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Kinetic Characterization and Partial Purification of Peroxidase in Eucalyptus F_1 Hybrids

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Abstract

A differential pattern of peroxidase (E.C. 1.1.11.7) has been observed in reciprocal F_1 hybrids of Eucalyptus (FRI-4 and FRI-5) produced using parental combination of E. tereticornis and E. camaldulensis. The hybrids were characterized for the qualitative, kinetic parameters (total and specific activity, units, K_m and V_{max}) and partial purification of peroxidase. The qualitative study showed the presence of high molecular weight peroxidase isoforms i.e. 260 KD, 250 KD and 110KD bands. A strong maternal inheritance was observed for 260 KD loci in FRI-5. Kinetic characterization of peroxidase revealed that F_1 genotypes had efficient peroxidase system over their parental combination resulting in 8 -10 times more heterotic vigour. The FRI-5 genotypes were found to be having efficient peroxidase system with lowest K_m values (0.12mM-0.17mM) and higher V_{max} values ranging from 0.07-0.14mM min^{-1} $mg\ prot^{-1}$. The study revealed the role of maternal inheritance and was found that better genotypes are produced when superior parents are used as seed parent. The results suggest the efficient use of peroxidase marker for the identification of the better genotypes in forest tree species.

Keywords: *Peroxidase, Enzyme Purification, E. tereticornis, E. camaldulensis, enzyme kinetics*

1. Introduction

Eucalyptus, a diverse genus of flowering trees and shrubs native to Australia, is renowned for its rapid growth and adaptability to various ecological niches. These characteristics have positioned *Eucalyptus* species as vital resources for timber, paper, pulp, and essential oils in global forestry and agroforestry industries. The attributes making *Eucalyptus* spp. so useful for research are their rapid growth and ease of vegetative propagation, these features also underlie the significant economic importance for example, artificial hybrids of Eucalyptus grown in large-scale plantations are becoming increasingly important for pulp production in Indian Sub-continent. However, the successful cultivation and propagation of Eucalyptus hybrids, particularly in regions with suboptimal soil and climatic conditions, remain challenging due to the vulnerability of these hybrids to various biotic and abiotic stresses.

To address these challenges and to enhance the resilience and growth of Eucalyptus F1 hybrids, it is imperative to gain a deeper understanding of the mechanisms underlying their stress response and adaptability. One such mechanism of paramount importance is the enzymatic antioxidant defense system, which plays a critical role in mitigating the adverse effects of oxidative stress. Among the antioxidant enzymes, peroxidases, which catalyze the reduction of hydrogen peroxide and organic hydroperoxides, hold a central place in the plant's defense against oxidative damage (Faria *et al*, 2023). In the present study the kinetic characterization of peroxidase that is the key enzyme for regulation of cell elongation and lignification, in Eucalyptus F_1 hybrids has been reported that can be correlated to the better growth performance of the F1 hybrids over their parental combinations.

E.C. 1.1.11.7, or peroxidase, is a vital metabolic enzyme that is a member of a broad family of enzymes found in plants, animals, and fungus. In the presence of hydrogen peroxide, these proteins, which typically have a

ferroprotoprophyrin prosthetic group, oxidise a variety of substrates (Leon *et al*., 2002; Jouili *et al*., 2011). Numerous isoenzymes present in various plant cell compartments demonstrate the diversity of roles played by peroxidase enzyme systems. According to Morales and Barcelo (1997), they are involved in the regulation of plant hormones, defense mechanisms, control of cell elongation, manufacture of lignin, and suberization process. The current study reports on the qualitative and quantitative characterization of peroxidase enzyme systems using reciprocal F1 hybrids of *Eucalyptus tereticornis* and *Eucalyptus camaldulensis*.
These hybrids produced at Forest Research Institute, Dehradun, India, have shown pronounced degree of hybrid vigour both with respect to height and diameter, the two major parameters resulting in the volume yield (Venkatesh and Sharma, 1977). Thus these hybrids prove to be valuable source for the tree improvement programs, paper and pulp industries etc. The present investigation was aimed to determine the physiological and metabolic levels using peroxidase enzyme system. Further these reciprocal hybrids were tested for the efficient peroxidase system. It was anticipated that this study would provide information regarding the performance of hybrids with respect to kinetic properties of enzyme system under study. Given the diverse ecological range and adaptability of Eucalyptus hybrids, it is reasonable to hypothesize that their peroxidase enzymes play a crucial role in mediating stress responses. However, to date, a comprehensive kinetic characterization of peroxidases in Eucalyptus F1 hybrids and their partial purification remains an unexplored area of research. This knowledge gap limits our understanding of the specific roles of peroxidases in Eucalyptus hybrid physiology and their potential applications in stress mitigation strategies.

