

Differential expression of biochemicals with reference to the degree of juvenility in *Casuarina equisetifolia*

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Abstract

Biochemical studies including total phenol content, peroxidase activity, total chlorophylls, total crude protein content and DNA content were carried out to understand the tissue characteristics between juvenile and adult tissues of *Casuarina equisetifolia*. Phylloclad cuttings collected from four different positions from lower (juvenile) to upper (mature) parts of 9 year old male, female and monoecious trees were subjected to the analyses at the Institute of Forest Genetics and Tree Breeding, Coimbatore. Total phenol content and peroxidase activity exhibited an increasing trend when tissues from lower to upper positions were examined (7.43 to 14.30 mg g⁻¹ and 27.35 to 39.31 enzyme units (mg protein)⁻¹ respectively) whereas, chlorophylls, total crude proteins and DNA content recorded a decreasing trend (3.95 to 2.85 mg g⁻¹, 37.05 to 33.99 mg g⁻¹ and 354.09 to 292.59 µg g⁻¹ respectively). They could be used as biochemical indicators of juvenility. Degree of juvenility plays an important role in successful clonal propagation of this species.

Keywords : Biochemical Indicators, *Casuarina*, Juvenility, Phenol, Peroxidase, Chlorophyll, Protein Content and DNA Content

Introduction

Casuarinas are widely planted in the tropics, subtropics and Mediterranean countries because of their ready adaptability to a variety of environmental conditions and also for their rapid growth performance. Among the 96 species of trees and shrubs in the family Casuarinaceae (Turnbull, 1990), *Casuarina equisetifolia* L. has gained much attention due to its multiple end-uses. India is the largest *Casuarina* growing country in the world. Its amenability to short rotation and a sustained market demand as scaffolding in the building industry, cheap housing material, banana stakes and excellent fuel wood are keys for its success. It is an ideal tree for agro forestry practices. *Casuarinas* have also gained importance as a major pulpwood species.

Propagation by seeds is the major practice this species. However, the seedling raised plantations show considerable variations resulting in unpredictable yield. Though cloning can easily be carried out in *Casuarina*, there exists variability in rooting with respect to the degree of juvenility of the material. Venkataramanan et al. (1998) reported a decreasing trend in rooting ability as cuttings were collected progressively from lower to upper parts of the tree. The juvenile material responded fully to rooting whereas in mature tissues, the rooting response was reduced but not completely suppressed. An experiment was carried out at the Institute of Forest Genetics and Tree Breeding, Coimbatore to understand the difference in tissue characteristics between the juvenile and adult materials

of *C. equisetifolia* with reference to biochemical characteristics.

Materials and Methods

Generally, in a population of *C. equisetifolia* 48 percent of trees are male and 49 percent female with 2 to 3 percent being monoecious. Three *C. equisetifolia* trees, all 9-years-old, were selected for the study. They included a female, a male and a monoecious individual grown in the Forest Campus, Coimbatore, Tamil Nadu.

Plant tissues obtained from the four different positions (Position 1 to 4) mentioned in table – 1, were subjected to various studies as under. Three samples each were collected from the four positions of the male, female and monoecious trees for various biochemical analyses.

Total Phenols

Plant sample (0.5 g) was grounded in 10 ml of 80 percent ethanol and the homogenate was centrifuged at 10,000 rpm for 20 minutes. After collecting the supernatant, the residue was re-extracted five times, pooled the supernatants, evaporated to dryness and dissolved the residue in 5 ml of distilled water. Aliquots (25 μ l) were pipetted out into test tubes, made up the volume to 3 ml with distilled water and added 0.5 ml of Folin-Ciocalteu reagent. After 3 minutes, 2 ml of 20 per cent sodium carbonate (Na_2CO_3) solution was added, mixed thoroughly, placed the tubes in

boiling water bath for one minute, cooled and measured the absorbance at 650 nm against a reagent blank. The concentration of the phenols in the test sample was found out from the standard (catechol) curve and expressed on dry weight basis after estimating the moisture content of the plant samples (Sadasivam and Manickam, 1996).

