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Salicylic Acid Induces Somatic Embryogenesis from Mature Trees of *Pinus roxburghii* (Chir pine) using TCL Technology

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ABSTRACT

Several physiological and biochemical effects of salicyclic acid (SA), when applied to plants, have been known for a long time. This paper highlights for the first time the role of SA as a signaling molecule in inducing embryogenic tissue derived from mature trees of *Pinus roxburghii*. External pre-treatment of explants with different concentrations (0.1, 0.2, 0.4, 0.5, 1.0, 2.0, 5.0 mg^{-1}) of SA for 5 min could not induce somatic embryogenesis effectively when plated on DCR induction medium. All explants of 10 genotypes failed to induce embryogenic tissue, and resulted in the browning of explants with callusing during pre-treatment of explants with SA. On the other hand, incorporation of 1.0 mg^{-1} SA in full DCR medium, i.e. containing 2,4-D, NAA and BA, was optimum for all Chir pine genotypes by increasing the percentage of somatic embryogenesis significantly more than the control. The highest percentage (31%) of somatic embryogenesis was recorded in genotypes PR-821 and PR-46. SA proved to be a powerful synergistic activator of somatic embryogenesis in conifers when placed with other plant growth regulators.

Keywords: clonal forestry, India, somatic embryo, shoot apical meristem, Western Ghat Forest
Abbreviations: ABA, abscisic acid; **ASA**, acetyl-salicyclic acid; **BAP**, 6-benzyl aminopurine; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **DCR**, Gupta and Durzan medium, **H_2O_2**, hydrogen peroxide; **NAA**, α-naphthalene acetic acid; **SA**, salicyclic acid; **SDW**, sterile distilled water; **TCL**, thin cell layer; **tTCL**, transverse thin cell layer

INTRODUCTION

The plant growth regulator (PGR) salicyclic acid (SA), when applied to plants, affects diverse physiological processes (Dean and Delaney 2008) including stomatal closure in *Phaseolus vulgaris* (Larque-Saavedra 1978, 1979), induction of flowering in duckweeds (Cleland and Ajami 1974), increased cell division and growth during somatic embryogenesis in tissue culture of *Coffea arabica* (Quiroz *et al*. 2001), increase in the accumulation of nitrates in roots of *Pinus patula* (San Miguel *et al.* 2002), inhibition of the biosynthesis of ethylene in cell suspension cultures of pears and seed germination in species (Leslie and Romani 1988) and root growth stimulation of soybean (Gutierrez-Coronado *et al.* 1998). In African violet, the application of SA at low concentrations affects plant size, and the number of leaves and flowers (Martin-Mex *et al.* 2005). Moreover, application of SA to the growth medium induced flowering in several species of Lemnaceae (Oota 1975; Cleland *et al.* 1982). SA has also been reported to increase the activity of superoxide dismutase (Rao *et al.* 1997), and inhibit activities of ascorbate peroxidase (Durner and Klessig 1995), and catalase (Conrath *et al.* 1995), thus leading to endogenous H_2O_2 accumulation. Thus, SA inhibits the decomposition of H_2O_2 produced in plants. SA is an important signaling molecule involved in plant defense responses to pathogens and abiotic stress, as well as in plant growth and development (Raskin *et al*. 1987; Raskin 1992). On the other hand, there are several reports describing exogenous SA and acetyl-salicylic acid (ASA) enhancing 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), naked oat (*Avena nuda*) (Hao *et al*. 2006) and *Pinus roxburghii* (Malabadi *et al*. 2008a).

Thin cell layers (TCL) of plant tissues have been used successfully as explants for *in vitro* plant regeneration in many plant species (Mulin and Tran Thanh Van 1989; Tran Thanh Van and Van Le 2000; Nhut *et al.* 2003a, 2003b, 2003c, 2003d, 2003e, 2003f; Teixeira da Silva 2003; Malabadi and van Staden 2003; Malabadi *et al*. 2004a, 2004b; Teixeira da Silva 2005; Malabadi *et al*. 2005; Nhut *et al.* 2006; Malabadi and Nataraja 2007a, 2007b, 2007d; Malabadi *et al.* 2008e; Teixeira da Silva 2008, 2009; Teixeira da Silva and Tanaka 2009). This culture method was first developed by Tran Thanh Van for programming different patterns of morphogenesis (Tran Thanh Van 1981). Moreover, one of the most important developmental building blocks of TCLs, cells, are responsible for the success of this technology (Teixeira da Silva *et al*. 2007). TCL systems allow for the isolation of specific cell or tissue layers, which, depending on the genetic state and epigenetic requirements, and in conjunction with strictly controlled growth conditions (light, temperature, pH, PGRs, media additives and others) may lead to the *in vitro* induction of morphogenic programs (Teixeira da Silva *et al*. 2007). The capacity of a TCL to enter a program depends upon a number of factors, including correct signal perception and transduction, the capacity of the internal genetic machinery to respond and react to these signals and in the latter case, may depend on the physiological state and origin (tissue and organ) of the TCL, environmental stress, chemical stress factors applied to the TCL (Teixeira da Silva *et al.* 2007). Successful initiation of embryogenic tissue using TCL technology has been reported in many recalcitrant pines, e.g. *P. kesiya* (Malabadi *et al*. 2004a), *P. roxburghii* (Malabadi 2006;

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Malabadi and Nataraja 2006a, 2006b), *P. wallichiana* (Malabadi and Nataraja 2007a), *P. patula* (Malabadi and van Staden 2003; Malabadi and van Staden 2005a, 2005b, 2005c; Malabadi and van Staden 2006), *P. sylvestris* (Aronen *et al.* 2007), *P. pinea* (Portuguese stone pine) and *P. pinaster* (Portuguese Maritime pine) (Malabadi *et al*. unpublished work).

