Chapter III

Development of polyclonal antibody to purified Eucalyptus peroxidase and its applications
Immunology, the scientific study of the immune response in vertebrates, has contributed greatly to contemporary biological ideas and practice. This is due to uniqueness of concepts as well as the usefulness and wide applications of its technique in the analysis of plant macromolecules. Immunology has as its fundamental basis the response of animals to immunization with foreign cells, tissues, microorganisms, and proteins. Immunochemical methods have become widely employed because they permit the analysis of both unknown antigens mixtures (substance capable of inducing antibody formation in an animal) and highly purified antigens in terms of their cellular and tissue activity, sites of localization, and significance in chemotaxonomy and evolution. In the absence of other measurable activity (i.e. enzyme activity), immunochemical methods may provide the sole means for detecting, quantifying, and localizing a particular antigen. Immunochemistry represents a powerful research tool in the study of plant physiology/biochemistry and plant pathology. Plants do not produce antibodies in response to foreign antigens. Nevertheless, plant components, proteins, glycoproteins, and polysaccharides are usually excellent antigens.

The capacity of the mammalian immune system to produce serum glycoproteins (antibodies) in direct response to immunization with foreign proteins or polysaccharides (antigens) has been exploited in many creative ways in plant pathology and biochemistry. Once harvested, these antibodies possess the capacity to recognize, bind to, and neutralize the immunizing agent (antigen). Additional uses for specific antibodies include localization of enzymes or microbial agents in plant cells, affinity chromatography, and quantitative determination of antigen concentration.

The advantages of immunological methods have been enumerated (Clausen et al., 1997) as follows
I. Immunizing microbes or cells do not need to be viable

II. Confidence in sensitivity and specificity of antigen antibody distribution is high

III. Most techniques are straightforward and simple, often using commercial kits

IV. Antigen purification usually employs conventional protein methodology

V. Microgram quantities of antigens are amplified into milligrams of antibody which can be preserved for many years

VI. Many samples can be tested simultaneously with minute amounts of antigen and antibody and

VII. Costs for production of antibody—commercial antibody production is readily available.

Antibodies have been widely used in research for many years and for diagnostic and therapeutic applications. They have been identified as very promising candidates for commercial production by transgenic plant. Clinical traits of therapeutical plant antibodies have already been reported (Ma et al., 1998), they assist in identification, purification, and characterization of proteins, cloning relevant genes, and investigation of functions and interaction with other proteins.

Chargelegue et al., (2000) showed that plant recombinant antibodies could be used as therapeutic agents and vaccines in humans. In this work mice were immunized subcutaneously with a recombinant mouse monoclonal antibody produced in tobacco plants with alum as adjuvant. Two control groups were immunized in the same way with either the original murine monoclonal antibody or horseradish peroxidase (a plant glycoprotein). Analysis by direct immunoassay, competition immuno assay and real-time surface plasmon resonance showed undetectable levels of antibody directed against both the protein and the glycan part of the plant recombinant antibody.
The use of antibodies in detecting and characterizing plant enzymes and proteins has been fairly extensive.

Five different murine monoclonal antibodies specific for cationic isoperoxidase (C1) prepared from spinach leaves have been produced and characterized. Immunoassay showed that they differed in their affinities for the purified C1 and that they recognized different epitopes of the peroxidase molecule (Penel et al., 1990)

A simple but highly efficient method was developed (Desmyter et al., 2001) for the purification of monospecific antibodies against the plant glycoprotein Sambucus nigra lectin related protein. Monoclonal antibodies were raised (and selected) against recombinant Plantago major PmSUC2 sucrose carrier protein. Structural analysis of the sucrose carrier was carried out using these antibodies (Stolz et al. 1999).

Monoclonal antibodies have been useful in investigating homology among plant components from different species, and therefore, a phylogenetic relationship, through cross-reactivity experiments. Thus, monoclonal antibodies to hordeins of cultivated barley (Hordeum vulgare L.) have been used to study hordein variation in the genus Hordeum (Pelger & Von Bothmer, 1992).

In their investigation Murillo et al. (1997) used an antibody raised against the maize PRms (Pathogenesis-related proteins raised against maize) protein was used to localize the protein in fungal-infected maize radicals. The PRms protein was found to be localized at the contact areas between parenchyma cells of the differentiating protoxylem elements.

