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# Spatio-Temporal Accumulation of Peroxidases and Assessment of Clonal Identity of Somatic Seedlings by ISSR and RAPD in *Pinus roxburghii*

Ravindra B. Malabadi and K. Nataraja
Division of Plant Biotechnology, Department of Botany, Karnatak University,
Pavate Nagar, Dharwad-580003, Karnataka State, India

**Abstract:** This study highlights the key role of peroxidases influencing somatic cells towards embryogenic pathway. Further the clonal identity of somatic seedlings was assessed using RAPD and ISSR markers. The lowest peroxidase activity was observed in embryogenic cultures on maintenance medium showing elongated cells with cleavage polyembryony as compared to control. Highest peroxidase activity was observed in non-embryogenic cultures showing round, globular and oval cells. Peroxidase activity is not only involved in reducing the accumulation of toxic amounts of $H_2O_2$ but might also influences the conversion of somatic cells into an embryogenic state and, therefore, can be used as a biochemical marker to differentiate embryogenic and non-embryogenic cultures. RAPD and ISSR analysis found no evidence of genetic variation either within or between the embryogenic lines established from three of these trees, or between these lines and the trees of origin, or between somatic embryos derived plantlets and the trees of origin.

**Key words:** DNA fingerprinting, mature tree, peroxidase, shoot apices, somatic embryogenesis

## INTRODUCTION

Peroxidases normally exist in plants as multiple isoenzymes and have been known for a long time to be involved in several biophysical functions in plants (Gasper *et al*., 1982; Malabadi and Nataraja, 2002, 2003). Peroxidases are oxidoreductases that catalyze the oxidation of diverse group of organic compounds using hydrogen peroxide as the ultimate electron acceptor (Dawson, 1988). Peroxidases have been suggested to be involved in various metabolic steps such as auxin catabolism (Normanly *et al*., 1995), the formation of isodi-Tyrosine bridges in the cross-linking of cell wall proteins (Schnabelrauch *et al*., 1996) and the cross-linking of pectins by diferulic bridges (Amaya *et al*., 1999). They have also been shown to be involved in responses to stress and defense mechanisms against pathogens (Sherf and Kolattukudy, 1993; Malabadi and Nataraja, 2002).

The genetic stability of *in vitro* regenerated plants is an essential requisite for large-scale clonal forestry. One of the PCR-based molecular markers, RAPD has recently been shown to be very useful for DNA fingerprinting (Williams *et al*., 1990), detection of genetic polymorphism, varietals identification and mapping. RAPD was also used to ascertain the genetic stability of embryogenic systems for tree species such as *Picea mariana* (Isabel *et al*., 1993), *Picea glauca* (De Verno *et al*., 1999), *Picea abies* (Fourre *et al*., 1997), *Pinus taeda* (Tang, 2001) and for assessment of clonal identity of somatic seedlings in *Pinus patula* (Malabadi *et al*., 2006). Similarly, inter-simple sequence repeat (ISSR) (Zietkiewicz *et al*., 1994) is simple and does not require previous knowledge of the sequences of the genome being tested. ISSR is widely used to detect intraspecific polymorphisms in plants such as peanut (Raina *et al*., 2001), rice (Joshi *et al*., 2001) and chickpea (Iruela *et al*., 2002).

**Corresponding Author:** Ravindra B. Malabadi, Forest Biotechnology Lab (6.06), ITQB/IBET,
Universidade Nova de Lisboa, Av. Da Republica, Apt. 127, 2781-901, Oeiras, Portugal

In the present study, the activity of peroxidase was investigated to study its key role in determining whether a somatic cell should enter into embryogenic state or not. Present study indicates for the first time that peroxidase activity is influencing the cell morphology and might be involved in programming the cells towards embryogenesis. Further both ISSR and RAPD analysis was performed to assess the genetic fidelity within embryogenic lines established from three genotypes and molecular conformity to the trees of origin of these lines and plantlets generated therefrom.

