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Root Induction in Microshoots of *Simarouba glauca* L. In Vitro: Peroxidase as a Marker for Rooting

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Abstract

Induction of rooting in microshoots of *Simarouba glauca* L. was achieved within 12 to 15 days of culture on MURASHIGE and SKOOG's (1962) medium supplemented with 1.0 mg/l IBA and 3% (w/v) sucrose. There was no spontaneous rooting observed without the application of auxins. Peroxidase activity was the minimum at the induction phase and maximum at the initiation and expression phase grown on medium containing 1.0 mg/l IBA. Rooting was associated with selective expression or repression of isoforms of peroxidase during induction, initiation and expression phase. This study indicates a key role of peroxidase in rooting of microshoots of *Simarouba glauca in vitro*.

Key words: biochemical marker, *in vitro*, peroxidase activity, rooting, *Simarouba glauca*, tree.

FDC: 165.44; 161.4; 181.36; 176.1 *Simarouba glauca*.

Abbreviations: IBA, indole-3-butyric acid; MS, MURASHIGE and SKOOG's (1962); PVP, Polyvinyl-pyrrolidone; BA, 6-benzyladenine; NAA, a-naphthaleneacetic acid.

Introduction

Simarouba glauca L. (Simaroubaceae), a fast growing multipurpose tree, grows even on marginal lands under water stress conditions and yields edible oil to the extent of about 60 % of kernels (ROUT and DAS, 1994). *In vitro* micropropagation of *Simarouba glauca* was reported by ROUT and DAS (1995). Rooting of microshoots is critical in plant production systems *in vitro*. Induction of rooting for a long time has been considered as a single-phase process but successively there were several reports where the adventitious rooting depended on a series of interdependent phases (induction, initiation and expression) (MONCOUSIN *et al.*, 1988; GASPAR *et al.*, 1992, 1994). Various studies on adventitious root formation in microshoots have shown the fundamental role played by peroxidases in controlling rooting *in vitro* (QUOIRIN *et al.,* 1974; VAN HOOF and GASPAR, 1976; MONCOUSIN and GASPAR, 1983; BERTHON *et al.,* 1987; HAUSMAN,

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1993; RIVAL *et al.,* 1997). The role of auxin in relation to peroxidase activity in rooting of various plant species was also reported by HAUSMAN *et al.* (1997) and KEVERS *et al.* (1997). The present investigation was conducted to monitor the rooting behaviour in shoots of *Simarouba glauca* through *in vitro* and the role of peroxidase and isoenzyme patterns during rooting.

Material and Methods

Plant material

Healthy branches (12 cm to 15 cm long) were collected from 10-year-old mature tree of *Simarouba glauca* growing in the experimental garden of the Regional Plant Resource Centre, Bhubaneswar. Leaves were removed from the branches and were cut into 6 to 7 segments, having one node in each segment. Explants were then washed with 2 % (v/v) 'Labolene' detergent solution (Glindia, India) for 5 min to 10 min and rinsed in running tap water for 15 min. The internodal segments were surface disinfected by 0.1 % (w/v) mercuric chloride aqueous solution for 15 min and subsequently washed in sterile distilled water at least three times under aseptic condition. The internodal segments, were further cut into 0.5 cm to 1.0 cm pieces having one node in each segment, used as explant material.

Culture medium and condition

The nutrient media consisted of MS (MURASHIGE and SKOOG, 1962) basal salts supplemented with various concentrations of 6-benzyladenine (BA; 0 mg/l, 0.5 mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l, 2.5 mg/l and 3.0 mg/l) and a-naphthaleneacetic acid (NAA; 0 mg/l, 0.1 mg/l and 0.25 mg/l) singly or in combination for axillary bud proliferation and multiplication. The pH of the media was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl before gelling with 8 g/l (w/v) of agar (Qualigen, India). Routinely, 20 ml of the molten medium was dispensed into culture tubes (25 mm x 150 mm), plugged with non-absorbent cotton wrapped in one layer of cheese cloth and autoclaved at 121 °C and 104 kPa for 15 min. The cultures were maintained by regular subculture at 6-week intervals on fresh medium with the same composition.

For root induction, the 6-week-old microshoots (1 cm to 2 cm) were separated from the mother cultures and transferred to MS basal salts supplemented with various concentrations of IBA (0 mg/l, 0.5 mg/l, 1.0 mg/l, 1.5 mg/l and 2.0 mg/l) with 3 % (w/v) sucrose. One excised shoot was placed in each culture tube (25 mm x 150 mm) having 20 ml of the culture media. All the cultures were maintained at 25 ± 2 °C under 16-h photoperiod with cool, white fluorescent lamps (55 µmol $m^{-2}s^{-1}$) (Phillips, Bombay, India).