2. MATERIAL AND METHODS

Plant material

Foliar sample from three genotypes each of Eucalyptus reciprocal F_1 hybrids i.e. FRI-4 (*E. tereticornis* X *E. camaldulensis*) i.e. FRI-4:13, FRI-4:14, FRI-4:15 and FRI-5 (*E. camaldulensis* X *E. tereticornis*) i.e. FRI-5:16, FRI-5:17, FRI-5:18 were collected from the campus of Forest Research Institute, Dehradun. Tips bearing 4-5 young leaves were harvested and were stored at –20^{o}C for further experimentation.

Enzyme extraction

Varying the time of extraction, composition and pH of extraction buffer, the extraction of peroxidase enzyme was carried out with the proportion of sample of leaves to the extraction buffer being 1:5 (w/v). All the steps were performed at 4^{0}C. Finally, the enzyme systems were extracted in sodium phosphate buffer (0.2 M, pH 7.2). The extract was then centrifuged at 10,000 rpm for 5 min. The resulting peroxidase containing supernatant was kept for further analysis.

Native PAGE of peroxidase

Non-denaturing gel electrophoresis of the crude extract was done using horizontal gel electrophoresis apparatus, Phast System® (Pharmecia Biotech Ltd.). The separation was performed according to the manufacturer's instruction using three gel concentrations i.e. 7.5 12.5 and 20% acrylamide. Following gel electrophoresis, the gel was rinsed in deionized water and stained for the activity staining according to Tanskley and Ortan (1983), using 3-amino 9-ethyl carbazole as a redox dye system.

Iso-Electric Focusing of peroxidase

Iso-Electric focusing was performed using Phast System® (Pharmecia Biotech Ltd.). The focusing was done according to the manufacturer's instruction in a pH gradient of 3-9. The peroxidase bands were stained according to the method described above.

Protein Determination

Protein concentration was estimated using standard method of Lowry *et al*. (1951). The absorbance was measured at 660 nm and compared with the standard curve of Bovine Serum Albumin.

Estimation of molecular weight in native form

Molecular weight estimation in native form was determined using seven standard proteins (Sigma- molecular weight marker kit) and was subjected to Ferguson plot analysis (Pharmacia Biotech Instruction Manual no. 121).

Enzyme activity assays

Frozen leaves were extracted into extraction buffer (100 mM Na Pi, pH 7.0, 0.1% [v/v] Triton X-100, and 5% [w/v] polyvinylpyrolidone), clarified by centrifugation, and the supernatant was immediately assayed for peroxidase activity.

Optimum temperature and pH for enzyme stability and functionality
The aliquots of crude extract were incubated at different temperatures as; 20^0, 27^0, 35^0, 40^0 and 50^0C for 5 min and pH values of phosphate buffer i.e. 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 pH. Subsequently, the activity of peroxidase was determined for each aliquot using o-dianisidine as the redox system.

Total activity of peroxidase enzyme system
Initial velocity of reaction was determined according to Demetrios *et al*. (2002). Ortho-dianisidine, 3-amino 9-ethyl carbazol and catechol were used as redox systems for the estimation of total activity of peroxidase in crude extract. Phosphate buffer (0.2 M, pH 6.5) was used as the blank for calibration of spectrophotometer. Reaction mixture containing phosphate buffer (pH 6.5), 0.1 ml H_2O_2 (0.2 M) and 0.2 ml o-Dianisidine and no enzyme extract was used as the control for enzyme reaction. All the reaction mixtures were incubated at the experimental temperature for at least 2 min before the reaction was started by the addition of enzyme. In 5 ml cuvette the reaction mixture was poured and crude extract was added to start the reaction. Subsequently the change in absorbance at 470 nm was recorded at 30 sec interval for 5 min. The experiment was performed in triplicate.