Pinus roxburghii (Chir pine) is one of the most important pine species distributed throughout all parts of India. Induction of somatic embryogenesis using TCLs of apical shoots and secondary needles of mature trees has been well established in *P. roxburghii* (Malabadi and Nataraja 2006, 2007). In addition, the first report of genetic transformation using biolistic method including the isolation of cDNA clones of genes has also been reported using TCL-induced embryogenic tissue in *P. roxburghii* (Malabadi and Nataraja 2007c, 2007d). However, there are currently no reports on the effect of SA on induction of somatic embryogenesis in pines despite several reports in angiosperms. Therefore, the first objective of the present study was to determine the effect of SA on the initiation of embryogenic tissue from apical shoot sections of mature (14-years old) *P. roxburghii* trees. Further, in our previous study we were able to establish the embryogenic system using TCL technology in only three *P. roxburghii* genotypes (Malabadi and Nataraja 2006). Therefore, the second objective of this study was to test whether TCL technology could be applicable to more *P. roxburghii* genotypes for the establishment of embryogenic tissue from mature trees.

MATERIALS AND METHODS

Plant material

Shoot apical domes (**Fig. 1A**) from 10 genotypes (PR811, PR805, PR821, PR32, PR76, PR193, PR46, PR51, PR05, and PR92) of mature *P. roxburghii* trees (14-years old) 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. Apical domes were harvested during April. They were cleansed with 1% citramide (sodium hypochlorite, 3.5% (v/v)) for 5 min and rinsed thoroughly with sterilized distilled water (SDW). 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 double SDW. Transverse thin cell layers (tTCLs) approximately 0.5-1.0 mm thick (**Fig. 1B**) were cut using a sharp sterilized blade or scalpel from shoot apical domes (upper part with 2 to 4 sections only) for the initiation of embryogenic tissue. tTCLs of all 10 genotypes were kept in separate sterile Petri dishes under aseptic conditions prior to their use in the following three separate experiments.

Experiment 1. Effect of pretreatment of explants with SA only

During this experiment, the tTCLs of all 10 genotypes were pretreated with SA (Sigma, USA) (ACS reagent grade) at different concentrations (0.1, 0.2, 0.4, 0.5, 1.0, 2.0, 5.0 mg^{-1}) for 5 min and subsequently explants were cultured individually on full-strength DCR basal medium (Gupta and Durzan 1985) containing full-strength inorganic salts and 0.2 g l^{-1} polyvinyl pyrollidine (PVP) (Sigma), 2 g l^{-1} Gellan gum (Sigma), 30 g l^{-1} maltose (Sigma) and 0.3% activated charcoal (Sigma) without PGRs (i.e., pre-culture medium as defined in Malabadi and van Staden 2003; Malabadi *et al*. 2004; Malabadi and Nataraja 2006). These cultures were first treated with SA for 5 min then incubated in the dark at 4°C for 3 days (Malabadi and van Staden 2003; Malabadi *et al*. 2004; Malabadi and Nataraja 2006). The SA/cold-pretreated explants at 4°C for 3 days were then subcultured on induction medium, namely full-strength DCR basal medium containing 0.2 g l^{-1} PVP, 2 g l^{-1} Gellan gum, 1 g l^{-1} L-glutamine (Sigma), 1 g l^{-1} casein hydrosylate (Sigma), 1 g l^{-1} meso-inositol (Sigma), and supplemented with 22.6 μM 2,4-dichlorophenoxy acetic acid (Sigma), 26.8 μM α-naphthalene acetic acid (Sigma), and 8.9 μM 6-benzyl aminopurine (Sigma) for the initiation of embryogenic tissue as described previously (Malabadi and van Staden 2003; Malabadi *et al*. 2004; Malabadi and Nataraja 2006; Malabadi *et al*. 2008). The pH of the medium was adjusted to 5.8 with 1 N 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 (Whatman filter paper, pore size = 0.45 μm; diameter = 25 mm) and added to the media after it had cooled to below 50°C. All the cultures were maintained for 20-30 days in the dark at 25 ± 2°C. The presence of embryonal masses (cells undergoing division showing the formation of a head) and cleavage poly-embryony (cells undergoing division showing the formation of head and suspensors, which is an advanced stage) showing embryonal suspensor masses (ESMs) (nothing but pro-embryos with developed heads and suspensors) was determined by squash preparation of embryogenic tissue under the microscope at 45X, 65X or 100X magnification. After identification of embryogenic cells, the embryogenic mass of cells was separated from the rest of the tissue, and subcultured on maintenance medium (for at least 4 to 6 weeks) for the further proliferation of callus. Maintenance medium consisted of full-strength DCR basal medium containing 30 g l^{-1} maltose, 2 g l^{-1} Gellan gum supplemented with 2.26 μM 2,4-D, 2.68 μM NAA and 0.88 μM BA (Malabadi and van Staden 2003; Malabadi *et al*. 2004; Malabadi and Nataraja 2006). During the maintenance of embryogenic cultures, the embryogenic callus forming proembryonal masses were broken into small similar-sized pieces and then subcultured onto maintenance medium every two weeks. In all these experiments, a control was maintained, and all the results were compared against the control, which was induction medium without SA.