## MATERIALS AND METHODS

### Plant Material and Initiation of Embryogenic Cultures

Apical shoots from mature (14 years-old) trees of *Pinus roxburghii* from 3 genotypes (PR11, PR105 and PR521) were collected from the Western Ghat forests, India (Latitude-14° 5'-15° 25'; Longitude-74° 45'-76°E with an average rainfall of 80 cm). The mature material was cleansed with commercial NaOCl (3.5% sodium hypochlorite as the active ingredient) for 5 min and then washed 3 times with sterilized distilled water. They were then surface decontaminated with 70% ethanol for 5 min followed by immersion in 0.2% $HgCl_2$ for 2 min and rinsed 4 times with sterile double-distilled water. Transverse sections of approximately 0.5-1.0 mm thick were cut, using a scalpel or a sharp sterilized razor blade from the decontaminated apical shoots for the initiation of embryogenic callus. These apical shoot sections were cultured individually on full strength inorganic salts DCR (Gupta and Durzan, 1985) basal media containing 0.2 g $L^{-1}$ polyvinyl pyrollidine (PVP) (BDH Chemicals), 1.5 g $L^{-1}$ Gellan gum (Sigma), 90 mM maltose (Analar grade, ACE Chemicals) and 0.3% activated charcoal (Sigma) without growth regulators. The cultures were placed in 25×75 mm glass culture tubes (Borosil) containing 10 mL of nutrient medium. These cultures were incubated in the dark at 2°C for 3 days.

After cold pretreatment, cultures were subcultured on to full strength DCR basal media for the initiation of embryogenic tissue. DCR basal medium containing 0.2 g $L^{-1}$ PVP, 1.5 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 20 μM 2,4-D, 25 μM NAA and 9 μM BA was used as an initiation medium (Malabadi and Nataraja, 2006; Malabadi and Van Staden, 2003, 2005a-d). The pH of the medium was adjusted to 5.8 with NaOH or HCl before the Gellan gum was added. The media were then sterilized by autoclaving at 121° C and 1.05 kg $cm^{-2}$ for 15 min. The L-glutamine and casein hydrolysate were filter sterilized and added to the media after it had cooled to below 50°C. All the cultures were maintained in the dark at 25±2°C.

### Maintenance of Embryogenic Cultures

The callus producing embryonal suspensor masses or early embryos was subcultured onto maintenance medium. DCR basal medium containing 120 mM maltose, 2 g $L^{-1}$ Gellan gum supplemented with 2 μM 2,4-D, 2.5 μM NAA and 1 μM BA (maintenance medium) was used for this purpose (Malabadi and Nataraja, 2006; Malabadi and van Staden, 2003, 2005a-d). All the cultures were maintained in the dark at 25±2°C. On this medium, embryonal suspensor masses or early embryos were cultured for 30 days with 2 subcultures. The presence of embryonal suspensor masses was determined by morphological and cytological observations. In all experiments, a control was maintained and all the results were compared against this. As control, the apical shoot sections were cultured directly on induction medium with activated charcoal at 2°C for 3 days, without preculture.

### Partial Desiccation

To study the effect of partial desiccation, one gram fresh weight of the calli, after 15 days on the maintenance medium, was transferred to sterile petri dishes; containing two Whatman No. 1 filter paper disks (Malabadi *et al*., 2004). The Petri dishes were sealed with parafilm and kept at 25±2°C in the dark for 24 h to obtain the desired degree of desiccation (Malabadi and Nataraja, 2006; Malabadi and van Staden, 2003, 2005a-d). The relative water content of calli was calculated as:

$$\frac{\text{Initial weight of calli} - \text{Final weight of calli after desiccation}}{\text{Initial weight}} \times 100$$

**Maturation of Somatic Embryos**

After desiccation, the partially desiccated calli were transferred to maturation medium to induce cotyledonary development. DCR basal medium with 175 mM maltose, 80 μM ABA and 9 g $L^{-1}$ Gellan gum (maturation medium) (Malabadi and Nataraja, 2006; Malabadi and van Staden, 2003, 2005a-d; Malabadi *et al*., 2004) was tested for this purpose. All the cultures were placed in the dark at 25±2°C and maintained for 8 to 12 weeks.

**Germination and Plantlet Recovery**

After 8 to 12 weeks of maturation in the presence of ABA and higher concentrations of maltose and Gellan gum, advanced cotyledonary somatic embryos were taken from the cultures for germination. The germination medium used was DCR basal medium with 2 g $L^{-1}$ Gellan gum (Malabadi and Nataraja, 2006; Malabadi *et al*., 2004). Somatic embryos were considered germinated as soon as radicle elongation occurred. Conversion to a plantlet was based on the presence of an epicotyl. After 4-6 weeks on germination medium, plantlets were transferred to vermiculite. Plantlets were placed in a growth room with a 16 h photoperiod (50 μmol $m^{-2}$ $sec^{-1}$) for acclimatization.