To determine the total units of enzyme present in crude preparation of peroxidase in different genotypes
For routine assays, the conversion of O-dianisidine to its oxidized form was measured spectrophotometrically as described Demetrios *et al*. (2002). Protein concentrations were determined by the method of Lowery et al. (1951) using BSA as a standard. Specific activity was calculated using an extinction coefficient of $e = 11.3$ M^{-1} cm^{-1} for H_2O_2. The number of units of enzymes in the crude extracts was calculated for each genotype, which was as follows**:** Enzyme Units (Kat Cm^{-3}) = $\Delta E_{460} / \varepsilon$ X a /1000 X 1000/ x. Where ΔE_{460} is the change in the absorbance at 460 nm. S^{-1} (total activity), **a** is the total volume of the reaction mixture in a cuvette of 1cm light path, **x** is the volume of crude preparation included in the reaction mixture and **ε** is the molar extinction coefficient for o- dianisidine (11.3 $mol^{-1}.cm^{-1}$).

To determine the Michaelis constant (K_m) and V_{max} for peroxidase enzyme
To determine the experimental value of K_m and V_{max}, a series of substrate concentrations [S] were used to measure the velocity of reaction, [V]. To determine the rate of reaction/ total activity [V], crude extract was used as the peroxidase enzyme source and o-dianisidine was used as a redox substrate. Five concentrations of o-dianisidine viz. 0.102 mM, 0.204 mM, 0.306 mM, 0.408 mM and 0.510 mM were used to evaluate the rate of reaction. Reaction was carried out in a total of 4ml mixture containing phosphate buffer (0.1 M, pH- 6.5), H_2O_2 (0.2 M), varying concentrations of o-dianisidine and crude plant extract. The change in absorbance was recorded at 470 nm and the reaction was initiated by the addition of crude enzyme fraction (0.2 ml). To determine the K_m and V_{max} values, the double reciprocal curve (Lineweaver Burk Plot) was plotted for reciprocal values of substrate concentrations (1/[S]) versus reciprocal values of initial velocity (1/ [V]).

Partial purification of Enzyme
Phosphate buffer (pH-6.5, 0.2M) was used to extract the peroxidase enzyme from the leaves of F_1 hybrids. 10 ml of the crude enzyme extract was precipitated using ammonium precipitation upto 95% salt concentration. The concentrated extract was then dialyzed for about 24 hours against 0.2M phosphate buffer. The total activity and specific activity of peroxidase was measured in the extract before loading. This dialyzed enzyme extract was loaded on pre-equilibrated Sephadex G-100 gel filtration column. The eluting fractions of 5ml were collected from the bottom of the column and were tested for the activity of peroxidase and specific activity. The eluted fractions containing peroxidase activity were pooled and precipitated by ammonium acid precipitation upto 40% and dialyzed against phosphate buffer (0.2M, pH-6.5). The dialyzed sample were treated with Sodium Dodecyl Sulphate (SDS) and heated at 100^0c in water bath for 5 minutes and were separated using SDS-PAGE.

3. RESULTS AND DISCUSSION

Isoenzyme analysis:
The zymogram of peroxidase as revealed after staining with 3-amino 9-ethyl carbazol, resulted in the presence of both the monomeric and dimeric forms of high molecular weight peroxidase. Out of the three genotypes of FRI-4 studied, FRI-4:14 displayed a heterozygous dimeric condition exhibiting three bands of molecular weight corresponding to approximately 260 kD, 250 kD and 110 kD with Rf values of 0.32, 0.37 and 0.42 respectively (Fig 1). The other two genotypes i.e. FRI-4:13 and FRI-4:15 showed a heterozygous monomeric condition revealing two bands of activity with molecular weight 260 KD and 250 KD bands (Figure1-A). Zymogram of FRI-5 genotypes revealed the presence of monomeric and dimeric isoforms of peroxidase. The genotype FRI-5:16 was found to be hetero-monomeric revealing two bands of activity and having the relative

Rf value of 0.32 and 0.42 and molecular weight of 260 KD and 110 KD respectively. The IEF zymogram showed only one band of peroxidase of molecular weight 260KD at pI 9, which was different from other peroxidase isoforms detected in the gel. The two other peroxidase isoforms of molecular weight 250KD and 110KD were found to have the pI approximately 4-5 (Figure 1-B), denoting a clear difference between the two enzymes.