There are many ways to make antibodies, but polyclonal antibodies against a given antigen are more versatile than monospecific (monoclonal and combinatorial) or phage - display derived antibodies. Polyclonals are heterogeneous population of antibodies with
different affinities and targeting different portions of a given antigen, while monospecific antibodies are homogeneous, with a single affinity and targeting one portion of a given antigen. Combinatorial antibodies and similar products are randomly engineered in vitro, and as such lack the in vivo maturation of naturally made polyclonals, and have unknown reactivity, thus requiring extensive screening. (Valle et al., 2002).

For screening purposes, polyclonal antibodies with their broader reactivity range (i.e. on larger portions of target molecules) possess a greater advantage over monospecific antibodies. Indeed, polyspecific antibodies against the same protein capture all the different isoforms of that portion, and are thus well suited for identifying novel disease related targets (Valle et al., 2002).

Antibodies against both pathogen-related proteins and nucleic acid are currently being utilized for detecting plant pathogens such as bacterial blight in tomato, soybean and geranium (Flynn, 1994). Polyclonal- antibody based immunoassays was developed for detection of Sclerotiana sclerotiorum on canola petals and prediction of stem rot (Bom & Boland, 2000). Recently, aculeatisides was detected using a polyclonal antibody against aculeatiside A (Patalun et al., 2002).

Lignins are complex cell wall phenolic heteropolymers covalently associated with both polysaccharides and proteins. They are mainly localized in the xylem and are formed by the oxidative polymerization of cinnamyl alcohols in a reaction that can be mediated by both peroxidase and \( \text{H}_2\text{O}_2 \) independent phenoloxidase, leading to an optically inactive heteropolymers (Lewis & Yamamoto, 1990; Ros Barcelo, 1997). Polyclonal antibodies against a newly found lignin substructure, dibenzodioxocin antibodies have been used for more accurate localization of lignin in the cell wall during their development (Kukkola et al., 2003)
Plant peroxidases are composed of a peptide and associated heme, calcium and glycans. Class III plant peroxidase (Px), a plant-specific oxidoreductase, is one of the many types of peroxidases that are widely distributed in animals, plants and microorganisms. Pxs exist as isoenzymes in individual plant species, and each isoenzyme has variable amino acid sequences and shows diverse expression profiles, suggesting their involvement in various physiological processes. Indeed, studies have provided evidence that Pxs participate in lignifications, suberization, auxin catabolism, wound healing and defense against pathogen infection (Hiraga et al., 2000, Grace & Logan, 2000). Little, however, is known about the signal transduction for inducing expression of the POX genes. Recent studies have provided information on the regulatory mechanisms of wound- and pathogen-induced expression of some POX genes. These studies suggested that POX genes were induced via different signal transduction pathways from those of other known defense-related genes.

Secretory class III plant peroxidases (Pxs) catalyze the oxidation of various reductants, and are encoded by a large multigene family (Fujiyama et al. 1995). In rice, 42 independent expressed sequence tags for Pxs have been identified, showing that a variety of peroxidase genes are involved in different physiological processes (Hiraga et al., 2000). Px genes from many plant species have been cloned and their gene sequences have been deposited in the Genbank at www.ncbi.nlm.nih.gov.

Plant peroxidases possess a wide range of substrate specificity (McEldoon & Dordick, 1996). The reaction catalyzed is the reduction of peroxidase at the expense of electron donating substrates. These characteristics make peroxidase useful in a number of industrial and analytical applications, for example as reagents in clinical diagnosis and in enzyme immuno assay (Agostini et al., 2002).
In most reports, the quantitation and identification of peroxidases was based on the measurement of the enzyme activity, using various hydrogen donors. The need for more accurate methods has led to the preparation of antibodies raised against peroxidases. This has been used to obtain more reliable results (Conroy et al., 1982; Espelie et al., 1986; Carbonera et al., 1987; Kim et al., 1988; Abeles et al., 1989).

In the present investigation a method for the development of polyclonal antibodies against purified Eucalyptus peroxidase has been described and it has been shown that these antibodies can be used very effectively as field screening tools for future breeding programmers as well as screening low/high peroxidase containing superior Eucalyptus clones.

