2.0 and 5.0 mg^{-1}) of SA resulted in the browning of cultures, and thus had an inhibitory effect. These results indicate a positive role of SA as a growth regulator in conifer somatic embryogenesis.

Keywords: embryogenic culture, forestry, somatic embryogenesis

OVERVIEW

Forests are very important resources to the world economy, an integral part of human life, and a vital component of biodiversity. Forest trees in particular are renewable sources of fuel wood, timber and energy. Due to the rapid growth of populations and humans' desire to progress, often at the expense of nature, there has been a tremendous reduction in forest cover. There are alarming threats to forests in particular and biodiversity in general. To maintain and sustain forest vegetation, conventional approaches have been exploited in the past for propagation and improvement. However, such efforts are confronted with several inherent bottlenecks because forest trees are generally slow-growing, long-lived, sexually self-incompatible and highly heterozygous plants. Due to the prevalence of high heterozygosity in these species, a number of recessive deleterious alleles are retained within populations, resulting in high genetic load and inbreeding depression (Giri *et al*. 2004). This limits the use of traditional breeding methods such as selfing and backcrossing, and makes it difficult to fix desirable alleles in a particular genetic background. Zygotic rejuvenation occurs naturally in trees and happens when mature tissues undergo a series of genetic changes during meiosis that result in the reversal of epigenetic changes that occur during maturation. The zygote that is formed following gamete fusion demonstrates juvenile characters. Rejuvenation presents a difficult obstacle to regenerating mature woody conifers. One of the major problems facing the implementation of clonal forestry is the phase change. Phase change is defined as the series of changes that occur when a tree passes from the juvenile phase in which there is the ability to initiate growth of the plant and a general absence of flowering to the mature phase where flowering is common, and the ability to initiate any plant growth or development is lost or dramatically reduced. These changes are epigenetic and have a large effects on the physiology of tree. Tissues that have undergone a phase change are not easily converted back to a juvenile state (Wareing 1987). Juvenile characteristics such as rooting/shooting potential may be preserved at the base of plants in ontogenetically young tissues (meristems), while maturation occurs in the periphery of the plant in ontogenetically older but chronologically young tissues.

Against the background of the limitations of long juvenile phases and life span, cloning of mature trees using apical meristematic tissue has been recently achieved in many recalcitrant pines such as *P. kesiya* (Malabadi *et al.* 2004), *P. wallichiana* (Malabadi and Nataraja 2007a), *P. patula* (Malabadi and van Staden 2003), *P. sylvestris* (Aronen *et al.* 2007), *P. pinea* (Malabadi *et al*. unpublished), *P. pinaster* (Malabadi *et al*. unpublished), including *Pinus roxburghii* (Chir pine) (Malabadi 2006; Malabadi and Nataraja 2006, 2007a, 2007b). Apical meristematic cells may retain a high enough degree of juvenility to make them more amenable to cloning of mature conifers. The capacity of excised meristems with actively dividing cells undergoing an embryogenic pathway was greatly influenced by the time of explant collection immediately after bud break. Somatic rejuvenation does not occur naturally in trees. To achieve somatic rejuvenation, it would be necessary to reverse the epigenetic changes that occurred during maturation by repeated application of a physical (cold or heat treatment) or chemical (growth regulators) stimulus to the mature tissue during cloning. To ensure that somatic rejuvenation had occurred, the tissue would need to be matured to ensure that the tissue underwent all the normal stages of maturation in the correct sequence over the normal time scale under *in vitro* conditions.

P. roxburghii is one of the most important pine species distributed throughout all parts of India. *P. roxburghii* not only provides timber, fuel wood and pulpwood, but also meets the demand for packing cases, stakes for vegetable cultivation, bedding for cattle sheds, and cushion material

Received: 10 June, 2008 Accepted: 23 June, 2008.

Original Research Paper

for packaging of fruits and vegetables. Resin obtained from Chir pine is a product of great industrial importance as it is used in soap, paper and pharmaceutical and paint industries (Sharma *et al*. 2007). The present investigation reports for the first time the role of salicyclic acid (SA) as a growth regulator for the induction of somatic embryos and the regeneration of plantlets from slices (0.5-1.0 mm) taken from the domes (zones) of vegetative shoot apices of mature *P. roxburghii* trees.