**Enzyme Extraction and Assay of Peroxidase Activity**

The activity of peroxidase was determined at different developmental stages of culture from isolated shoot tip explants to somatic seedlings via initiation, maintenance, maturation and germination of somatic embryos respectively. Samples of 1 g (FW) of tissue were taken at different time intervals (0, 3, 5, 10, 15, 20 days) and were homogenized separately in 2 mL 50 mM sodium phosphate buffer (pH 6.0) in a mortar and pestle at 4°C. Homogenates were then centrifuged at 15,000 rpm for 5 min at 4°C, peroxidases that were ionically-bound to the cell wall were obtained by extracting the pellet with the same buffer containing 1 M KCl after shaking for 2 h at 4°C. The supernatant was used for the peroxidase activity assay (Malabadi and Nataraja, 2002, 2003). Peroxidase activity was determined at 30°C following the formation of tetraguaiacol in a 3 mL reaction mixture containing 1 mL 50 mM sodium phosphate buffer (pH 6.0), 1 mL of 20 mM O-methoxyphenol (guaiacol), 0.9 mL 3 mM $H_2O_2$ and 0.1 mL of crude enzyme extract. Activity was measured spectrophotometrically (Cary 50 Conc UV-Visible Spectrophotometer, Varian Australia) following the increase in Absorption at 470 nm for 3 min and expressed in arbitrary units (U $g^{-1}$ FW). The total peroxidase activity values reported are the average of three measurements. One unit of peroxidase activity (U) represents the amount of enzyme catalyzing the oxidation of 1 mol guaiacol in 1 min, which gives an absorbance of 0.1 at 28±2°C in a 3 mL reaction mixture.

**DNA Fingerprinting by RAPD and ISSR**

DNA was extracted from donor apical shoots of mature field-grown plants, embryogenic callus and somatic seedlings of three genotypes (PR11, PR105 and PR521) for RAPD and ISSR analysis. DNA was extracted using a modified CTAB protocol (Hills and Van Staden, 2002).

**RAPD Fingerprinting**

A prescreening of 96 random decamer RAPD primers kits (OPB, OPC, OPD, OPE and OPG from Operon Technologies, USA) was performed using three genotypes of *Pinus roxburghii*. Amplification was achieved according to the protocol outlined by Williams *et al*. (1990), with some modifications. Of these 62 produced amplification with at least one band. During preliminary primer selection, 17 out of the 62 primers, each yielding more than three bands were selected. Finally, 10

primers (OPB-1, OPB-14, OPC-6, OPD-11, OPD-14, OPE-3, OPG-4, OPG-6, OPG-10, OPG-12), which produced strong, intense and unambiguous bands were selected for RAPD analysis (Table 2) (Malabadi *et al.* 2006). Reproducibility of the selected primers was tested by repeating the PCR amplification twice under the same amplification conditions. PCR reactions were carried out in a final 25 μL reaction mixture containing 25 ng template DNA, 0.2 μL (1 U) of *Taq* DNA polymerase (Roche, Germany), 2.5 μL of 10× PCR buffer (Roche, 100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 0.5 μL of 10 mM dNTP stock (Operon Technologies). The negative control mixture contained all reagents except the DNA template. Each reaction mixture was overlaid with 25 μL of mineral oil (Sigma) to prevent evaporation. The amplification was performed in a Hybaid Theremal Reactor (Hybaid, UK) programmed for 45 cycles, as described in Malabadi *et al.* (2006).

**ISSR Fingerprinting**

Thirty nine selected Simple Sequence Repeat (SSR) primers from primer 800-899 (University of British Columbia (UBC), Biotechnology Laboratory, Vancouver, BC, Canada) were used for PCR amplification. Of these 18 produced amplification with at least one band. During preliminary primer selection, 10 out of the 18 primers, each yielding more than three bands were selected. These primers were mostly 16 to 20 decamer oligonucleotides. Finally, 6 primers which produced strong, intense and unambiguous bands were selected for ISSR analysis (Table 3). The amplification was carried out in a 25 μL reaction volume containing 25 ng template DNA, 0.2 μL (1 U) of *Taq* DNA polymerase (Roche, Germany), 2.5 μL of 10× PCR buffer (Roche, 100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 0.5 μL of 10 mM dNTP stock (Operon Technologies). Initial denaturation was for 2 min at 94° C; followed by 45 cycles of 30 s at 94°C, 45 sec at 52°C and 2 min at 72°C; with a final 5 min extension at 72°C.