The data pertaining to mean percentage of rooting and number of roots/shoot were recorded over a period of 15 days from the start of the experiment. There were twenty replicates in each treatment and the experiment was repeated thrice.

Sample collection for peroxidase activity

Microshoots were collected from the mother cultures prior to inoculation into rooting medium (0-day) and at every three day intervals up to 15 days. Usually, 60 cultures were used per treatment for sample preparation. The experiment was performed three times.

Peroxidase activity

Fresh tissue (100 mg) was taken from the rooting zone (~ 0.5 cm) of the microshoots grown on various treatments at 3-day intervals (0, 3, 6, 9, 12 and 15 days) and homogenised with mortar and pestle in 4 ml of cold 0.1 M phosphate buffer (pH 6.1) containing 30 mg of insoluble PVP and 15 mg sodium ascorbate. The homogenate was filtered through four layers of miracloth and centrifuged at 12,000 g for 10 min at 4 °C. The supernatant was used for the peroxidase assay. The assay mixture contained 0.1 M phosphate buffer (pH 6.1), 4 mM guaiacol, 3 mM H_2O_2 and 0.4 ml of crude enzyme extract. The total reaction volume was 1.2 ml. The rate of absorbance (OD) at 420 nm was measured using a double beam UV-Spectrophotometer (Jasco, UVIDEC-650, Japan). The levels of enzyme activity were expressed as µmoles H_2O_2 destroyed/min/mg protein (BERGMEYER et al., 1974). Protein contents were determined according to the method of BRADFORD (1976) using bovine serum albumin as standard.

Enzyme extraction and detection

Fresh tissue (100 mg) was taken from the rooting zone (~ 0.5 cm) of the microshoots at 3-day intervals (0, 3, 6, 9, 12 and 15 days) and homogenised with 0.2 M Tris-HCl at pH 8.5, containing 1 M sucrose and 0.056 M 2-mercaptoethanol (WETTER and DYCK, 1983). The crude homogenates were then centrifuged at 12,000 g for 30 min to remove cellular debris. The supernatant was used directly for electrophoresis. All the extractions were made at a temperature of 4 °C.

Isozymes were performed by tube polyacrylamide gel electrophoresis (PAGE) using a stacking gel density of 0.6 % (w/v) N,N'-methylene bis-acrylamide and 2.5 % (w/v) acrylamide and 0.2 % (w/v) and 7.5 % (w/v) bis-acrylamide and acrylamide respectively for resolving gel. The running buffer consisted of 0.05 M tris-glycine, pH 8.3. Gels were precooled to 2 °C to 5 °C prior to the time of electrophoresis. Extracts were prepared by the addition of 0.005 ml bromophenol blue (BPB) (0.05 % m/v) and 0.050 ml of this extract added to each tube. Electrophoresis was performed in the dark at 5 °C using 4 milliampere per tube for 120 min. Immediately, after each eletrophoretic run; gels were stained for peroxidase (PXR) activity at room temperature using 2 mM O-dianisidine, 2.01 mM β-napthol in 0.1 M Tris-acetate buffer pH 4.0, 3.44 mM acetone, 0.029 mM 30 % H_2O_2 and 100 ml distilled water (EDUARDO, 1983). After staining, the gels were photographed, diagrams made and stored in 7 % (v/v) acetic acid. The position of the isoenzyme band in the gel was expressed as relative mobility (R_f) by measuring the distance migrated by the particular band in relation to that of bromophenol blue used as tracking dye.

Results and Discussion

In vitro shoot multiplication was achieved on MS medium containing 2.5 mg/l BA with 0.1 mg/l NAA and a maximum of 5.83 shoots were produced per nodal explant within 6-week of culture (ROUT and DAS, 1995). Elongated shoots were rooted on MS medium supplemented with 1.0 mg/l to 1.5 mg/l IBA. Our results indicate that the rooting occurred between the 12th and the 15th day of culture on MS medium supplemented with 1.0 mg/l IBA. The percentage of rooting was the maximum (82.45 %) on medium having 1.0 mg/l IBA; rooting was inhibited on the devoid of IBA *(Table 1)*. The number of roots/shoot significantly varied with different concentration of IBA *(Table 1).* Roots produced in 1.0 mg/l IBA were healthier than that produced in higher concentration of IBA (1.5 mg/l to 2.0 mg/l). The media containing auxin stimulated the induction of rooting as reported earlier in other plant species (BLAKSLEY *et al.,* 1991; BLAKESLEY, 1994; HAUSMAN *et al.,* 1997; GASPAR *et al.,* 1997; KEVERS *et al.,* 1997).