METHODOLOGY

Shoot apical domes from mature trees (14-years old) of 10 *P. roxburghii* genotypes (these are some local genotypes of *P. roxburghii* from India provided by local breeders), namely PR811, PR805, PR821, PR32, PR76, PR193, PR46, PR51, PR05, and PR92 (explanation of nomenclature: e.g. PR811 = *P. roxburghii* line No. 811) were collected from the Western Ghat Forests (material was collected from these forests according to the forest pine breeders since plantation were created inside the Western Ghat Forest area), India (14°5′ to 15°25′ N latitude and 74°45′ to 76°15′ E longitude with an average rainfall of 80 cm). Apical domes were harvested during April, surface-sterilized with 1% citramide (sodium hypochlorite with 3.5% (v/v) active chlorine) for 5 min and rinsed thoroughly with sterile double distilled water (SDDW). A second surface decontaminated was performed with 70% ethanol for 5 min followed by immersion in 0.5% $HgCl_2$ for 2 min and rinsed 4 times with SDDW. Transverse-thin sections or thin cell layers (tTCLs) approximately 0.5-1.0 mm thick were cut using a sharp sterilized blade or scalpel from shoot apical domes (only 2 to 4 sections from the apical section only) and cultured on Petri dishes individually on DCR (Gupta and Durzan 1985) basal medium containing 0.2 gl^{-1} polyvinylpyrollidine (PVP), 1.5 gl^{-1} Gellan gum (Sigma), 87 mM maltose (Analar) and 0.3% activated charcoal (Sigma), without plant growth regulators. These cultures were incubated in the dark at 4°C for 3 days. Cold-pretreated explants (Malabadi and van Staden 2003; Malabadi *et al*. 2004; Malabadi and Nataraja 2007a, 2007b, 2007c, 2007d) were then subcultured on induction medium in Petri-dishes, i.e. 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} *myo*-inositol supplemented with 22.6 µM 2,4-D, 26.8 µM NAA, 8.9 µM BAP (Malabadi and van Staden 2003; Malabadi *et al.* 2004) and different concentrations of SA (Sigma) (0.1, 0.2, 0.4, 0.5, 1.0, 2.0, 5.0 mg l^{-1}), for the initiation of embryogenic tissue. The control was induction medium without SA. All cultures were maintained for 20 to 30 days in the dark at 25 ± 2°C. Control treatments did not contain any SA. In all the experiments, each replicate contained 50 cultures and one set of experiments consisted of two replicates (total 100 cultures for one experiment) for each *P. roxburghii* genotype (PR811, PR805, PR821, PR32, PR76, PR193, PR46, PR51, PR05, and PR92). All the experiments were repeated in triplicate and **Table 1** represents the average of three independent experiments. Data presented in **Table 1** were analyzed for significance using ANOVA (analysis of variance, $p<0.05$) or evaluated for independence using the Chi-square test. Further, the differences in means were contrasted using Duncan's multiple range test (α = 0.05) following ANOVA. All statistical analyses were per-formed using the SPSS statistical software package.

After the microscopic identification of embryogenic cells showed cleavage polyembryony, which is a pre-requisite for somatic embryogenesis of conifers, the embryogenic mass of cells was separated from the rest of the tissue, and subcultured on maintenance medium (full-strength DCR basal medium containing 87 mM maltose, 2 g l^{-1} Gellan gum supplemented with 2.26 µM 2,4-D, 2.68 µM NAA, 0.88 µM BA (Malabadi and Nataraja 2006, 2007a, 2007b) and 0.5 mg l^{-1} SA (maintenance medium lacking SA was the control) for the further proliferation of callus. Maintenance of embryogenic cultures involved the subculture of embryogenic callus with proembryonal masses onto maintenance medium every three weeks. The percentage of somatic embryogenesis in **Table 1** was calculated as the responsive callus-based embryogenesis. Therefore, 5 g of embryogenic tissue of each genotype was aseptically chopped into 100 pieces and subcultured on maintenance medium for the growth of callus. From 100 pieces, the number of pieces that grew and formed SE was recorded and calculated as a percentage. Before maturation, the embryogenic callus was partially desiccated for 24 h. During partial desiccation, 1 g fresh weight of embryogenic tissue of each genotype was transferred to a sterile empty Petri dish (60 mm diam.) containing two sterile Whatman filter paper disks (50 mm) (Schleicher and Schuell, qualitative circles). The Petri dishes were sealed with Parafilm and kept at 25 ± 2°C in the dark for 24 h to obtain the desired extent of desiccation (Malabadi and van Staden 2003; Malabadi and Nataraja 2006a, 2006b). Partially desiccated tissue was transferred to maturation medium (DCR basal salts containing 175 mM maltose, 80 µM ABA and 9 gl^{-1} Gellan gum) for further development (Malabadi and Nataraja 2006a, 2006b). After 8 to 12 weeks of ma-