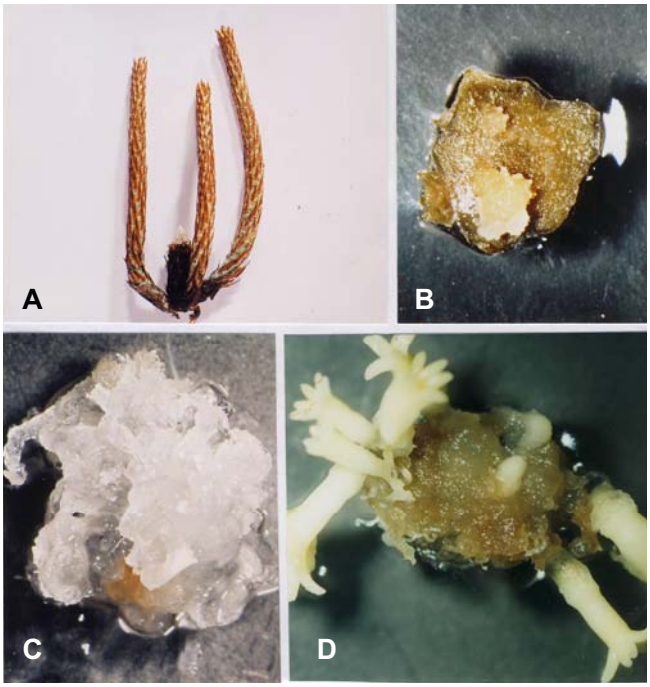

Fig. 1 Effect salicyclic acid on somatic embryogenesis from vegetative shoot apices of mature *Pinus roxburghii* trees. (**A**) Apical shoots from mature trees (scale 10 mm = 0.9 mm). (**B**) Shoot apical dome thin section showing the initiation of white mucilaginous embryogenic callus (scale: 10 mm = 4 mm). (**C**) Proliferation of white embryogenic callus on maintenance medium (scale: 10 mm = 7.5 mm). (**D**) Development of somatic embryos on maturation medium seen under the microscope (scale: 10 mm = 11 mm).

Experiment 2: Effect of incorporation of SA in induction medium

In this experiment, the tTCLs of all 10 genotypes were cultured individually on pre-culture and induction media under the exact same conditions as defined in Experiment 1, i.e., without any SA. Although culture on maintenance was identical, in this experiment it was supplemented with different concentrations of SA (0.1, 0.2, 0.4, 0.5, 1.0, 2.0, 5.0 mg^{-1}) was used for this purpose. The controls were the same as for Experiment 1.

Maturation of somatic embryos

For maturation, embryogenic tissue clumps of each of the 10 genotypes from experiments 1 and 2 were incubated in the dark at room temperature (28 ± 2°C). The percentage somatic embryogenesis was calculated as responsive callus-based embryogenesis (expressed in terms of number of responsive regrowth of callus pieces per 100 since a total of 100 pieces of calluses were subcultured). Therefore, 5 g of embryogenic tissue of each genotype was taken aseptically and chopped into 100 pieces under aseptic conditions by using a normal scalpel and blade and subcultured on maintenance medium for the growth of callus. The number of pieces re-callusing from the 100 sub-cultured embryogenic tissue indicated the percentage of somatic embryogenesis. This was estimated (i.e. the total number of somatic embryos, germinated embryos, and somatic seedlings produced per gram FW of tissue was calculated) before transferring the embryogenic tissue onto maturation medium. One gram fresh weight of embryogenic tissue of each genotype was transferred to sterile empty Petri dishes (60 mm diameter) 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. After desiccation, the partially desiccated embryogenic tissue (1 g-pieces ×5 per Petri dish) of each genotype was transferred to maturation medium to induce cotyledonary embryo development. Maturation medium consists of full strength DCR medium with 60 g l^{-1} maltose, 37.84 µM abscisic acid (ABA; Sigma, ACS grade) and 5 g l^{-1} Gellan gum (Malabadi and van Staden 2003; Malabadi *et al*. 2004; Malabadi and Nataraja 2006). All the cultures were placed in the dark at 25 ± 2°C and maintained for 8-12 weeks (Malabadi 2006b; Malabadi and Nataraja 2006a, 2006b).

Germination and plantlet recovery

After 12 weeks of maturation in the presence of ABA and higher concentrations of maltose (60 g l^{-1} maltose), the cotyledonary somatic embryos were recovered from the cultures for germination. Before germination, cotyledonary somatic embryos of all 10 genotypes were cold pre-treated at 2°C and kept in the dark for 25 days. The germination medium consisted of half DCR medium with 2 g l^{-1} Gellan gum (Malabadi and Nataraja 2006a, 2006b). In the first week of germination, cultures were kept in the dark then transferred to diffuse light (30 µmol m^{-2} s^{-1}) in the second week, and thereafter to a 16-hr photoperiod under a light intensity of 50 µmol m^{-2} s^{-1} for hardening. Somatic embryos were considered germinated as soon as radicals elongated and conversion to plantlets was based on the presence of epicotyls. After 4-6 weeks on germination medium, plantlets were transferred to vermiculite in a controlled growth room.

Statistical analysis

In experiments 1 and 2, each replicate contained 50 cultures and one set of experiments consisted of two replicates (i.e. total of 100 cultures per experiment) for each genotype. All the experiments were repeated in triplicate. Data in **Tables 1** and **2** represent the average of three independent experiments. Data was arcsine transformed before being analyzed for significance using ANOVA ($p<0.05$) or evaluated for independence using the Chi-square test. Further, the differences in means were contrasted using Duncan's multiple range test ($a=0.05$) following ANOVA. All statistical analyses was performed using SPSS (Microsoft Windows ver. 13.0.1.1) statistical software package.