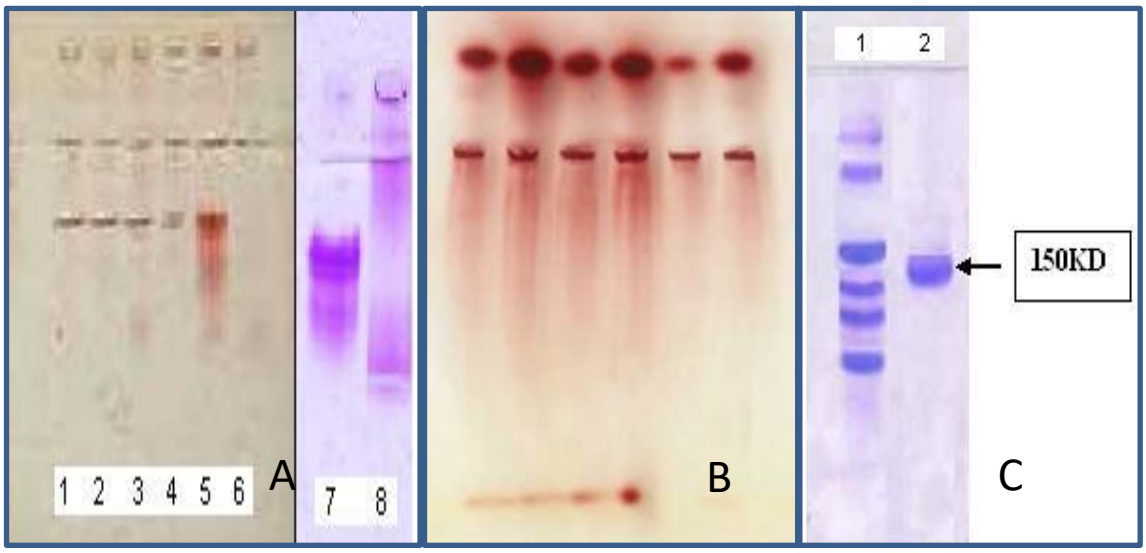

Figure 1: Isoforms of peroxidase enzyme system of Eucalyptus F1 Hybrids. **A**- F_1hybrids as resolved on Phast gel Homogeneous 20. Lane**:** 1- FRI-4: 13, Lane: 2- FRI-4: 14, Lane: 3- FRI-4: 15, Lane: 4- FRI-5: 16, Lane: 5- FRI-5: 17, Lane: 6- FRI-5: 18, Lane**:** 7- *E. camaldulensis*, Lane: 8- *E. tereticornis.*; **B-** Isoelectric focussing of peroxidase enzyme system of F_1 genotypes; C- SDS-PAGE of partially purified protein as resolved on Phast Gel Homogeneous 7.5 using SDS buffer strips.