Table 1 Recovery of somatic embryos, germination and establishment of somatic seedlings from embryogenic tissue in ten genotypes of *Pinus roxburghii* following the incorporation of 1.0 mg l^{-1} SA in the DCR basal medium (initiation medium) compared against control lacking SA.

Genotypes	Somatic embryogenesis (%)	№ of somatic embryos recovered per gram fresh wt of embryogenic callus	№ of somatic embryos germinated per gram fresh wt of embryogenic callus	№ of somatic seedlings recovered per gram fresh wt of embryogenic callus
PR-811	28.0 ± 1.2 a	11.0 ± 0.5 b	5.0 ± 0.2 b	3.0 ± 0.1 b
control	7.0 ± 0.3 b	3.0 ± 0.1 b	0.0 ± 0.0 c	0.0 ± 0.0 c
PR-805	30.0 ± 1.8 a	17.0 ± 2.3 b	13.0 ± 1.2 b	10.0 ± 0.5 b
control	6.0 ± 0.2 b	2.0 ± 0.1 b	1.0 ± 0.1 b	0.0 ± 0.0 c
PR-821	31.0 ± 1.6 a	16.0 ± 1.9 b	12.0 ± 0.5 b	8.0 ± 0.3 b
control	11.0 ± 0.4 b	4.0 ± 0.2 b	2.0 ± 0.1 b	1.0 ± 0.1 b
PR-32	14.0 ± 0.8 b	6.0 ± 0.4 b	3.0 ± 0.2 b	2.0 ± 0.1 b
**control	3.0 ± 0.1 b	2.0 ± 0.1 b	0.0 ± 0.0 c	0.0 ± 0.0 c
PR-76	26.0 ± 1.6 a	15.0 ± 1.8 b	10.0 ± 1.5 b	7.0 ± 0.3 b
control	13.0 ± 1.0 b	6.0 ± 0.4 b	3.0 ± 0.2 b	1.0 ± 0.1 b
PR-193	15.0 ± 1.3 b	8.0 ± 1.0 b	5.0 ± 0.1 b	3.0 ± 0.2 b
control	2.0 ± 0.1 b	3.0 ± 0.1 b	1.0 ± 0.1 b	0.0 ± 0.0 c
PR-46	31.0 ± 1.8 a	18.0 ± 2.3 b	15.0 ± 1.8 b	10.0 ± 0.7 b
control	12.0 ± 0.5 b	5.0 ± 0.2 b	3.0 ± 0.4 b	1.0 ± 0.1 b
PR-51	8.0 ± 0.3 b	11.0 ± 2.0 b	7.0 ± 0.2 b	4.0 ± 0.3 b
control	2.0 ± 0.1 b	4.0 ± 0.1 b	2.0 ± 0.1 b	1.0 ± 0.1 b
PR-05	3.0 ± 0.1 b	6.0 ± 0.3 b	3.0 ± 1.0 b	1.0 ± 0.1 b
control	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
PR-92	12.0 ± 0.7 b	25.0 ± 1.8 a	17.0 ± 1.8 b	9.0 ± 0.2 b
control	3.0 ± 0.1 b	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c

*Mean (±SE) followed by the same letter in each column were not significantly different at P≤0.05 using DMRT. All the experiments were repeated in triplicate and data represents the average of three independent experiments.