The amplification products in both cases, along with DNA molecular weight marker (Gene Ruler™ 100bp DNA Ladder Plus, Fermentas, Lithuania) were separated on 1.5% (w/v) agarose gels. The gels were stained with ethidium bromide and photographed under UV light.

**Statistical Analysis**

RAPD and ISSR bands were scored as discrete variables, using 1 to indicate presence and 0 to indicate absence of a band (Table 4) and the binary data were used to calculate similarity coefficients as described by Sneath and Sokal, (1973). In all experiments; each culture tube contained a single explant. Each replicate contained 25 cultures and each treatment had at least 3 replicates (75 cultures). All the experiments were repeated 3 times (total 225 cultures). Enzyme activity was determined at 0, 3, 5, 10, 15 and 20 days after subculture. Each treatment was replicated three times. Data represent the mean of three replicates in the table were analyzed for significance using ANOVA and the differences contrasted using Duncan's multiple range test. All statistical analysis was performed at the 5% level.

## RESULTS AND DISCUSSION

**Peroxidase Activity**

In the present investigation, peroxidase activity was estimated at the different developmental stages of tissue cultures during embryogenesis of *P. roxburgii*. Higher peroxidase activity compared to control was recorded in the isolated shoot apical dome sections when cultured on preculture DCR basal medium containing 0.3% activated charcoal without growth regulators following incubation in the dark at 2°C for 3 days (Table 1). Microscopic observation of explants after this cold pretreatment showed signs of initiation of callus in the central part of the majority of explants, whereas the peripheral part remained green. High peroxidase activity may be associated with stress or mechanical

Table 1: Influence of various culture media conditions on peroxidase activity (U/g fresh weight, FW). Enzyme activity was determined at the following respective days after subculture. Each treatment was replicated three times. Data represent the mean of three replicates

| | | Peroxidase activity (U $g^{-1}$ fresh weight, FW) expressed after respective days | | | | | |
|---|---|---|---|---|---|---|---|
| Genotype | Culture stages | 0 | 3 | 5 | 10 | 15 | 20 |
| PR11 | Control | 60.3±5.3a | 66.6±6.8a | 66.7±6.3a | 66.7±6.3a | 62.1±5.3a | 67.7±7.0a |
| | Initiation | 46.9±3.9b | 36.0±3.0c | 32.9±3.6c | 29.0±3.0c | 22.4±2.5h | 20.6±2.1h |
| | Maintenance | 14.7±1.2d | 12.6±0.1d | 11.3±0.1d | 11.0±0.1d | 6.4±0.2f | 5.7±0.1f |
| | Maturation | 5.7±0.1f | 9.6±0.1d | 10.8±0.1d | 17.9±1.4h | 55.4±5.0a | 61.3±5.5a |
| | Germination | 59.7±5.2a | 63.7±5.1a | 54.0±5.0a | 66.4±5.9a | 61.0±5.9a | 60.2±5.2a |
| PR105 | Control | 63.1±5.8a | 57.0±5.1a | 63.4±5.4a | 57.0±5.1a | 60.1±5.2a | 61.2±5.2a |
| | Initiation | 39.2±5.6b | 31.3±3.0c | 29.1±2.6c | 23.7±2.0h | 19.7±1.8h | 17.4±1.9h |
| | Maintenance | 16.8±1.3d | 15.3±1.3d | 13.8±0.3e | 12.4±0.3e | 11.1±0.1e | 9.2±0.1e |
| | Maturation | 9.1±0.2e | 13.1±1.2d | 16.8±1.6d | 33.4±3.3c | 59.0±5.5a | 67.0±7.6a |
| | Germination | 54.7±5.0a | 53.7±4.9a | 55.1±4.8a | 52.1±4.6a | 54.9±4.6a | 62.6±5.9a |
| PR521 | Control | 61.3±5.4a | 52.8±5.0a | 55.2±5.3a | 57.8±5.9a | 58.7±5.0a | 50.7±4.3a |
| | Initiation | 36.4±3.0c | 30.7±3.0c | 22.3±2.7h | 15.5±1.3h | 8.5±0.2e | 8.1±0.2e |
| | Maintenance | 6.8±0.2f | 2.4±0.2g | 2.2±0.1g | 1.6±0.1g | 1.5±0.1g | 1.6±0.1g |
| | Maturation | 9.2±0.2e | 15.7±1.3d | 29.9±3.1c | 44.5±3.8b | 69.3±7.5 a | 66.7±7.5a |
| | Germination | 58.7±5.1a | 58.4±5.6a | 57.0±5.5a | 55.4±4.8a | 60.0±5.7a | 60.4±5.5a |