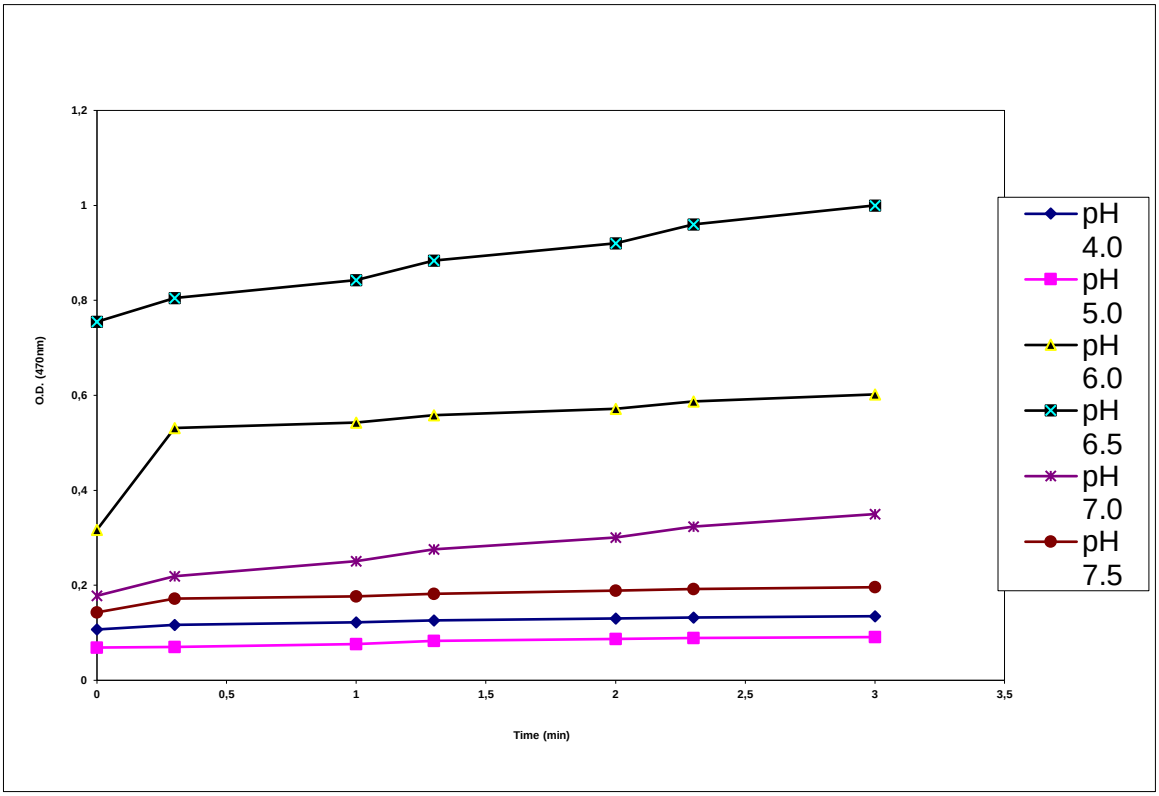

Figure 2: Effect of pH on activity of peroxidase enzyme isolated from Eucaltptus F1 hybrids