Chlorophylls

Plant tissue (250 mg) was ground well to a fine pulp using 5 ml of 80 percent acetone and the supernatant transferred to a 25 ml volumetric flask. The process was repeated until the residue was colourless and the final volume was made up to 25 ml with 80 per cent acetone. The absorbance of the solution was read at 645 and 663 nm against the solvent (80 per cent acetone) blank. The amount of chlorophyll present in the extract was calculated based on the following equations (Sadasivam and Manickam, 1996).

$$\text{Chlorophyll } a \text{ (mg g}^{-1} \text{ tissue)} = 12.7 (A_{663}) - 2.69 (A_{645}) * (V / 1000 * W)$$

$$\text{Chlorophyll } b \text{ (mg g}^{-1} \text{ tissue)} = 22.9 (A_{645}) - 4.68 (A_{663}) * (V / 1000 * W)$$

$$\text{Total Chlorophyll (mg g}^{-1} \text{ tissue)} = 20.2 (A_{645}) + 8.02 (A_{663}) * (V / 1000 * W)$$

Where A = absorbance at specific wavelengths,

V = final volume of chlorophyll extract in 80 per cent acetone (25 ml) and

W = fresh weight of tissue extracted (250 mg)

Table – 1. Different positions in tree from where samples were collected

Positions	Description	Remarks
Position 1	Upto 60 cm from the ground level	No flowering in any branch
Position 2	Between 60 cm and 95 cm from the ground level	Occasional flowering in some branches
Position 3	Between 95 cm and 145 cm from the ground level	Many branches showing flowering
Position 4	Between 145 cm and 165 cm from the ground level	All branches showing flowering

Peroxidase (E.C. 1.11.1.7)

Extracted 0.5 g of the fresh tissue in 1.5 ml of 0.1M phosphate buffer pH 7 by grinding with a pre-cooled mortar and pestle. The homogenate was centrifuged at 18,000 rpm at 5°C for 15 minutes and the supernatant was used as the enzyme source. Pipetted out 2 ml of the buffer solution, 0.05 ml of 20 mM guaiacol solution, 0.02 ml of the enzyme extract and 0.03 ml of the 12.3 mM hydrogen peroxide in a cuvette, mixed well and placed in the spectrophotometer. Waited till the absorbance has increased by 0.05 and noted the time required in minutes (Δt) to increase the absorbance by 0.1. Since the extinction coefficient of guaiacol dehydrogenation product at 436 nm under the conditions specified is 6.39 per micromole, the enzyme activity per litre of the extract was calculated as below.

$$\text{Enzyme activity units / litre} = (3.18 \times 0.1 \times 1000) / (6.39 \times 0.5 \times \Delta t \times 0.02).$$

The enzyme activity was expressed as units per mg protein based on the estimation of total crude proteins (Sadasivam and Manickam, 1996).

Total Crude Proteins

Extracted 0.5 g of the fresh tissue in 5 ml of 0.1M phosphate buffer (pH 7) by grinding with a mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 15 minutes and the supernatant was used for protein estimation. Pipetted out 0.1 ml of the sample extracts into test tubes and the volume were made up to 1 ml with distilled water. A tube with 1 ml of distilled water served as the blank. Added 5 ml of alkaline copper solution (prepared by mixing 50 ml of 2% sodium carbonate in 0.1 N sodium hydroxide and 1 ml of 0.5% copper sulphate in 1% potassium sodium tartrate) and mixed well. After allowing to stand for 10 minutes, 0.5 ml of Folin-Ciocalteu reagent was added, mixed well, incubated at room temperature in the dark for 30 minutes and measured the absorbance at 660 nm. The

amount of proteins in the test sample was found out from the standard graph (bovine serum albumin) and expressed on dry weight basis after estimating the moisture content of the plant samples (Sadasivam and Manickam, 1996).