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

injury, since wounding and environmental stress responses itself is a significant signal in the induction of dedifferentiation. These cold stress responses may involve the release of polyphenols from the vacuole as well as phenol synthesis in the cytoplasm. Elevated levels of peroxidases catalyze the reduction of $H_2O_2$ and protect the explants from oxidative damage (Brown *et al*., 1993).

On the other hand, the peroxidase activity of cultures of three genotypes of *P. roxburghii.* decreases with an increase in the number of days of subculture (15 days) on initiation medium (Table 1). The subculture of shoot apical dome sections on initiation medium was characterized by 3 different growth phases namely a short *lag* phase not exceeding 5 days, followed by an *exponential* phase between 5-20 days and finally a slow *stationary* phase from 20-30 days before senescence occurs. During the first 5 days of culture of shoot apical dome sections on initiation medium, few cells might have been programmed towards embryogenesis and showed a very slow growth of callus and correlated with higher peroxidase activity. Microscopic observation of cells showed actively-dividing round, globular and oval cells with a prominent nucleus and cytoplasm, with a very few showing signs of elongation. These cells showed higher peroxidase activity, correlated with an increase in cell wall material. During the *exponential* phase between 5-20 days, programmed cells might have shifted their developmental pathway due to cold pretreatment. These samples had an increase in the fresh weight of callus showing embryonal suspensor masses with decreased peroxidase activity compared with the control. In general, during cell division cellular proteins differ, not only with respect to their function but also in terms of their timing and extent of expression. It has been shown in a number of plant systems that during organogenesis or embryogenesis, functionally-related proteins seem to be encoded by groups of co-ordinately expressed genes and that plant growth regulators are key moderators of this gene expression (Schnabelrauch *et al*., 1996; Malabadi and Nataraja, 2002, 2003). Another reason for the decrease of peroxidase activity after cold pretreatement could be the release of substances necessary for embryogenesis, mainly amino acids and stress-induced proteins (Xie *et al*., 1997; Malabadi and Van Staden, 2005a, b). Acquisition of embryogenic competence largely relies on dedifferentiation, a process whereby existing transcriptional and translational profiles are erased or altered to allow cells to set a new developmental program. The activation of cell division is required to maintain the dedifferentiated cell fate, as well as for embryo differentiation (Malabadi, 2002). Establishment of a new cellular state is not only governed at the level of gene expression, but requires modifications and/or removal of unnecessary polypeptides, as well as the proper folding of newly-