RESULTS AND DISCUSSION

In the present study, the incorporation of SA into DCR induction medium influenced somatic embryogenesis in a few of the Chir pine genotypes which were previously considered to be recalcitrant. This study for the first time highlights the importance of SA as a signaling molecule. Therefore, SA can be used as growth regulator in conifer somatic embryogenesis and its use might help to solve the low initiation frequencies of many other recalcitrant pines. The primary goal of *in vitro* culture of forest trees has always been mass clonal propagation of the most desirable genotypes, although very recently the provision of target material for gene transfer has assumed prominence (Malabadi and Nataraja 2007e; Malabadi *et al.* 2008c, 2008d). A simple and efficient transformation procedure has been developed for embryogenic tissue of *P. roxburghii* using disarmed *Agrobacterium* strain EHA105 containing copies of *vir*B, *vir*C and *vir*C genes from the supervirulent plasmid pToK47 (Malabadi *et al.* 2008b). This robust and reliable regeneration system has been the basis of a programme for genetic engineering *P. roxburghii* using *A. tumefaciens*-mediated gene transfer (Malabadi *et al.* 2008b). This is the first report of *Agrobacterium*-mediated T-DNA integration in *P. roxburghii* using embryogenic lines derived from apical meristematic tissue of mature trees (14-years old) (Malabadi *et al.* 2008b). This procedure will therefore, permit Chir pine (*P. roxburghii*) improvement via genetic engineering and facilitate physiological studies through the use of genetic manipulation. Embryogenic cultures have been generated for most of the conifers. For the most part, however, even the best of these systems lack commercial viability for two reasons: first, a low frequency of regeneration for many of the most desirable clones; and secondly, unproven genotypes, as starting material for the cultures is derived from seeds or seedlings. Therefore, the current approach of cloning mature trees of conifers using SA has many practical applications particularly in clonal forestry schemes. Use of SA might be helpful in solving many problems of conifer somatic embryogenesis. On the basis of findings by several research groups, SA is an important signaling molecule not only involved in defense responses but also in somatic embryogenesis of many plant species (Luo *et al.* 2001; Komaraiah *et al.* 2004; Hao *et al*. 2006). SA is a mobile molecule, which is capable of acting as a cell signal that senses, amplifies, and transmit information from a cell and might help in programming towards embryogenesis during cloning. Secondly, SA is involved (together with nitrogen oxide, hydrogen peroxide, and other metabolites) in the function of several signal systems, unifying them into an intricate network of regulatory interactions (Vasyukova and Ozeretskovskaya 2007). Perhaps embryo differentiation may sharing some of the intermediates in the salicylate signal pathway.

Pre-treatment with SA (Experiment 1)

In the present study, pre-treatment (5 min) of shoot-tip tTCL explants of 10 different Chir pine genotypes with different concentrations of SA did not induce embryogenic tissue any more than the control (**Table 1**). All genotypes showed a mixed response in embryogenic tissue induction following pretreatment of tTCLs with SA (in general). The pre-treatment of tTCLs from any of the 10 genotypes with 0.1, 0.2 and 0.4 mg^{-1} SA could not effectively increasing the percentage of somatic embryogenesis when compared to the control (**Table 1**). PR-05 and PR-92 failed to induce embryogenic tissue following pre-treatment of explants with any concentration of SA (**Table 1**), i.e. these two genotypes were completely recalcitrant to this treatment. Pretreatment of explants with higher concentrations (2.0-5.0 mg^{-1}) of SA might have had a toxic effect and resulted in the browning of explants without callus formation in all 10 genotypes (**Table 1**). The percentage of responsive explants that could induce embryogenic tissue increased (significantly in some cases) from 7 to 12% in PR-811, 3 to 5% in PR-32, 6 to 8% in PR-805, and 11 to 16% in PR-821 following the pretreatment of 1.0 mg^{-1} SA when compared with the control (**Table 1**). This trend was also similar with 0.5 mg^{-1} SA where the percentage of responsive based explants for inducing embryogenic tissue increased from 7 to 9% in PR-811 and 11 to 14% in PR-821, respectively. Therefore, pre-treatment with 0.5 or 1.0 mg^{-1} SA was optimum at least in a few *P. roxburghii* genotypes (PR-811, PR-32, PR-805, PR-821) for improving the percentage of somatic embryogenesis (**Table 1**).

Table 1 Effect of pre-treatment (5 min) of 10 *P. roxburghii* genotype tTCLs with different concentrations of salicyclic acid on the initiation of embryogenic tissue.