**Control= DCR basal medium lacking SA

(% of somatic embryogenesis) = 5 g of embryogenic tissue of each genotype was taken aseptically and chopped into 100 pieces and subcultured on the maintenance medium for the growth of callus. Out of 100 pieces, growth of the number of pieces was recorded and calculated in terms of percentage of SE).

turation, somatic embryos with developing cotyledons were picked out from the cultures for germination. The germination medium used was DCR basal medium with 2 g l^{-1} of Gellan gum (Malabadi and van Staden 2003; Malabadi 2006; Malabadi and Nataraja 2007a, 2007b, 2007c, 2007d). After a further 4 to 6 weeks on germination medium, plantlets were transferred to vermiculite and placed in a growth room (continuous light, 25 ± 3°C) for hardening.

RESULTS AND DISCUSSION

There are several reports describing how exogenously applied SA enhances somatic embryogenesis in plants, e.g. *Plumbago* (Komaraiah *et al.* 2004), *Avena* (Hao *et al*. 2006). Thus, one of the primary objectives of this study was to test the effectiveness of SA on somatic embryogenesis using vegetative shoot apices of mature trees of *P. roxburghii*. There are no reports of somatic embryogenesis in conifers using SA as a growth regulator. The present study reports for the first time the use of SA as an effective growth regulator in conifer somatic embryogenesis. Somatic embryogenesis could be been well established using SA as a growth regulator in most of the recalcitrant genotypes of *P. roxburghii*. This protocol is very simple and can be used as an improvement over our previous protocol of cloning mature trees of *P. roxburghii* (Malabadi and Nataraja 2006a, 2006b). TCLs of the apical dome (zone) precultured at 4°C for 3 days with 0.3% of activated charcoal formed elongated cells with embryonal suspensor masses (ESMs) which are typical of zygotic embryo-derived somatic embryogenesis in conifers (**Fig. 1**). Without a 3-day cold pretreatment the sections failed to produce ESMs as a result of cleavage polyembryony (embryonal suspensor masses) but instead yielded callus, confirming previous studies with *P. patula* (Malabadi and van Staden 2003). The use of SA at a low concentration (1.0 mg l^{-1}) increased the percentage of embryogenic tissue (12 to 31%) in all the tested genotypes, more than our previous protocols of *P. roxbughii* lacking SA (**Table 1**). On the other hand DCR basal medium lacking SA resulted in a lower percentage of somatic embryogenesis (0 to 13%) in all ten *P. roxbughii* genotypes (**Table 1**). Further incorporation of 0.5 mg l^{-1} of SA in the maintenance medium also improved (visually assessed) and favored the development of proembryonal masses against the control lacking SA (**Fig. 1**). SA, in our study, acted as a growth stimulant. SA is an important signaling molecule involved in plant defense responses to pathogens and abiotic stress, as well as in plant growth and development (Hayat and Ahmad 2007). SA and acetylsalicyclic acid (ASA) enhanced somatic embryogenesis in plants viz. carrot (*Daucus carota*) (Roustan *et al.* 1990), pearl millet (*Pennisetum americanum*) (Pius *et al.* 1993), geranium (*Pelargonium* × *hortorum* Bailey) (Hutchinson and Saxena 1996), *Astragalus adsurgens* Pall (Luo *et al.* 2001), *Plumbago rosea* L. (Komaraiah *et al.* 2004) and naked oat (*Avena nuda*) (Hao *et al*. 2006). Our results are in agreement with these findings. The differential response of SA and ASA in somatic embryo development was unexpected as for most physiological responses both compounds are interchangable. ASA is spontaneously hydrolysed to SA releasing the acetyl group for transacetylation blocking prostaglandin biosynthesis in animal tissues (Raskin 1992; Hutchinson and Saxena 1996). It may be speculated that the acetyl group changes the rate of uptake and metabolism of SA. SA is endogenously produced in many plants in normal as well as during stress conditions (Raskin *et al*. 1990). SA is known to be involved in a number of physiological and developmental responses in plants (Dean and Delaney 2008). However, the mechanism of salicyclate-induced differentiation in plants is not known, although SA induced somatic embryogenesis in wide variety of plants. Perhaps embryo differentiation may share some of the intermediates in the salicyclate signal pathway. Also, SA inhibited ethylene biosynthesis in cell suspension cultures of carrot (Roustan *et al*. 1990). It is well known that ethylene inhibits differentiation in plants. In the present study SA may be promoting embryo development by inhibiting ethylene biosynthesis, action or signal molecule in different recalcitrant genotypes of *P. roxburghii.* Another hypothesis is that SA has been reported to increase the activity of superoxide dismutase (Rao *et al.* 1997), and inhibit activites of ascorbate peroxidase and catalases, thus leading to endogenous H_2O_2 accumulation (Rao *et al*. 1997). Somatic embryogenesis in plants may be linked to reactive oxygen species level in callus, namely O_2 and H_2O_2 level favorable for induction of embryo development, and the maintenance of cell membrane integrity which is also critical in *P. roxburghii*.