The peroxidase activity in microshoots was determined on different treatments during the rooting process *(Fig. 1).* The activity became less apparent in microshoots derived from the

Table 1. – Effect of various concentrations of IBA on rooting of *Simarouba glauca* L. cultured on MS basal salts with 3% (w/v) sucrose after 15 days of culture. 20 cultures per treatment; repeated thrice. a-callusing at the basal end.

IBA concentration (mg/l)	Percentage of rooting (%) (Mean ± S.E.)	Av. number of roots/shoot (Mean ± S.E.)	Av. length of roots/shoot (cm) (Mean ± S.E.)
0	0	0	0
0.5	0	0	0
1.0	82.45 ± 1.3	6.15 ± 0.23	1.34 ± 0.11
1.5	46.34 ± 1.0 a	3.64 ± 0.33	1.02 ± 0.05
2.0	28.32 ± 0.4 a	1.45 ± 0.26	0.77 ± 0.08

media without the growth regulator. The peroxidase activity was also the minimum at primary (inductive) phase and maximum at secondary (initiative) phase in microshoots grown on medium having 1.0 mg/l IBA *(Fig. 1).* The minimum peroxidase activity was observed between the 0-day and the 9th day; maximum activity, however, was noted between the 12th and the 15th day. Similar trends were found in *Sequoiadendron giganteum* (BERTHON *et al.,* 1990) and oil palm (RIVAL *et al.,* 1997). HAND (1994) reported that there was minimum time required for a specific developmental pathway. The curves showed the changes in the levels of peroxidase activity in relation to auxin treatments (GASPAR *et al.,* 1990, 1992, 1994; MONCOUSIN and GASPAR, 1983; MONCOUSIN *et al.,* 1988).

Enzymes which are known as metabolic markers, change during development and differentiation (CHAWLA, 1989). Based on the peroxidase isozyme analysis at different intervals during the rooting process, it was observed that rhizogenesis accompanied the synthesis of certain proteins and enzymes. In the primary (induction) phase, four cathodic bands (R_f = 0.20, 0.28, 0.33 and 0.37) and anodic bands having R_f values ranging from 0.62 to 0.64 were observed *(Fig. 2).* After 3 days of culture on rooting media three cathodic bands disappeared and two anodic bands reappeared having R_f value 0.54 and 0.62. On the 6th and the 9th day of culture, the appearance and disappearance of the anodic and cathodic bands were noted *(Fig. 2).* During initiation of rooting, four cathodic bands (R_f = 0.20, 0.28, 0.33 and 0.37) and two thick anodic bands (R_f = 0.43, 0.48) became visible which might be an additional multiple molecular form of enzyme marker during rhizogenesis. The number and intensity of anionic peroxidases continuously increased during the process of rhizogenesis. This is also in agreement with earlier observations in other plant species (BERTHON *et al.,* 1989).

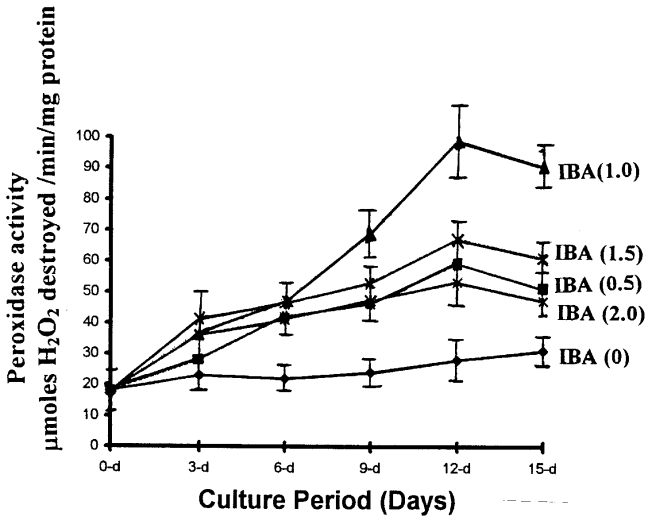

Fig.1. – Changes of peroxidase activity in microshoots of *Simarouba glauca* L. in the absence and presence of IBA (different concentrations) prior to inoculation on rooting media (0-day) and after inoculation on rooting media at 3-d, 6-d, 9-d, 12-d and 15-d of culture. Sixty cultures / treatment; repeated thrice. Bar represents the standard error of the mean of the three independent experiments.