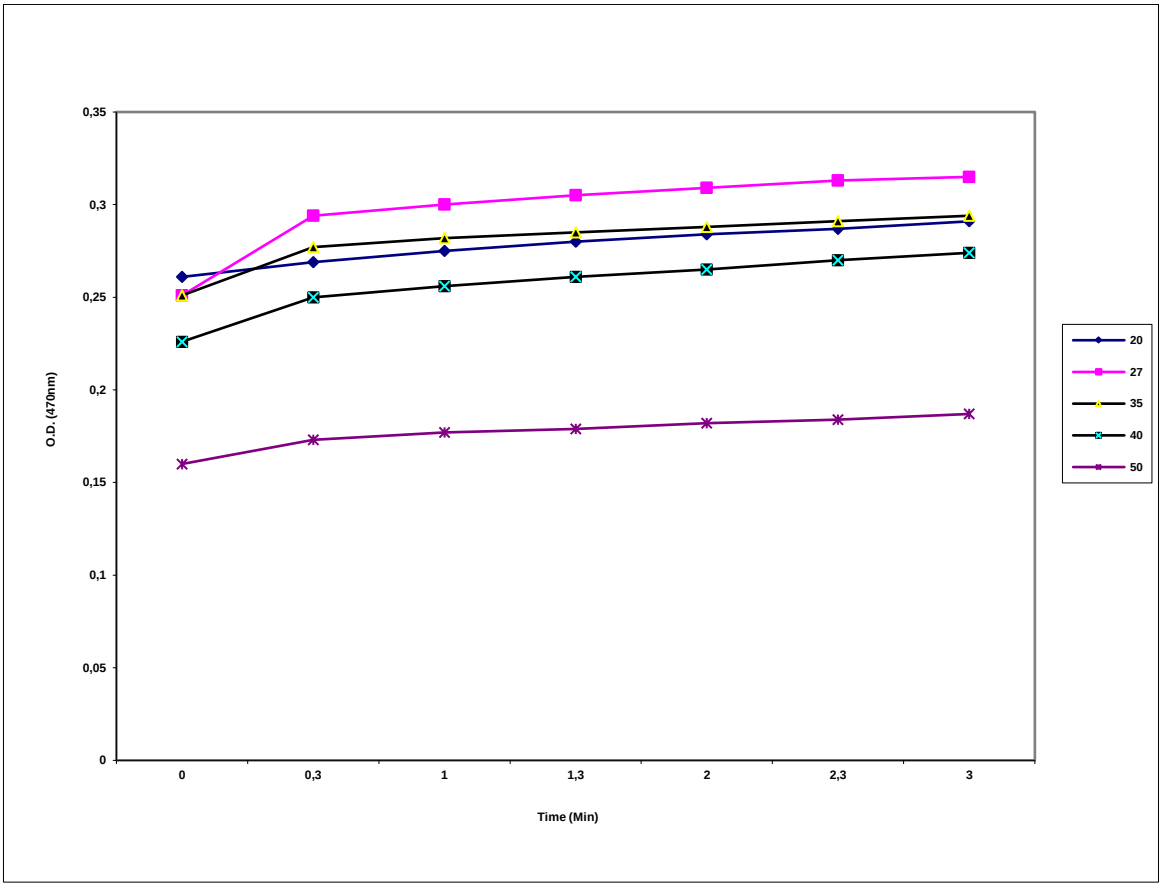

Figure 3: Effect of temperature on enzyme activity of peroxidase enzyme isolated from Eucaltptus F1 hybrids.

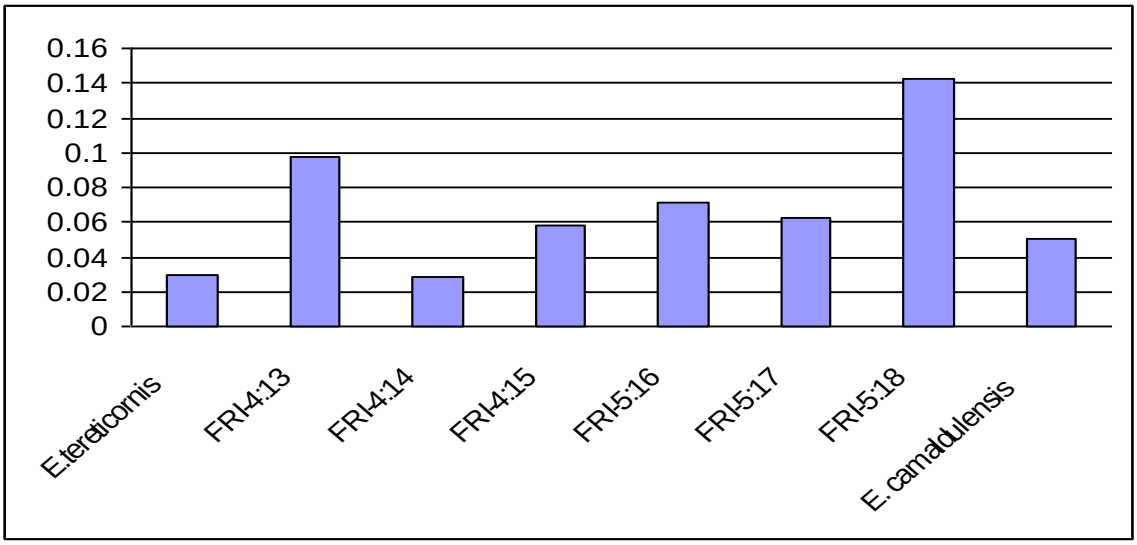

Figure 4: V_{max} values of peroxidase enzyme isolated form F_1 hybrids and their parental combinations.