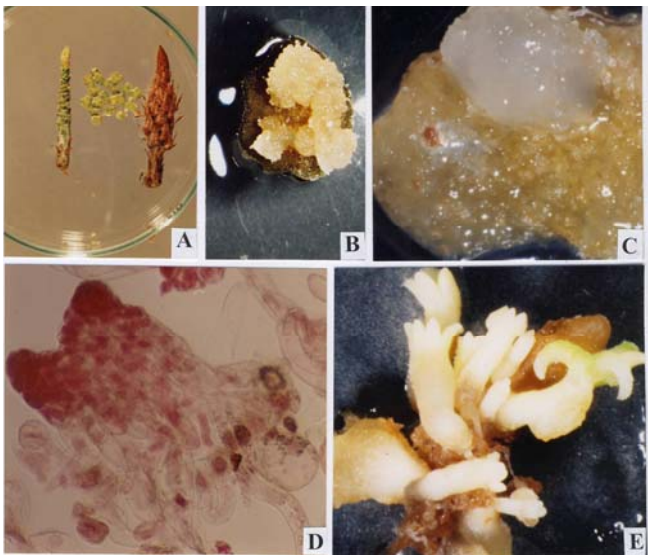

Fig. 1 Induction of somatic embryogenesis in *P. roxburghii* using SA. (**A**) Apical shoots harvested from mature trees of genotype PR-821 (1 cm = 0.92 mm); (**B**) Cold-pretreated apical shoot section showing the initiation of white mucilaginous embryogenic tissue on initiation medium containing 1.0 mg l^{-1} SA (1 cm = 5.3 mm); (**C**) Proliferation of embryogenic tissue on maintenance medium containing 0.5 mg l^{-1} SA (1 cm = 11 mm); (**D**) Cells with cleavage polyembryony showing the head and elongated suspensor (1 cm = 10.5 mm); (**E**) Well matured somatic embryos on maturation medium showing an advanced cotyledonary stage (1 cm = 10.8 mm).

In conclusion, this simplified cloning method of using TCLs of vegetative shoot apices of mature trees of *P. roxburghii* should influence breeding strategies by offering an alternative tool for accelerated production of plants through the use of SA to meet the demands of commercial forestry. All these clones could be used for the production of plants for clonal forestry.

REFERENCES

Aronen TS, Ryynanen L, Malabadi RB (2007) Somatic embryogenesis of Scots pine: initiation of cultures from mature tree explants and enhancement of culture system. In: *IUFRO Tree Biotechnology Conference*, June 3-8, 2007, Ponta Delgada, Azores, Portugal, No. SIX. 2 (Abstract)

Dean JV, Delaney SP (2008) Metabolism of salicyclic acid in wild-type, ugt74f1 and ugt74f2 glucosyltransferase mutants of *Arabidopsis thaliana*. *Physiologia Plantarum* **132**, 417-425

Giri CC, Shyamkumar B, Anjaneyulu C (2004) Progress in tissue culture, genetic transformation and applications of biotechnology to trees: an overview. *Trees* **18**, 115-135

Gupta PK, Durzan DJ (1985) Shoot multiplication from mature trees of Douglas fir and sugar pine. *Plant Cell Reports* **4**, 177-179

Hao L, Zhou L, Xu X, Cao J, Xi T (2006) The role of salicyclic acid and carrot somatic embryogenic callus extracts in somatic embryogenesis of naked oat (*Avena nuda*). *Plant Cell, Tissue and Organ Culture* **85**, 109-113