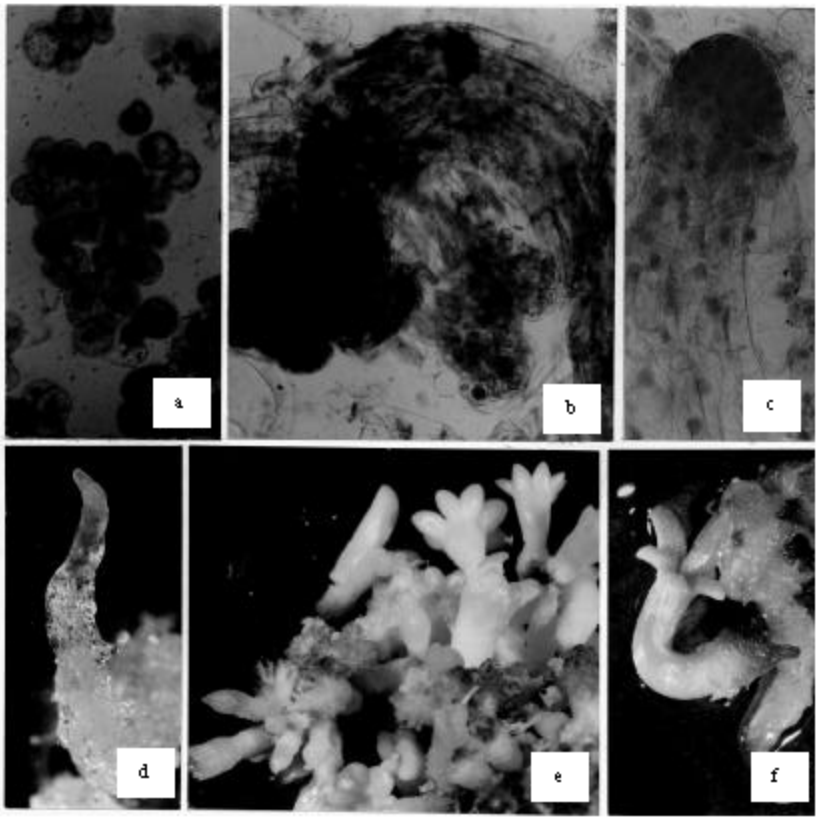


Fig. 1: Effect of peroxidase activity on cell morphology, a: A mixture of round, globular and oval cells showing higher peroxidase activity in non-embryogenic cultures; b, c: Elongated cells with cleavage polyembryony showing low peroxidase activity in embryogenic cultures on maintenance medium; d: Elongated cells showing the formation of embryonal head; e: Matured somatic embryos with higher peroxidase activity, f: Successful germination of somatic embryo on germination medium. (Scale bars: A-1 cm = 0.025 cm; B-1 cm = 0.016 cm; C-1 cm = 0.014 cm; D-1 cm =0.025 cm; E-1 cm = 0.076 cm; F-1 cm = 0.2 cm)

synthesized proteins and protein complexes (Feher *et al*., 2003). This investigation indicated that higher peroxidase activity corresponds to non-embryogenic cultures showing mostly round, globular and oval cells (Fig. 1a) whereas decreased peroxidase activity was correlated with elongated cells with cleavage polyembryony (Fig. 1b-c). The lowest peroxidase activity of cultures was recorded on maintenance medium (Fig. 1b-c) compared with the control (Table 1). Microscopic observation of cells on maintenance medium showed the formation of elongated cells with the development of proembryonal heads and cleavage polyembryony (Fig. 1b-d).

The matured somatic embryos (Fig. 1e) showed higher peroxidase activity similar to that of non-embryogenic cultures (Table 1). Higher peroxidase activity was also recorded with somatic seedlings (Fig. 1f) which clearly indicate that peroxidases catalyzed the reduction of $H_2O_2$ and protected the embryos from oxidative damage (Wakui *et al*., 1999). Polyphenols and peroxidases have been reported to associate with mechanical injury and many environmental stress responses such as radiation, desiccation, drought and wounding (Lagrimini, 1992; Vaugh and Duke, 1984). Plant tissue

necrosis and subsequent cell death are usually observed during *in vitro* regeneration of mature trees of conifers. Cell death is correlated with elevated levels of peroxidases during tissue browning. It was reported that desiccation-tolerant somatic embryos induced by abscisic acid (ABA) and or polyethylene glycol (PEG) 6000 have higher peroxidase activity, improved tissue organization after desiccation and improved survival (Fowke *et al.*, 1994; Iida *et al*., 1992; Tang, 2000). Hydrogen peroxide ($H_2O_2$) levels increase in plant cells after exposure to environmental stress and prolonged accumulation of toxic amounts of $H_2O_2$ within the cell is eventually lethal. To avoid these deleterious effects, all organisms express peroxidases, which function as detoxifying enzymes by catalyzing the reduction of $H_2O_2$ (Creissen *et al*., 1994). It has been shown that ABA affects stabilization of the cell membrane system. ABA and PEG 6000 also promoted the production of peroxidases in desiccation-tolerant somatic embryos (Attree and Fowke, 1993; Tang, 2000). In case of loblolly pine, peroxidase activity of desiccated somatic embryos increased sharply after 1 day of desiccation treatment at 87% relative humidity and desiccation-tolerant somatic embryos had higher peroxidase activity than sensitive somatic embryos (Tang, 2000). Present results are in agreement with the literature. Peroxidase activity might influences cell morphology by controlling the cell wall extensions and modifies the cell wall properties and can be thus used as a biochemical marker to differentiate embryogenic and non-embryogenic cultures during somatic embryogenesis of *Pinus roxburghii*.