SA	*Responsive explant-based somatic embryogenesis (%)									
(mg^{-1})	PR-811	PR-805	PR-821	PR-32	PR-76	PR-193	PR-46	PR-51	PR-05	PR-92
0.1	6.0 ± 0.2 a	5.0 ± 0.2 a	10.0 ± 0.6 a	2.0 ± 0.1 a	12.0 ± 1.3 a	2.0 ± 0.1 a	13.0 ± 0.6 a	2.0 ± 0.1 a	0.0 ± 0.0 b	0.0 ± 0.0 b
control	7.0 ± 0.3 a	6.0 ± 0.2 a	11.0 ± 0.4 a	3.0 ± 0.1 a	13.0 ± 1.0 a	2.0 ± 0.1 a	12.0 ± 0.5 a	2.0 ± 0.1 a	0.0 ± 0.0 b	3.0 ± 0.1 a
0.2	7.0 ± 0.1 a	4.0 ± 0.3 a	10.0 ± 0.3 a	2.0 ± 0.1 a	10.0 ± 0.9 a	2.0 ± 0.0 a	11.0 ± 2.6 a	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
control	7.0 ± 0.3 a	6.0 ± 0.2 a	11.0 ± 0.4 a	3.0 ± 0.1 a	13.0 ± 1.0 a	2.0 ± 0.1 a	12.0 ± 0.5 a	2.0 ± 0.1 a	0.0 ± 0.0 b	3.0 ± 0.1 a
0.4	3.0 ± 0.1 a	2.0 ± 0.1 a	12.0 ± 1.2 a	2.0 ± 0.1 a	11.0 ± 1.7 a	2.0 ± 0.1 a	11.0 ± 0.4 a	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
control	7.0 ± 0.3 a	6.0 ± 0.2 a	11.0 ± 0.8 a	3.0 ± 0.1 a	13.0 ± 1.0 a	2.0 ± 0.1 a	12.0 ± 0.5 a	2.0 ± 0.1 a	0.0 ± 0.0 b	3.0 ± 0.1 a
0.5	9.0 ± 0.2 a	3.0 ± 0.1 a	14.0 ± 0.7 a	3.0 ± 0.1 a	9.0 ± 0.3 a	0.0 ± 0.0 b	10.0 ± 0.0 a	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
control	7.0 ± 0.3 a	6.0 ± 0.2 a	11.0 ± 0.4 a	3.0 ± 0.1 a	13.0 ± 2.8 a	2.0 ± 0.1 a	12.0 ± 0.5 a	2.0 ± 0.1 a	0.0 ± 0.0 b	3.0 ± 0.1 a
1.0	12.0 ± 0.8 a	8.0 ± 0.1 a	16.0 ± 2.0 a	5.0 ± 0.3 a	11.0 ± 1.4 a	0.0 ± 0.0 b	8.0 ± 0.5 a	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
control	7.0 ± 0.3 a	6.0 ± 0.2 a	11.0 ± 0.4 a	3.0 ± 0.1 a	13.0 ± 1.0 a	2.0 ± 0.1 a	12.0 ± 0.5 a	2.0 ± 0.1 a	0.0 ± 0.0 b	3.0 ± 0.1 a
2.0	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
control	7.0 ± 0.3 a	6.0 ± 0.2 a	11.0 ± 0.4 a	3.0 ± 0.1 a	13.0 ± 1.0 a	2.0 ± 0.1 a	12.0 ± 0.5 a	2.0 ± 0.1 a	0.0 ± 0.0 b	3.0 ± 0.1 a
5.0	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
control	7.0 ± 0.3 a	6.0 ± 0.2 a	11.0 ± 0.4 a	3.0 ± 0.1 a	13.0 ± 1.0 a	2.0 ± 0.1 a	12.0 ± 0.5 a	2.0 ± 0.1 a	0.0 ± 0.0 b	3.0 ± 0.1 a

Means (±SE) followed by the same letter in each column were not significantly different at P≤0.05 using DMRT. All experiments were repeated in triplicate and data represents the average of three independent experiments.
Control = Explants without pre-treatment of different concentrations of SA.
* Responsive explant-based somatic embryogenesis = The percentage was calculated on the basis of explants producing embryogenic callus.

Table 2 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	Callus based-somatic embryogenesis (%)	Somatic embryos recovered per g fresh wt of embryogenic callus	Somatic embryos germinated per g fresh wt of embryogenic callus	Somatic seedlings recovered per g 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

Means (± SE) followed by the same letter in each column were not significantly different at P≤0.05 using DMRT. All 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. This represents callus-based somatic embryogenesis.

SA in induction medium (Experiment 2)

Incorporation of 1.0 mg^{-1} SA in the induction medium was optimum for all 10 genotypes by increasing the percentage of somatic embryogenesis compared to the control (**Table 2**). The highest percentage (31%) of somatic embryogenesis was recorded in PR-821 and PR-46. For PR-05, in particular, the addition of 1.0 mg^{-1} SA to the induction medium was very beneficial since in the control this genotype failed to induce somatic embryogenesis. This clearly indicates the positive role of SA as a signaling molecule during cloning of mature *P. roxburghii* trees. In this study SA alone (i.e. without PGRs) did not induce somatic embryogenesis and resulted in the browning of explants and callus. Microscopic observation showed simple, elongated parenchymatous cells without any sign of cleavage polyembryony. SA, when combined with 22.6 μM 2, 4-D, 26.8 μM NAA, 8.9 μM BAP in the induction medium, improved the percentage of somatic embryogenesis. However, in PR-05 this synergistic mix induced only 3%somatic embryogenesis while in PR-05 it failed to induce somatic embryogenesis. Hence, the combination of SA with other PGRs such as 2,4-D/NAA/BA might be beneficial in inducing somatic embryogenesis in Chir pine. In geranium (*Pelargonium* x *hortorum* Bailey), thidiazuron (TDZ) effectively induced somatic embryogenesis in cultured hypocotyls explants during only a 3-day period of induction (Hutchinson and Saxena 1996). The presence of acetylsalicyclic acid (ASA) during this period caused a two-fold increase in the number of somatic embryos an enhanced synchronization of embryo development compared to the TDZ treatment alone. However, in the same study, SA was ineffective in modulating similar embryogenic responses as ASA in geranium. Enhanced somatic embryogenesis and plant regeneration have been obtained using young leaf bases of naked oat (*Avena nuda*) as explants by including 0.5 mM SA and carrot embryogenic