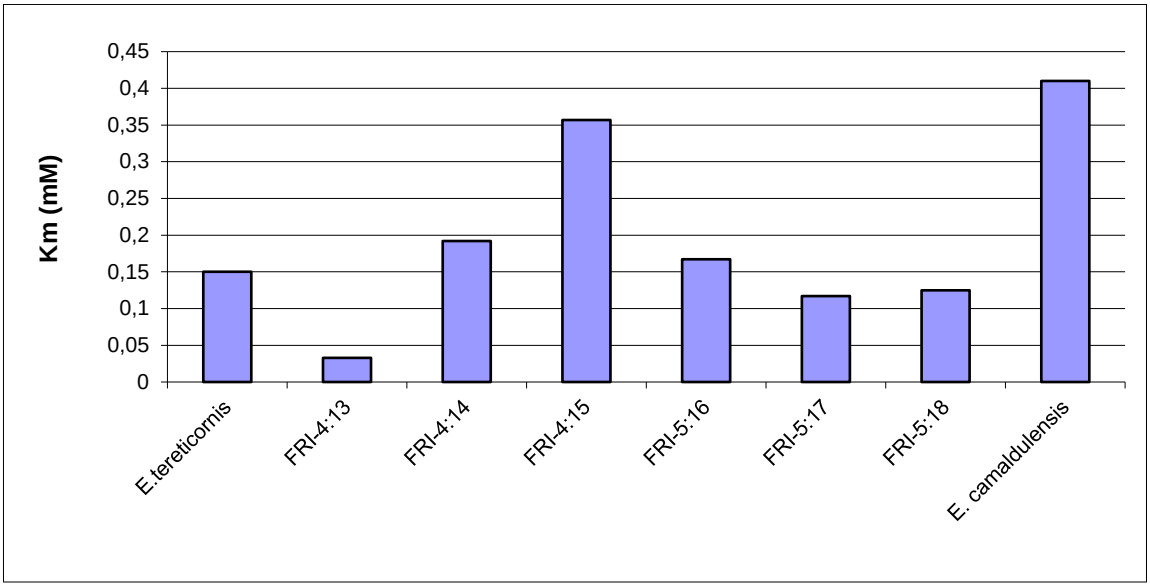

Figure 5: K_m values of peroxidase enzyme isolated form F_1 hybrids and their parental combinations.

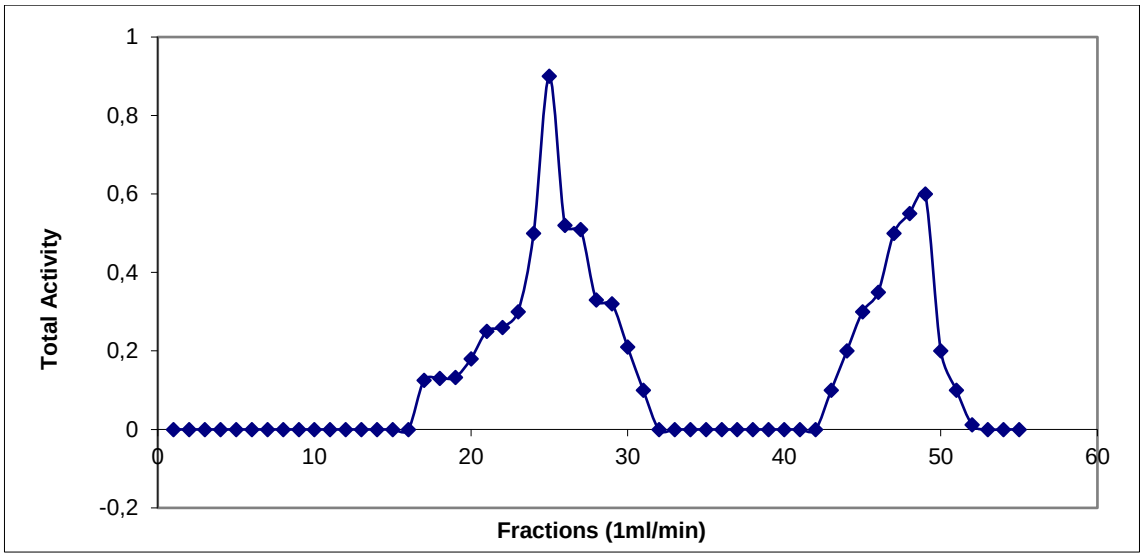

Figure 6: Elution profile of peroxidase after size exclusion chromatography. Proteins were separated on Sephadex G-100 by a flow rate of 1ml/min.

Physicochemical properties:

Substrate utilities of peroxidase have been investigated in terms of optimal pH, temperature and enzyme concentration in order to infer the physiological function of the enzyme. Eucalyptus peroxidase was found to be having a pH optimum of 6.5 when o-dianisidine and H_2O_2 were used as the substrates. The optimum pH with respect to guaicol and 3-amino 9-ethyl carbazol was also determined that ranged between 6-6.5 (Figure 2). These ranges are similar to the peroxidase forms isolated from various other lower plants (Lee & Kim, 1994; Lee *et al*., 2001). The optimum temperature for the stability and functionality was determined using o-dianisidine and H_2O_2 as the substrates that showed the optimal temperature to be 27^0C above which a sharp decline in the activity of peroxidase was observed (Figure 3).

Partial purification of peroxidase

The salt precipitated and dialyzed crude extract was subjected to the 35 cm X 1cm column and fractions of 5 ml were collected. The absorbance at 260 nm gave the peaks of protein which when analyzed for the activity of peroxidase the peak 1 and 2 showed the activity of peroxidase (Figure 6). The ascending portion of the peak was pooled and precipitated using ammonium acid precipitation. The precipitated protein was subjected to SDS-PAGE, which revealed the presence of a single band of molecular weight approximately 150KD when stained with commessie blue (Figure 1-C). The partially purified crude protein, which showed the activity of peroxidase, when subjected to SDS-PAGE revealed the presence of a low molecular weight peptide. This suggests the presence of two peptides of low molecular weight, which collectively forms the functional peroxidase enzyme system in Eucalyptus F_1 hybrids.