Hayat S, Ahmad A (Eds) (2007) *Salicylic Acid - A Plant Hormone*, Springer, Germany, 401 pp

Hutchinson MJ, Saxena PK (1996) Acetylsalicyclic acid enhances and synchronizes thidiazuron induced somatic embryogenesis in geranium (*Pelargonium* × *hortorum* Baily) tissue cultures. *Plant Cell Reports* **15**, 512-515

Komaraiah P, Jogeswar C, Ramakrishna S, Kavi Kishor P (2004) Acetylsalicyclic acid and ammonium induced somatic embryogenesis and enhanced plumbagin production in suspension cultures of *Plumbago rosea* L. *In Vitro*

Cellular and Developmental Biology – Plant **40 (2)**, 230-234

Luo JP, Jiang ST, Pan LJ (2001) Enhanced somatic embryogenesis by salicyclic acid of *Astragalus adsurgens* Pall: relationship with H_2O_2 production and H_2O_2-metabolizing enzyme activities. *Plant Science* **161**, 125-132

Malabadi RB, van Staten J (2003) Somatic embryos can be induced from shoot apical domes of mature *Pinus patula* trees. *South African Journal of Botany* **69**, 450-451

Malabadi RB, 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 RB, Nataraja K (2006a) RAPD detect no somaclonal variation in cryopreserved cultures of *Pinus roxburghii* SARG. *Propagation of Ornamental Plants* **6 (3)**, 114-120

Malabadi RB (2006) Effect of glutathione on maturation of somatic embryos derived from vegetative shoot apices of mature trees of *Pinus roxburghii*. *Journal of Phytological Research* **19 (1)**, 35-38

Malabadi RB, Nataraja K (2006b) Cryopreservation and plant regeneration *via* somatic embryogenesis using shoot apical domes of mature *Pinus roxburghii* Sarg. trees. *In Vitro Cellular and Developmental Biology – Plant* **42**, 152-159

Malabadi RB, Nataraja K (2007a) Smoke-saturated water influences somatic embryogenesis using vegetative shoot apices of mature trees of *Pinus wallichiana* A. B. Jacks. *Journal of Plant Sciences* **2**, 45-53

Malabadi RB, Nataraja K (2007b) Plant regeneration *via* somatic embryogenesis using secondary needles of mature trees of *Pinus roxburghii* Sarg. *International Journal of Botany* **3**, 40-47

Malabadi RB, Nataraja K (2007c) Gene transfer by particle bombardment of embryogenic tissue derived from vegetative shoot apices of mature trees of *Pinus roxburghii*. *American Journal of Plant Physiology* **2 (2)**, 90-98

Malabadi RB, Nataraja K (2007d) Isolation of cDNA clones of genes differentially expressed during somatic embryogenesis of *P. roxburghii*. *American Journal of Plant Physiology* **2 (6)**, 333-343

Pius J, George L, Eapen S, Rao PS (1993) Enhanced plant regeneration in pearl millet (*Pennisetum americanum*) by ethylene inhibitors and cefotaxime. *Plant Cell, Tissue and Organ Culture* **32**, 91-96

Rao MV, Paliyath G, Ormrod DP, Murr DP, Watkins CB (1997) Influence of salicyclic acid on H_2O_2 production, oxidative stress, and H_2O_2-metabolizing enzymes: salicyclic acid mediated oxidative damage requires H_2O_2. *Plant Physiology* **115**, 137-149

Roustan JP, Latche A, Fallot J (1990) Inhibition of ethylene production and stimulation of carrot somatic embryogenesis by salicyclic acid. *Biologia Plantarum* **32**, 273-276

Sharma K, Degen B, van Wuehlisch G, Singh NB (2007) An assessment of heterozygosity and fitness in Chir pine (*P. roxburghii*) using isozymes. *New Forests* **34**, 153-162

Wareing PE (1987) Phase change and vegetative propagation. In: Abbott AJ, Atkin RK (Eds) *Improving Vegetatively Propagated Crops*, Academic Press Ltd., San Diego, CA, pp 263-270