callus extracts in MS media. An improvement was achieved in somatic embryogenesis and plant regeneration on the corresponding media supplemented with 0.5 mM SA and carrot embryogenic callus extracts as compared to control (Hao *et al.* 2006). Somatic embryogenesis was induced from suspension cultures derived from leaf callus of an important medicinal plant, *Plumbago rosea* L. (Komaraiah *et al.* 2004) in which 8.32 µM ASA alone induced embryogenesis, but IAA, NAA or IBA alone failed to elicit a similar response. Optimal embryogenic response per culture (216 embryos per culture) was observed in MS medium containing a combination of ASA (8.32 µM) and IAA (5.06 µM), i.e. a similar synergistic response as observed in our study between SA/ASA and other PGR(s) in the medium. It was also observed that by increasing the concentration of ASA alone (without auxin) in the medium (up to 11.09 µM) the number of somatic embryos formed per culture increased (Komaraiah *et al.* 2004). The interactive effect of ASA and IAA appears to be essential for enhanced production of embryos per culture since no embryogenesis was noticed when IAA alone was added in *Plumbago rosea* L. (Komaraiah *et al.* 2004). SA is endogenously produced in many plants in normal as well as during stress conditions (Raskin *et al*. 1990). However, the mechanism of salicylate-induced differentiation in plants is not known, although salicylate is a signal molecule implicated in eliciting many physiological functions in plants (Komaraiah *et al.* 2004). Ping *et al.* (2001) reported that the inclusion of SA to differentiation medium below 200 µmol/L significantly enhanced somatic embryogenesis in *Astragalus adsurgens* Pall. callus cultures, the highest frequency of somatic embryogenesis occurring at 150 µmol/L SA. They also reported that enhanced somatic embryogenesis by SA was accompanied by an increase in the endogenous hydrogen peroxide (H_2O_2) level compared to controls. This increased endogenous H_2O_2 level was related to the inhibition of ascorbate peroxidase and catalase activities (Ping *et al*. 2001). Although the promoting effect of exogenous H_2O_2 was significantly lower than that of exogenous SA on the development of somatic embryos, the pre-treatment of callus cultures of *A. adsurgens* with dimethylurea (a trap for H_2O_2) significantly inhibited somatic embryogenesis, even if callus was cultured on the differentiation medium supplemented with 150 µmol/L SA, suggesting that endogenous H_2O_2 was required for SA-enhanced somatic embryogenesis in *A. adsurgens* (Ping *et al*. 2001). H_2O_2 and its role in plant stress have been reviewed by Cheeseman (2007). Therefore, one possible link between oxidative stress and plant regeneration in tissue culture could be H_2O_2. SA is endogenously produced in many plants in normal as well as during stress conditions (Raskin *et al.* 1990). SA also 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. Another hypothesis is that SA has been reported to increase the activity of superoxide dismutase (Rao *et al.* 1997), and inhibits the activities of ascorbate peroxidase and catalases, thus leading to endogenous H_2O_2 accumulation in *Arabidopsis thaliana* (Rao *et al*. 1997). The biosynthetic pathways of SA have been reviewed by Vasyukova and Ozeretskovskaya (2007).

CONCLUSION

The cloning of mature *P. roxburghii* trees was successful following the addition of 1.0 mg^{-1} SA in DCR induction medium in most *P. roxburghii* genotypes tested. On the contrary, the external application of SA to tTCL explants prior to cloning was not effective for inducing somatic embryogenesis. Therefore, SA was shown to play an important role in inducing somatic embryognesis in conifers, expanding this function already observed in a wider range of plants.

REFERENCES

Aronen T, 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 islands, Portugal, No. SIX. 2 (Abstract)

Beers EP, McDowell JM (2001) Regulation and execution of programmed cell death in response to pathogens, stress and developmental cues. *Current Opinion in Plant Biology* **4**, 561-567

Bozhkov PV, Filonova LH, Suarez MF (2005) Programmed cell death in plant embryogenesis. *Current Topics in Developmental Biology* **67**, 135-179

Caliskan M, Turet M, Cuming AC (2004) Formation of wheat (*Triticum aestivum* L.) embryogenic callus involves peroxide-generating germin-like oxalate oxidase. *Planta* **219**, 132-140

Cheeseman JM (2007) Hydrogen peroxide and plant stress: a challenging relationship. *Plant Stress* **1**, 4-15

Cleland CF, Ajami A (1974) Identification of the flower-inducing factor isolated from aphid honeydew as being salicyclic acid. *Plant Physiology* **54**, 909-906

Cleland CF, Tanaka O, Feldman IJ (1982) Influence of plant growth substances and salicyclic acid on flowering and growth in the Lemnaceae (duckweeds). *Aquatic Botany* **13**, 3-20

Conrath U, Chen Z, Ricigliano JR, Klessig DF (1995) Two inducers of plant defense responses, 2, 6-dichloroisonicotinic acid and salicyclic acid inhibit catalase activities in tobacco. *Proceedings of the National Academy of Sciences USA* **92**, 7143-7147

Cui K, Xing G, Liu X, Xing G, Wang Y (1999) Effect of hydrogen peroxide on somatic embryogenesis of *Lycium barbarum* L. *Plant Science* **146**, 9-16

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

Desikan R, Mackerness RS-H, Hancock JT, Neill SJ (2001) Regulation of the *Arabidopsis* transcriptome of *Lycium barbarum* L. *Plant Physiology* **127**, 159-172