Plants can defend themselves against pathogen infection through a variety of mechanisms that can be local, constitutive, or inducible (Fraceschi et al., 1998, 2000). Inducible resistance mechanisms such as systemic acquired resistance (SAR) are broad-spectrum plant defense responses. SAR can be induced biologically by challenging a plant with a weaker strain of a specific pathogen or exposing plant to natural or synthetic chemical compounds (Elliston et al., 1977). SAR has been studied by plant biologists for the past 100 years as a means to increase resistance to fungal, bacterial, and viral pathogens in crop plants such as potato, wheat, and rice (Agrios, 1997). Ward et al., (1991) demonstrated that at least nine gene families were induced in uninfected leaves of inoculated plants; these gene families are now known as SAR genes. Several of these SAR gene products have direct antimicrobial activity or are closely related to classes of antimicrobial proteins. Morphological and biochemical changes in SAR protected plants following infection include a significantly faster lignification response, which corresponds with an increase in peroxidase activity (Ajilan and Potter 1992). Other changes include increased glucose and
fructose concentration in systemic tissue (Chandra and Bhatt 1998) an accumulation of fungi-toxic \(\beta\)-ionone derivatives (Wyatt and Kuc, 1992), induction of lipooxygenase (Staub et al., 1992), antimicrobial fatty acid derivatives (Namai et al. 1993), phenylalanine ammonia lyase, phytoalexins (Elliston et al., 1977), and hydroxyproline–rich glycoprotein (Raggi 1998). For conifers, inducible defense systems include secondary resin production, synthesis of new phenolics, traumatic resin duct formation, and initiation of a wound periderm (Franceschi et al., 2000). Chemicals that are able to induce SAR offer a number of advantages over current conventional techniques for disease control in trees. SAR retains its efficiency irrespective of adverse environmental conditions such as low temperatures. Salicylic acid (SA) is recognized as an inducer of pathgeness-related proteins (PR proteins) accumulation and SAR resistance when sprayed onto plants. Enyedi and Raskin (1993) reported that SA mediated resistance is restricted to effects in the treated tissue, indicating that SA does not translocate efficiently throughout the plants when applied exogenously. Chemicals, such as 2, 6 dichloroisonicotinic acid (CGA 41396) and its methyl ester (CGA 41397), both referred to as INA, are more effective in mediating SAR (Jensen et al.1998) against fungal and bacterial pathogens of crops under glasshouse and field conditions. Plant responses to INA and INA analogs include induction of \(\beta\)-1, 3-glucanase, chitinases, 6-phosphogluconate-dehydrogenase, various phenylpropanoid-derived metabolites, accelerated lipid metabolism, and synthesis of peroxidase(s) (Seguchi et al.1992; Staub et al 1992). Peroxidase was found to be induced in melon and cotton following elicitation with *Penicillium janczewskii* (Madi and Katan, 1998). Several workers (Van Loon and Vannstriend 1999) have reported that peroxidase and lignification are important components of the resistance pathway (SAR) in plants infected with pathogens.

\(\beta\)-amino butyric acid (BABA) is also a primer or elicitor. Cohen (1999) first reported that BABA was a resistance-inducing compound against fungal diseases in plants. It was
observed that BABA was capable of potentiating resistance in grapes (leaf discs and cuttings) challenged with *Plasmopara viticola*. This occurred as early as 24 hours after priming with BABA. Lignin like deposits was found in the host cells. Various groups have demonstrated the efficacy of BABA in protecting different plant species not only against fungal infection but also against abiotic stress and nematode infestation (Sticher *et al.*, 1997; Zimmerli *et al.*, 2001; Conrath *et al.*, 2002).

The use of chemical elicitors for SAR or wound healing has been limited with respect to woody plants in general and forest trees in particular (Percival, 2001). The efficacy of BABA in the above context has also not been studied. Our group has been working to characterize the Eucalyptus peroxidase and it's possible role in lignin formation (Pal *et al.*, 2002). Though Eucalyptus is reportedly susceptible to both fungal pathogens and abiotic stress (Sankaran *et al.*, 1995; Barber *et al.*, 2003) the use of chemical inducers/primers in controlling such situations has not been undertaken. In particular the efficacy of BABA in this context has never been studied. Therefore the effect of BABA was investigated on post-wounding response in Eucalyptus leaf discs, using peroxidase as a marker enzyme. We report for the first time that BABA potentiates the expression of peroxidase in Eucalyptus as confirmed by enzyme assay and Western blotting. The implication of this, *vis-a-vis* systemic acquired resistance in Eucalyptus has been discussed.
Materials & Methods
Polyclonal antibody preparation:

Commercial preparation of Horseradish peroxidase (HRP) was purchased from Sigma Chemical Co., USA and purified 58.6 kDa peroxidase enzyme from *Eucalyptus sp.* were used as antigen. Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA) and secondary antibodies (Anti-Rabbit-IgG-HRP) were purchased from M/S Bangalore Genei Pvt. Ltd, India. Four female rabbits (6-8 weeks age and weighing 1-1.5 kg), were purchased from the local vendor. Pre-immune sera from the rabbits were collected from the ear vein using disposable single use syringes, following standard animal safety protocols. 100 µg of HRP and EP were injected into each of the two rabbits by intra-muscular injections as a first dose. The injection material was prepared by dissolving 100 µg of respective proteins in 200 µl of FCA. After one month of the first injection, the second (booster) dose of HRP and EP (@10 µg per rabbit) were injected using FIA as adjuvant. A third booster dose of 10µg HRP and EP were given using FIA, 21 days after the second booster. Fourth booster dose of 1µg HRP and EP using FIA were given 21 days after the third booster. Within 7-10 days after each booster injection, small sample of blood was collected from each of the rabbits and tested for antibody titre against the antigens by immuno dot blots. Sera collected from the rabbits seven days after the fourth booster injection.

Immuno Blotting

Nitrocellulose paper (HYBOND) was purchased from M/S Amersham Inc., USA. H$_2$O$_2$, o-dianisidine were purchased from M/S Sigma Chemical Co., USA. 5µg of total proteins extracted from the leaves of clonally propagated Eucalyptus plants using the SDS containing extraction buffer (7.75ml 10% SDS, 1.25ml 1M Tris-HCl, pH 7.0, 1ml β-mercaptoethanol in 10ml double distilled water) were dot blotted on the nitrocellulose. A mechanical press
machine, exerting equal amount of pressure on leaf, was used to blot proteins in a circular patch from 10 different plants and subjected to standard immunoblotting method. After spotting proteins all the blots were air dried for one hour and then treated with hot (>65°C) 1X PBS solution (8gm NaCl, 0.2gm KCl, 1.44gm Na₂HPO₄, 0.24gm KH₂PO₄ in 1 liter double distilled water; pH 7.4) containing 1% SDS for 15 minutes to remove the remaining background peroxidase activities. The nitrocellulose papers were then treated with blocking solution (1XPBS containing 2% BSA) for 1 hour. The blots were washed with 1X PBS thrice, each for 15 minutes, and then treated with 1:1000 dilution of the primary antibody in 1X PBS for 1 hour, followed by washes with 1X PBS as described earlier. Blots were then treated with 1: 2000 dilution of the secondary antibody in 1X PBS for 1 hour. Three washes with 1X PBS were done and the blots were developed with H₂O₂ and o-dianisidine following standard protocols (Sambrook and Russell, 2001).

Preparation of a protein immunoadsorbent and purification of peroxidase

Reagents

1. Starting Buffer:
0.1 M Borate saline buffer, pH 8.3 containing Boric acid (6.18 gm l⁻¹) H₃BO₃ Na₂B₄O₇, 10 H₂O (9.54 gm l⁻¹) NaCl (4.38 gm l⁻¹).

2. Washing Buffer
0.15 M Phosphate –buffer saline (PBS) pH 7.2 containing NaCl (8.00 gm l⁻¹), KCl (0.20 gm l⁻¹), 0.008M Na₂HPO₄ (1.15 gm l⁻¹), KH₂PO₄ (0.20 gm l⁻¹).

3. Elution buffer:
0.1 M Glycine –HCl buffer pH 2.5 containing 0.2 M glycine (15.01gm l⁻¹), 0.2 M HCl.
4. Anti rabbit IgG against purified Eucalyptus peroxidase (prepared as detailed earlier).
5. Crude Eucalyptus leaf extract.

Methods:

1 ml activated cyanogenbromide Sepharose 4B beads (0.28 gm/ml) were taken in a sintered glass funnel and washed with 100 ml water and then 100 ml of borate – saline buffer. Then anti rabbit IgG against purified Eucalyptus peroxidase was loaded on it. Activated beads with protein were kept overnight at 4°C. Next day the immunoabsorbent (Anti Eucalyptus peroxidase antibody coupled to Sepharose 4B) was poured into the column and equilibrated with 20 ml PBS. Unbound protein fraction was monitored at 280 nm wavelengths and the process was stopped when absorbance was <0.01. 500-μl crude Eucalyptus leaf extract was loaded on the column. The beads were washed thoroughly with PBS and absorbance was measured in the flow through at 280 nm and enzyme activity was monitored as A_{460/min} as detailed earlier. The column was eluted with glycine-HCL buffer and eluate was collected and quantitated in terms of A_{280} and A_{460/min}.