DNA Content

Extraction of DNA was carried out following the modified method of Murray and Thompson (1980). Fresh plant sample (1g) was ground in liquid nitrogen and sprayed onto 7.5 ml of hot (65°C) 2x CTAB buffer (2% w/v CTAB, 100 mM Tris-Cl (pH 8), 20 mM EDTA, 1.4 M NaCl, 100 mg PVPP and 0.2% β -mercaptoethanol) and retained at 65°C for 20 minutes. After cooling to room temperature, 3 ml of chloroform / isoamyl alcohol (24:1) was added, mixed gently but thoroughly and centrifuged at 5000 rpm for 10 minutes at room temperature. Collected the aqueous phase and added 0.1 ml of CTAB / NaCl and 2 ml of chloroform / isoamyl alcohol and centrifuged at 5000 rpm for 10 minutes. To the aqueous phase, equal volume of cold isopropanol was added and mixed by inversion. Centrifuged at 5000 rpm for 10 minutes to pellet nucleic acids and discarded the supernatant. The nucleic acid pellet was washed with 70 percent ethanol and added 50 μ g of RNase-A and incubated at 37°C for 2 hours. Then, the mixture was extracted with phenol followed by phenol-chloroform and finally with chloroform. After adding 0.1 ml of 3 M sodium acetate (pH 5.2) and 2.5 ml of ethanol to the aqueous phase, the contents were mixed by inversion. Centrifuged at 5000 rpm for 10 minutes, washed the DNA pellet with 70 percent ethanol, dissolved in 200 μ l of TE buffer and stored at 4°C.

Quantification of DNA was carried out spectrophotometrically by measuring the absorbance at 260 nm (Reddy and Haisler, 1995). The DNA sample (50 μ l) was diluted to 3 ml with distilled water and the absorbance at 260 nm and 280 nm were recorded.

DNA has a maximum absorbance at about 260 nm and an optical density of 1.0 corresponds to 50 $\mu\text{g ml}^{-1}$ for double stranded DNA.

DNA concentration $\mu\text{g ml}^{-1} = A_{260} \times \text{dilution factor} (60) \times 50$.

Data were analyzed by applying techniques for analysis of variance for Completely Randomized Design (Snedecor and Cochran, 1967). Treatment means were compared using Duncan's Multiple Range Test at 5 per cent level of significance.

Results

Total phenol content (expressed on a per gram dry weight basis) varied significantly among the four positions. Position 4 recorded the maximum phenol concentration of 14.30 mg g^{-1} whereas; position 1 recorded the minimum value (7.43 mg g^{-1}). Position 3 and position 2 registered 11.61 and 9.86 mg g^{-1} of total phenols respectively (Table - 2).

Position 1 registered the maximum values for chlorophyll *a* (2.78 mg g^{-1}), chlorophyll *b* (1.18 mg g^{-1}) and total chlorophyll (3.95 mg g^{-1}) and was found significantly superior to positions 3 and 4. Position 2 which recorded 2.52, 1.05 and 3.56 mg g^{-1} of chlorophyll *a*, chlorophyll *b* and total chlorophyll respectively was on par with position 1 (Table - 2).

The maximum peroxidase enzyme activity was observed in position 4 (39.31 enzyme units (mg protein^{-1})) and was found on par with position 2 and position 3. Significantly inferior level of this enzyme (27.35 enzyme units (mg protein^{-1})) was estimated in position 1 when compared to other positions (Table -2).

Total crude protein content (expressed on a per gram dry weight basis) differed significantly among the four positions (Table -2). Position 1 which recorded the maximum value for total proteins (37.05 mg g^{-1}) was on par with position 2 (36.13 mg g^{-1}). The minimum protein content was estimated by position 4

(33.99 mg g^{-1}) and was found on par with position 3 (35.06 mg g^{-1}).

The plant sample collected from position 1 yielded the maximum quantity of DNA (354.09 $\mu\text{g g}^{-1}$) and was found on par with position 2 (352.02 $\mu\text{g g}^{-1}$) and position 3 (327.61 $\mu\text{g g}^{-1}$). The minimum quantity of DNA was obtained from position 4 (292.59 $\mu\text{g g}^{-1}$). The details can be obtained from Table - 2.

Discussion

Growth of the plant shoot can be divided into several discrete phases based on the character of the organs produced during these phases and the capacity of the shoots for reproductive development (Zimmerman *et al.*, 1985). Traditionally, the transition from juvenile to adult phase has been explained as a change in the reproductive competence but it also is marked by species specific changes in a variety of vegetative traits including leaf shape, leaf anatomy, adventitious root production, disease resistance and a number of secondary compounds (Kerstetter and Poethig, 1998). Rooting potential has been used in several woody plants as a marker for juvenility (Huang *et al.*, 1992a). Several biochemical markers are also being employed to distinguish the juvenile from the adult developmental phases of certain plants.