Durner J, Klessig DF (1995) Inhibition of ascorbate peroxidase by salicyclic acid and 2, 6-dichloroisonicotinic acid, two inducers of plant defense responses. *Proceedings of the National Academy of Sciences USA* **92**, 11312-11316

Gutierrez-Coronado M, Trejo CL, Larque-Saavedra A (1998) Effects of salicyclic acid on the growth of roots and shoots in soybean. *Plant Physiology and Biochemistry* **36 (8)**, 563-565

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

Hutchinson MJ, Saxena PK (1996) Acetylsalicyclic acid enhances and synchronizes thidiazuron induced somatic embryogenesis in geranium (*Pelargonium* x *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

Larque-Saavedra A (1978) The antitranspirant effect of acetylsalicyclic acid on *Phaseolus vulgaris* L. *Physiologia Plantaram* **43**, 126-128

Larque-Saavedra A (1979) Stomatal closure in response to acetylsalicyclic acid treatment. *Zeitschrift für Pflanzenphysiologie* **93 (4)**, 371-375

Leslie CA, Romani RJ (1988) Inhibition of ethylene biosynthesis by salicyclic acid. *Plant Physiology* **88**, 833-837

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* **79**, 583-593

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 Staden 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 (2004a) 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 Mulgund GS, Nataraja K (2004b) Efficient regeneration of *Vanda coerulea*, an endangered orchid using thidiazuron. *Plant Cell, Tissue and Organ Culture* **76**, 289-293

Malabadi RB, Mulgund GS, Nataraja K (2005) Micropropagation of *Dendrobium nobile* from shoot tip sections. *Journal of Plant Physiology* **162**, 473-478

Malabadi RB, Nataraja K (2006) 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. *Inter-*

national Journal of Botany **3**, 40-47

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

Malabadi RB, Nataraja K (2007d) 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**, 90-98

Malabadi RB, van Staden J (2005a) Somatic embryogenesis from vegetative shoot apices of mature trees of *Pinus patula*. *Tree Physiology* **25**, 11-16

Malabadi RB, van Staden J (2005b) Role of antioxidants and amino acids on somatic embryogenesis of *Pinus patula*. *In Vitro Cellular and Developmental Biology – Plant* **41**, 181-186

Malabadi RB, van Staden J (2005c) Storability and germination of sodium alginate encapsulated somatic embryos derived from the vegetative shoot apices of mature *Pinus patula* trees. *Plant Cell, Tissue and Organ Culture* **82**, 259-265

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, van Staden J (2006) Cold-enhanced somatic embryogenesis in *Pinus patula* is mediated by calcium. *South African Journal of Botany* **72**, 613-618

Malabadi RB, Nataraja K (2007e) Genetic transformation of conifers: Applications in and impact on commercial forestry. *Transgenic Plant Journal* **1**, 289-313

Malabadi RB, Teixeira da Silva JA, Nataraja K (2008a) A new approach involving salicyclic acid and thin cell layers for cloning mature trees of *Pinus roxburghii* (Chir Pine). *The Americas Journal of Plant Science and Biotechnology* **2**, 56-59

Malabadi RB, Teixeira da Silva JA, Nataraja K (2008b) Stable and consistent *Agrobacterium*-mediated genetic transformation in *Pinus roxburghi* (Chir Pine). *Tree and Forestry Science and Biotechnology* **2**, 7-13

Malabadi RB, Teixeira da Silva JA, Nataraja K (2008c) *Agrobacterium*-mediated genetic transformation of *Pinus kesiya* Royle ex Gord (Khasi Pine). *The Asian and Australasian Journal of Plant Science and Biotechnology* **2**, 7-14

Malabadi RB, Teixeira da Silva JA, Nataraja K (2008d) Green fluorescent protein in the genetic transformation of plants. *Transgenic Plant Journal* **2**, 86-109

Malabadi RB, Teixeira da Silva JA, Mulgund GS, Nataraja K (2008e) Shoot tip transverse thin cell layers and 24-epibrassinolide in the micropropagation of *Cymbidium bicolor* Lindl. *Floriculture and Ornamental Biotechnology* **2**, 44-48

de Marco A, Roubelakis-Angelakis KA (1996a) The complexity of enzyme control of hydrogen peroxide concentration may affect the regeneration potential of plant protoplasts. *Plant Physiology* **110**, 137-145

de Marco A, Roubelakis-Angelakis KA (1996b) Hydrogen peroxide plays a bivalent role in the regeneration of protoplasts. *Journal of Plant Physiology* **149**, 109-114

Martin-Mex R, Villanueva-Couoh E, Herrera-Campos T, Larque-Saavedra A (2005) Positive effect of salicyclates on the flowering of African violet. *Scientia Horticulturae* **103**, 499-502

Mulin M, Tran Thanh Van K (1989) Obtention of *in vitro* flowers from thin epidermal cell layers of *Petunia hybrida* (Hort.). *Plant Science* **62**, 113-121

Neill SJ, Desikan R, Clarke A, Hurst RD, Hancock JT (2002a) Hydrogen peroxide and nitric oxide as signaling molecules in plants. *Journal of Experimental Botany* **53**, 1237-1247

Neill SJ, Desikan R, Hancock JT (2002b) Hydrogen peroxide signaling. *Current Opinion in Plant Biology* **5**, 388-395

Nhut DT, Aswath CR, Teixeira da Silva JA, Bui VL, Thorpe T, Tran Thanh Van K (2003a) Tobacco thin cell layer morphogenesis. In: Nhut DT, Van Le B, Tran Thanh Van K, Thorpe T (Eds) *Thin Cell Layer Culture System: Regeneration and Transformation Applications*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 65-134