**DNA Fingerprinting by RAPD and ISSR**

RAPD and ISSR analyses were successfully used as a tool to asses the clonal identity of somatic embryo-derived plantlets from the vegetative shoot apices of three genotypes of *Pinus roxburghii*. In case of RAPD, 10 primers (Table 2) generated reproducible RAPD profiles and as a result, 134 amplified products were produced, ranging in size from 200 to 2900 bp, with an average of 3.9 bands per primer per lane. Of the 134 fragments scored from these primers, 100 were monomorphic. On the other hand, in case of ISSR, 6 primers (Table 3) generated reproducible ISSR profiles and as a result, 70 amplified products were produced, ranging in size from 200 to 2900 bp, with an average of

Table 2: Sequences of the selected primers used in the RAPD analysis and the total number of RAPD bands generated for three genotypes (PR11, PR105, PR521) of *Pinus roxburghii*

| | | No. of bands in 3 genotypes | | | |
|---|---|---|---|---|---|
| Primers | Nucleotide sequences (5'-3') | PR11 | PR105 | PR521 | Total |
| OPB-01 | GTTTCGCTCC | 7 | 8 | 9 | 24 |
| OPB-14 | TCCGCTCTGG | 4 | 5 | 3 | 12 |
| OPC-06 | GAACGGACTC | 7 | 6 | 7 | 20 |
| OPD-11 | AGCGCCATTG | 4 | 6 | 3 | 13 |
| OPD-14 | CTTCCCCAAG | 3 | 2 | 5 | 10 |
| OPE-03 | CCAGATGCAC | 3 | 4 | 2 | 09 |
| OPG-04 | AGCGTGTCTG | 2 | 5 | 3 | 10 |
| OPG-06 | GTGCCTAACC | 3 | 5 | 4 | 12 |
| OPG-10 | AGGGCCGTCT | 5 | 3 | 2 | 10 |
| OPG-12 | CAGCTCACGA | 3 | 7 | 4 | 14 |
| Total | | 41 | 51 | 42 | 134 |

Table 3: Sequences of the selected primers used in the ISSR analysis and the total number of ISSR bands generated for three genotypes (PR11, PR105, PR521) of *Pinus roxburghii*

| | | No. of bands in 3 genotypes | | | |
|---|---|---|---|---|---|
| Primers | Nucleotide sequences (5'-3') | PR11 | PR105 | PR521 | Total |
| 809 | GAGGAGAGAGAGAGAGG | 3 | 2 | 4 | 09 |
| 820 | GTGTGTGTGTGTGTC | 2 | 3 | 5 | 10 |
| 825 | ACACACACACACACACT | 4 | 5 | 3 | 12 |
| 843 | CTCTCTCTCTCTCTCTRA | 5 | 3 | 2 | 10 |
| 880 | GGAGAGGAGAGGAGA | 3 | 2 | 6 | 11 |
| 891 | ACTACGACTTGTGTGTGTGTGTG | 7 | 9 | 2 | 18 |
| Total | | 24 | 24 | 22 | 70 |

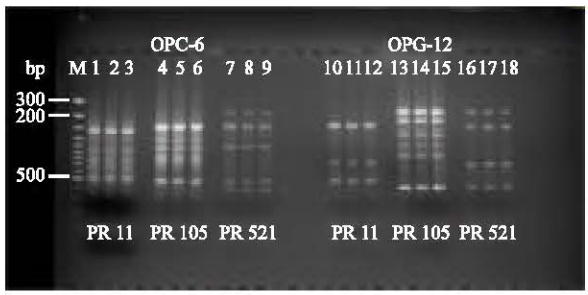


Fig. 2a: RAPD profiles of *Pinus roxburghii* genotype PR11 (Lanes 1-3, 10-12), PR105 (Lanes 4-6, 13-15) and PR521 (Lanes 7-9, 16-18) generated from primers OPC-6 and OPG-12 after agarose gel electrophoresis, Lane M represents molecular weight markers, Lanes 1, 4, 7, 10, 13, 16 = DNA from shoot tips of mature trees; lanes 2, 5, 8, 11, 14, 17 = DNA from callus; Lanes 3, 6, 9, 12, 15, 18 = DNA from somatic seedlings