Kinetic characterization of peroxidase

The total activity and arbitrary units of peroxidase was assessed spectrophometrically at 460nm using o-dianisidine as a redox system. In all the genotypes under study, a similar trend in the activity was observed which showed the maximum conversion of the substrate and the exponential rise in the slope in first 30 second of the reaction, following which there was a sigmoid saturation curve was observed. The total activity and enzyme units were significantly correlated with the total protein and specific activity. Out of the two parental species, *E. camaldulensis* showed the higher values of enzyme units as compared to *E. tereticornis*. Among the F_1 hybrids, FRI-5 genotypes showed the maximum units of peroxidase enzyme (data not shown).

Out of the two parental species the highest K_m values with respect to H_2O_2 was observed in *E. camaldulensis* i.e. 0.41mM and V_{max} of 0.05mM min^{-1}mg $prot^{-1}$ while *E. tereticornis* had lower values for K_m i.e. 0.15mM and V_{max} of 0.03mM min^{-1}mg $prot^{-1}$. Among the F_1 hybrids, K_m for peroxidase in FRI-5 genotypes ranged from 0.12mM – 0.17mM while the V_{max} values ranged from 0.07- 0.14mM min^{-1}mg $prot^{-1}$ (Fig 4) while FRI-4 genotypes, K_m of peroxidase ranged from 0.03- 0.36mM and V_{max} from 0.03- 0.09mM min^{-1}mg prot-1 (Figure 4). The result suggests that FRI-5 genotypes had more affinity towards the substrate having lower K_m and higher V_{max} values (Figure 5). These values are in agreement with the values of ascorbate peroxidase from Japanese radish that utilized mainly ascorbate as a substrate (Ohya *et al*., 1997).

Because forest trees often have a longer juvenile growth period, there is little relationship between a trait's juvenile expression and its mature form. Because of the lengthy generation period and challenges associated with breeding trees, biochemical markers have been widely employed in forest trees to gather information on genetics and metabolism. This has made traditional methods of genetic analysis challenging. According to reports, peroxidase enzyme is involved in a number of physiological processes, including lignification and cell elongation (Tomotaka et al., 2016; Nishida et al., 1991), phenolic oxidation (Henriksen et al., 1999), host-pathogen interaction (Linde et al., 1999), and potential use as a marker for identifying hybridity in different plant species (Manjunath et al., 2003).

Conclusion

The hybrids under investigation were previously described using their morphological and chemical characteristics, and it was discovered that the heterotic vigour increased when the superior parent was utilised as the seed parent (Kapoor and Sharma, 1984). In our investigation, we discovered that the genotypes of the F1 generation had higher plant peroxidase catalytic efficiency (Km and Vmax) than their parental combinations (Fig 5). The results made it evident that E. camaldulensis had a rather high affinity for the substrate and that it only needed a very small amount of substrate (0.15 mM) to reach the half maximum velocity. Thus using *E. camaldulensis* as seed parent, the F_1 genotypes of FRI-5 had maximum values of K_m as compared to FRI-4 genotypes and showed high affinity towards substrate thus contributing to more cell elongation resulting in better growth with respect to height and diameter. The values of V_{max} were significantly correlated with K_m and height of hybrids that indicates the possible role of peroxidase enzyme in the cell elongation, growth and volume production of the said hybrids. It has also been suggested that the greater heterotic vigour in the F1 generations is partly due to the cytoplasmic impact. It was discovered that the hybrids with the same maternal loci in the F1 generations had a more effective peroxidase enzyme system due to their improved expression and kinetic characteristics. The results were in the agreement with the work done by Kumar *et al*. (2001). Out of the two hybrid combination FRI-5 genotypes showed strong maternal inheritance of 260 Kd loci and resulted in more volume yield while this loci was not expressed in the FRI-4 genotypes. Similar types of reports on physiological characterization have not been reported for forest tree species, therefore these results provides a strong base for the use of peroxidase in hybridity confirmation and in assessing the growth and performance of hybrids at an early stages.

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