Nhut DT, Teixeira da Silva JA, Bui VL, Tran Thanh Van K (2003b) Thin cell layer studies of vegetable, leguminous and medicinal plants. In: Nhut DT, Van Le B, Tran Thanh Van K, Thorpe T (Eds) *Thin Cell Layer Culture System: Regeneration and Transformation Applications*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 387-425

Nhut DT, Teixeira da Silva JA, Bui VL, Tran Thanh Van K (2003c) Organogenesis of cereals and grasses by using thin cell layer technique. In: Nhut DT, Van Le B, Tran Thanh Van K, Thorpe T (Eds) *Thin Cell Layer Culture System: Regeneration and Transformation Applications*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 427-449

Nhut DT, Teixeira da Silva JA, Bui VL, Thorpe T, Tran Thanh Van K (2003d) Thin cell layer technology in fruit crop regeneration. In: Nhut DT, Van Le B, Tran Thanh Van K, Thorpe T (Eds) *Thin Cell Layer Culture System: Regeneration and Transformation Applications*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 451-472

Nhut DT, Teixeira da Silva JA, Bui VL, Thorpe T, Tran Thanh Van K (2003e) Woody plant micropropagation and morphogenesis by thin cell layers. In: Nhut DT, Van Le B, Tran Thanh Van K, Thorpe T (Eds) *Thin Cell Layer Culture System: Regeneration and Transformation Applications*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 473-493

Nhut DT, Teixeira da Silva JA, Bui VL, Tran Thanh Van K (2003f) Thin cell layer (TCL) morphogenesis as a powerful tool in woody plant and fruit crop micropropagation and biotechnology, floral genetics and genetic transformation. In: Jain SM, Ishii K (Eds) *Micropropagation of Woody Trees and Fruits*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 783-814

Oota Y (1975) Short-day flowering of *Lemna gibba* G3 induced by salicyclic acid. *Plant Cell Physiology* **16**, 1131-1135

Papadakis AI, Roubelakis-Angelakis KA (2002) Is oxidative stress responsible for plant protoplast recalcitrance? *Plant Physiology Biochemistry* **40**, 549-559

Papadakis AI, Siminis CI, Roubelakis-Angelakis KA (1999) Generation of active oxygen species in tobacco and grapevine protoplasts. *Plant Physiology* **121**, 197-205

Papadakis AI, Siminis CI, Roubelakis-Angelakis KA (2001) Reduced antioxidant machinery correlates with suppression of totipotency in plant protoplasts. *Plant Physiology* **126**, 434-444

Ping LJ, Tong JS, Jun PL (2001) Enhanced somatic embryogenesis by salicyclic acid of *Astragalus adsurgens* Pall: relationship with hydrogen peroxide production and hydrogen peroxide-metabolizing enzyme activities. *Plant Science* **161**, 125-132

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

Quiroz FM, Mendez ZM, Larque-Saavedra A, Loyola-Vargas VM (2001) Picomolar concentration of salicyclic induce cellular growth and enhance somatic embryogenesis in *Coffea arabica* tissue culture. *Plant Cell Reports* **20**, 679-684

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

Raskin I, Ehmann A, Melander WR, Meeusen BJD (1987) Salicyclic acid: a natural inducer of heat production in *Arum lilies*. *Science* **2373**, 1601-1602

Raskin I (1992) Role of salicyclic acid in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **43**, 439-463

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

San Miguel R, Gutierrez M, Larque-Saavedra A (2002) Low concentration of salicyclic acid increase nitrate accumulation in roots of *P. patula*. *Phyton* **71**, 79-82

Simins CI, Kanellis AK, Roubelakis-Angelakis KA (1994) Catalase is differentially expressed in dividing and non-dividing protoplasts. *Plant Physiology* **105**, 1375-1383

Teixeira da Silva JA (2003) Thin cell layer technology for induced response and control of rhizogenesis in chrysanthemum. *Plant Growth Regulation* **39**, 67-76

Teixeira da Silva JA (2008) Plant Thin Cell Layers: Challenging the concept. *International Journal of Plant Developmental Biology* **2**, 79-81

Teixeira da Silva JA (2009) Thin Cell Layers: Power-tool for organogenesis of floricultural crops. In: Mohan Jain S, Ochatt SJ (Eds) *Methods in Molecular Biology: Protocols for in Vitro Propagation of Ornamental Plants*, Humana Press, Totowa, NJ, USA, in press

Teixeira da Silva JA, Tanaka M (2009) Thin Cell Layers: The Technique. In: Davey M, Anthony P (Eds) *Plant Cell Culture: Methods Express*, Scion Publishing Ltd., Bloxham, UK, in press

Teixeira da Silva JA, Tran Thanh Van K, Biondi S, Nhut DT, Altamura MM (2007) Thin cell layers: developmental building blocks in ornamental biotechnology. *Floriculture Ornamental Biotechnology* **1**, 1-13

Tran Thanh Van K, Van Le B (2000) Current status of thin cell layer method for the induction of organogenesis or somatic embryogenesis. In: Mohan SJ, Gupta PK, Newton RJ (Eds) *Somatic Embryogenesis in Woody Plants* (Vol 6), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 51-92

Vasyukova NI, Ozeretskovskaya OL (2007) Induced plant resistance and salicyclic acid: A review. *Applied Biochemistry and Microbiology* **43**, 367-373