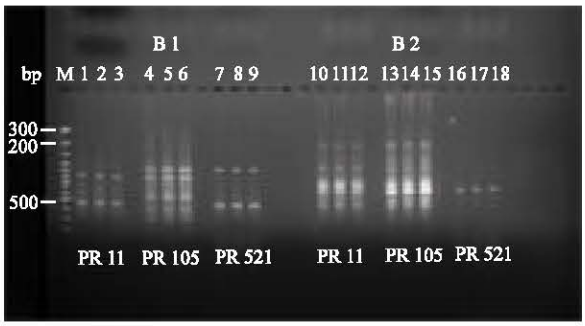


Fig. 2b: ISSR profiles of *Pinus roxburghii* genotype PR11 (Lanes 1-3, 10-12), PR105 (Lanes 4-6, 13-15) and PR521 (Lanes 7-9, 16-18) generated from primers 825 (B1) and (891) (B2) after agarose gel electrophoresis, Lane M represents molecular weight markers, Lanes 1, 4, 7, 10, 13, 16 = DNA from shoot tips of mature trees; lanes 2, 5, 8, 11, 14, 17 = DNA from callus; Lanes 3, 6, 9, 12, 15, 18 = DNA from somatic seedlings

2.3 bands per primer per lane. Out of 70 fragments scored from these primers, 50 were monomorphic. The RAPD and ISSR banding pattern in all three genotypes showed complete homology between donor parental field grown plants, callus and somatic seedlings (Fig. 2a, b). There was no deviation between field grown mother plants, callus and somatic seedlings in the three genotypes of *Pinus roxburghii*, which indicates uniformity within the clone. Furthermore, similarity matrix values of the three genotypes of *Pinus roxburghii* using Sneath and Sokal, (1973) method showed a clear remarkable genetic distance of 1.0, 0.53 and 0.68 respectively (Table 4). Many workers reported that RAPD markers indicated the genetic integrity among meristem-derived plants in *Picea mariana* (Isabel *et al*., 1993), *Pinus patula* (Malabadi *et al*., 2006), *Picea deltoids* (Rani *et al*., 2001), *Phoenix dactylifera* (Javouhey *et al*., 2000), *Zingerber officinale* (Rout *et al*., 1998) and *Eucalyptus camadulensis* (Rani and Raina, 1998). The absence of polymorphic patterns between donor-field-grown mother plants and the respective somatic seedlings are also in accordance with RAPD comparisons of *Pyrus* propagated *in vitro* with donor plants and suggested that no major chromosome rearrangements occurred (Oliveira *et al*., 1999). On the basis of results it is confirmed that RAPD and ISSR were appropriate markers for the detection of polymorphisms due to the simplicity

Table 4: Similarity matrix of the three genotypes of *Pinus roxburghii* (PR11, PR105 and PR521) based on Sneath and Sokal (1973) coefficient values determined from 123 RAPD and 60 ISSR products

| | PR11a | PR11b | PR11c | PR105a | PR105b | PR521c | PR11a | PR105b | PR521c |
|---|---|---|---|---|---|---|---|---|---|
| PR11a | 1.00 | | | | | | | | |
| PR11b | 1.00 | 1.00 | | | | | | | |
| PR11c | 1.00 | 1.00 | 1.00 | | | | | | |
| PR105a | 0.53 | 0.53 | 0.53 | 1.00 | | | | | |
| PR105b | 0.53 | 0.53 | 0.53 | 0.53 | 1.00 | | | | |
| PR105c | 0.53 | 0.53 | 0.53 | 0.53 | 0.53 | 1.00 | | | |
| PR521a | 0.68 | 0.68 | 0.68 | 0.68 | 0.68 | 0.68 | 1.00 | | |
| PR521b | 0.68 | 0.68 | 0.68 | 0.68 | 0.68 | 0.68 | 0.68 | 1.00 | |
| PR521c | 0.68 | 0.68 | 0.68 | 0.68 | 0.68 | 0.68 | 0.68 | 0.68 | 1.00 |

a: DNA from apical shoot (donor field grown plants), b: DNA from callus, c: DNA from somatic seedlings

of technique and information on template DNA sequence is not required (Malabadi *et al*., 2006). Therefore, we hereby ascertain clonal identity of somatic seedlings with its respective field grown parental clone in all the three genotypes of *Pinus roxburghii* on the basis of RAPD and ISSR profiles. Nevertheless, it is not excluded that the reliability of RAPD and ISSR as a marker system to certify genetic stability of *in vitro* woody plant material after long periods of micropropagation needs to be confirmed by adult phenotypic characteristics. It would be interesting to study the ability of other markers such as RFLP, AFLP, DNA methylation studies and microsatelites in this regard. In spite of above-mentioned limitations of RAPD and ISSR analyses, this consistency and the observation of considerable polymorphisms between genotypes, supports the conclusion that *Pinus roxburghii* plantlets regenerated by the method used in this study are likely to be genetically true to their trees of origin.

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