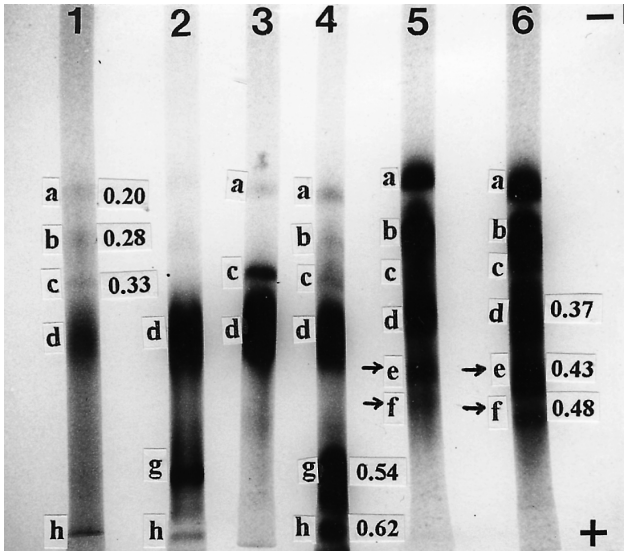

Fig. 2. – PAGE zymograms of peroxidase (PXR) isozyme patterns of Simarouba glauca L. cultured on rooting medium (MS + 1.0 mg/l IBA + 3% (w/v) sucrose) at different intervals. Samples were obtained from microshoots prior to inoculation onto rooting medium (0-day; Tube-1), and after inoculation onto rooting medium (3-day; Tube-2), (6-day; Tube-3), (9-day; Tube-4), (12-day; Tube-5) and (15-day; Tube-6).

The present results confirm that during rhizogenesis, peroxidase activity was the minimum in the primary (inductive) phase and maximum at secondary (initiation) phase in relation to auxin treatment. The variations in number and intensity of anodic and cathodic bands during rhizogenesis confirm the observations of other researchers in different plant species (DRUART *et al.,* 1982; MONCOUSIN and GASPAR, 1983; MONCOUSIN, 1991; KEVERS and GASPAR, 1992). It may be useful to monitor the rooting behaviour in microshoots for mass cloning of a wide range of woody plant species and recalcitrant clones.

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Genetic Variation Among and Within Populations of Four Swedish Hardwood Species Assessed in a Nursery Trial

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Abstract

Four broadleaved tree species, *Acer platanoides, Alnus glutinosa, Fagus sylvatica,* and *Fraxinus excelsior,* which vary with respect to pollen vectors or succession stage, were studied in a nursery trial in Uppsala, latitude 59°50', 12 m asl, at ages 2 to 5. Growth rhythm, growth capacity and damage were assessed in 3 to 7 autochthonous Swedish populations. Generally the family variance components were estimated with higher precision than the population components. There was a considerable variation in bud flushing both at the population and within-population level except for *Fagus sylvatica* with no variation at the population level. The family variance components for bud flushing were on average larger for *Acer platanoides* than for the other species. For budset in *Acer platanoides* (age 2 to 3) and *Fagus sylvatica* (age 3) the family variance components were mostly low. For all species the population variance components for plant height were significant. Except for *Alnus glutinosa* there is a trend that the family variance components for height decrease with age. On average the highest family components were obtained for *Fraxinus excelsior.* Mostly there was limited variation in damage among populations and families. The family mean correlations of the same trait studied different years were significant and positive except for budset in *Acer platanoides.* Correlations between pairs of traits and with meteorological variables were in many cases significant but the correlations never explained more than 50% of the variation. The comparatively large family variance components in *Fraxinus excelsior* and *Acer platanoides* were attributed to non-random mating in their populations.

Key words: Acer platanoides, Alnus glutinosa, Fagus sylvatica, Fraxinus excelsior, populations, families, growth rhythm, growth capacity, genetic variation.

FDC: 165.5; 181.525; 232.1; 176.1 *Acer platanoides;* 176.1 *Alnus glutinosa;* 176.1 *Fagus sylvatica;* 176.1 *Fraxinus excelsior;* (485).

Introduction

Broadleaved tree species from the genera *Acer, Alnus, Fagus, Fraxinus, Quercus, Tilia,* and *Ulmus* play a minor role in Swedish forestry. One reason for this is that these species have their northern limit of distribution in southern Sweden south of latitude 60° and in consequence they constitute approximately 1% of the total forest area in Sweden. Some of the species may play a greater role in the future owing to customer resistance to tropical timber for furniture. There is also a desire to utilize domestic seed sources in landscaping (LAGERSTRÖM and ERIKSSON, 1997). Thus there are incentives for

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