Total Phenols

Phenolic compounds are contained in plant tissues in different concentrations and the age related changes in their levels have been studied in many plants. Khazaal *et al.* (1993) reported an increase in the concentration of phenolics with the maturity stage in *Phillyrea latifolia*. In *Salix babylonica*, total phenol content was maximum in adult leaf tissues when compared to the juvenile ones (Sharma and Vaid, 1997). Higher amount of phenolic exudations of mature plant tissues when compared to juvenile materials was found to hinder the culture response and subsequent

rooting in *Dalbergia latifolia* (Warriar and Vijayakumar, 1999). An increasing trend in the concentration of total phenolics with maturity was observed in the current study also. Phenol content was found to increase when tissues were collected progressively from lower (juvenile) to upper (mature) positions within the same tree.

Phenolic compounds in plants are strong reducing agents and when they react with enzymes (usually separated by cell vacuoles) the hydroxy group in the phenolic compound get oxidised to form quinones and this irreversible process of hydrogen bonding to proteins inhibits enzyme activity and leads to cell death (Taji and Williams, 1996). Oxidation and browning of the tissue occurs instantaneously when the cut plant surface is exposed to air with damage appearing on and around the cut surface. This may be one of the reasons for the poor rooting response with respect to adult plant materials.

Chlorophylls

Several investigations have been made in different species to study the difference in concentrations of chlorophylls between juvenile and adult tissues. A reduction in the chlorophyll content with maturity was observed in Japanese persimmon, when evaluated at eight different stages of development (Forbus *et al.*, 1991). Analysis of juvenile and mature foliage of English ivy (*Hedera helix*) indicated that there was more chlorophyll present in the former (Greenwood and Hutchinson, 1993). Staden (1996) also observed similar trend in *Salix babylonica*. In *Sequoia sempervirens*, concentrations of chlorophyll *a*, chlorophyll *b* and total chlorophyll were significantly higher in the juvenile and rejuvenated shoots than in the adult ones (Huang *et al.*, 2003). Concentrations of chlorophyll *a*, chlorophyll *b* and total chlorophyll were significantly higher in juvenile tissues when compared to the adult ones in the

present study. A decreasing trend was recorded from lower to upper positions within a tree. The most juvenile plant material (position 1) contained 36, 46 and 39 per cent more of chlorophyll *a*, chlorophyll *b* and total chlorophyll respectively when compared to the tissues obtained from position 4 (most mature).

Peroxidase

Peroxidases represent a class of ubiquitous enzymes widely distributed throughout the plant kingdom (Flocco *et al.*, 1998). As multifunctional enzymes, PODs have been associated with several physiological processes including the regulation of growth and cell expansion (Goldberg *et al.*, 1987), auxin metabolism (Grambow and Langenbeck-Schwich, 1983) and lignifications (Fry, 1986). Matschke (1985) explaining the use of peroxidase as a marker in tree breeding programmes pointed out its relevance in various physiological studies including ageing. Higher peroxidase activity is reported to be closely associated with reduced growth of plants (Zheng and Van-Huystee, 1992). In *Sequoiadendron giganteum*, peroxidase activity was higher in mature tissues than juvenile ones (Berthon *et al.*, 1991). Charyulu (1995) observed increased peroxidase activity in mature tissues of *Spathodea companulata*. Peroxidase activity was noticed to increase with organ maturity in *Eucalyptus* (Li *et al.*, 2000). Lack of adventitious root formation in adult tissues of *Eucalyptus gunnii* was substantiated by the increased peroxidase and polyphenol oxidase activity of phenolic substances (Curir *et al.*, 1992). Peroxidase as a marker for rhizogenesis has been described by several workers (Rout *et al.*, 1999; Li *et al.*, 2000 and Palanisamy, 2001). The present investigation showed that the peroxidase activity followed an increasing trend when cuttings were examined from lower to upper positions within a tree. Significant difference in enzyme activity was recorded between position 1

Table - 2. Difference in various biochemical characteristics with reference to degree of juvenility in *Casuarina equisetifolia*

Position	Total Phenol Content (mg g ⁻¹ dry weight)	Chlorophyll Content (mg g ⁻¹ fresh tissue)			Peroxidase Activity (enzyme activity units (mg protein ⁻¹))	Total Proteins (mg g ⁻¹ dry weight)	DNA Quantity (µg g ⁻¹ dry weight)
		Chlorophyll <i>a</i>	Chlorophyll <i>b</i>	Total Chlorophyll			
Position 1	7.43 ^c	2.78 ^a	1.18 ^a	3.95 ^a	27.35 ^b	37.05 ^a	354.09 ^a
Position 2	9.86 ^{bc}	2.52 ^{ab}	1.05 ^{ab}	3.56 ^{ab}	33.72 ^a	36.13 ^{ab}	352.02 ^a
Position 3	11.61 ^b	2.47 ^b	0.97 ^b	3.44 ^b	34.34 ^a	35.06 ^{bc}	327.61 ^a
Position 4	14.30 ^a	2.04 ^c	0.81 ^c	2.85 ^c	39.31 ^a	33.99 ^c	292.59 ^b
SEM	0.84	0.09	0.05	0.13	2.00	0.47	7.82

Means with the same letter in a column do not differ significantly as per Duncan's Multiple Range Test at 5 per cent level of significance.

(juvenile) and position 4 (mature), the values being 27.35 and 39.31 enzyme units (mg protein)⁻¹. Significant difference with respect to rooting was also noticed between these two positions. Sprigs obtained from position 1 with low peroxidase activity gave the highest rooting response (80.75%) when compared to position 4 (46.15%).

Total Crude Proteins

Studies to understand the juvenility–maturity phase change have been undertaken at the protein level by several researchers. Bon *et al.* (1994) reported a difference in rooting characteristics and in protein abundance between juvenile and adult tissues, collected from the base and the crown respectively in a 90-year-old *Sequoia sempervirens* tree. Huang *et al.* (1992b) found leaf proteins associated with juvenility in the rejuvenated plants of the same species. A reduction in the amount of crude proteins was associated with maturity in *Leucaena leucocephala* (Tangendjaja *et al.*, 1986) and *Morus alba* (Sharma, 1999). A significant difference was observed between the juvenile and adult tissues with respect to total crude protein content in the current study. The content of crude proteins

exhibited a decreasing trend from position 1 (juvenile) to position 4 (mature).

DNA Content

In *Hedera helix*, Kessler and Reches (1977) reported that the mature shoots had a smaller genome than the juvenile shoots and the nuclear genome size was found to be larger in the lower portion of the bole throughout the year in *Abies balsamea* (Mellerowicz *et al.*, 1989). Comparisons between juvenile and mature cambial tissues with reference to DNA concentration, nuclear genome size, nuclear shape, number and volume of nucleoli per nucleus, extranuclear RNAs, proteins and insoluble carbohydrates in *Larix laricina* (Mellerowicz *et al.*, 1995) revealed that mature tissues exhibited lower DNA concentration, lower genome size, fewer nucleoli per nucleus and a higher extranuclear concentration of insoluble carbohydrates than the juvenile ones. Mazza and Viana (1998) studied the variation in quantity of DNA with reference to maturity and found that juvenile materials contained higher quantity of DNA in *Araucaria angustifolia*. The results of the present study also indicated similar findings. The maximum quantity of DNA was obtained from the

juvenile tissues whereas, the mature tissues yielded the minimum. The DNA concentration was found to decrease as the plant samples were collected from lower to upper positions within a tree.

Conclusion

Understanding the degree of juvenility is the key in any successful clonal forestry programme. Though cloning can easily be carried out in *Casuarina*, there exists variability in rooting with respect to the degree of juvenility of the material. A decreasing trend in rooting ability exists in *Casuarina equisetifolia* as cuttings are collected progressively from lower to upper parts of the tree. The present study revealed that the biochemical characteristics namely, total phenol content, chlorophylls, peroxidase activity, total crude protein content and DNA content could be used as biochemical indicators of juvenility. With reference to total phenol content and peroxidase activity an increasing trend was observed when tissues from the lower (juvenile) to upper (matures) positions were examined (7.43 to 14.30 mg g⁻¹ and 27.35 to 39.31 enzyme units (mg protein)⁻¹ respectively) whereas, chlorophylls, total crude proteins and DNA content recorded a decreasing trend (3.95 to 2.85 mg g⁻¹, 37.05 to 33.99 mg g⁻¹ and 354.09 to 292.59 µg g⁻¹ respectively).

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