Experiment with β-amino butyric acid

Leaf samples were collected from two-year-old clonally propagated Eucalyptus plants named as P4. Matured basal leaf was collected from lowest secondary branch of the identified tree and the leaf sample was cut into small disc (0.8cm) using a cork borer. The leaf discs were washed thoroughly in double distilled water and blotted properly to remove surface water. Six discs each were floated (upper surface down) on distilled water, 20μg/ml (T20) or 40 μg/ml (T40) BABA solutions in Petri dishes. Sampling was followed by enzyme extraction and assay. The 0- hour system refers to leaf discs that were processed immediately after excision.
Preparation of a protein immunoadsorbent

Reagents

Cyanogen bromide

Phosphate-buffered saline (PBS)

0.1 M Borate-saline buffer (pH 8.3)

Crude leaf extract

0.1 M Glycine-HCl buffer, pH 2.5

Trichloroacetic acid (TCA), 10%

Methods:

Activated cyanogen bromide beads was taken in a sintered glass funnel and washed with 100 ml water and then 100 ml of borate-saline buffer, then 100 mg of sample was added. Activated beads were kept overnight at 4°C. Next day activated beads was washed on a sintered glass funnel with 10 ml PBS and collected the washing fraction. Unbound protein fraction was measured at 280 nm wavelengths.

Western blot analysis

After running SDS-PAGE, the proteins were transferred onto nitrocellulose (NC) membrane (pore size 0.45 μm) by electroblotting at 0.8 V/cm2 of the gel surface using Pharmacia-LKB electrobloittiing apparatus. After transfer, the NC membrane was blocked overnight with 5% BSA solution 1X PBS (Phosphate buffer Saline) at 4°C. The membrane was washed thrice with PBST (1X PBS with 0.05% Tween-20) for 10 minutes each followed by addition of primary antibody with proper dilution. After incubation 2 hours at room temperature, the membrane washed thrice with PBST for 10 minutes each. Secondary antibody conjugated
with Horse Radish Peroxidase (HRP) was added at proper dilution and incubated at room temperature. Membrane was then washed extensively with PBST and the blot was photographed.
Results
&
Discussion
In Plate 3a panel IMM-EP, lanes 1-6 depicts the specificity and detection range of polyclonal antibodies raised against EP. The antibody bound to 20 µg, 10 µg, 6 µg, 5 µg, 2 µg and 1 µg of the denatured EP proteins. In panel PI-EP, lanes 1-6 shows that the pre immune sera bound very feebly or not at all to 20 µg, 10 µg, 6 µg, 5 µg, 2 µg and 1 µg of the denatured EP protein spotted on the blot respectively. If we consider further, that the antibody was used at a dilution of 1:999 and that 1 µg of EP corresponds to (8 X 10^-4 units of enzyme activity) units the specificity and sensitivity of the assay becomes evident. In panel control-EP, same amount of denatured proteins was blotted as in panel Imm-EP and PI-EP, but were not treated with any of the primary and secondary antibodies. This blot was directly developed with H_2O_2 and orthodianisidine. Negative results in this experiment clearly showed that denaturation of the EP proteins was complete and there were no background peroxidase activities from the blotted protein sample.

The applicability of this antiserum in the development of a protocol to screen various clonally propagated Eucalyptus plants was further tested using the clones derived from various CPT (Plate 3b).

Panel 1, 2 and 3 depict the blot pattern of J5, A2 and S5 leaf samples, similarly 4, 5 and 6 depict the blot pattern with leaf samples from A3, S1 and A4 plants. For every leaf sample 10 µg of protein was blotted on to the nitrocellulose paper. The intensity of every spot was analyzed in Easy gel to quantitate the intensity value (Table 3a). On the basis of intensity values the plants were arranged in the order J5 > A2 > S5 > S1 > A3 > A4 (the symbols refer to parent plants).

From the scan data it was obvious that J5 plant has the highest and A4 the lowest peroxidase activity as compared to other plants. Analysis of peroxidase activity by the routine spectrophotometric method (and the subsequent gradation) was in agreement with antibody...
blot technique (Table 3b). It may be mentioned that while the enzyme assay method required 25-75µg of protein the blot assay could be performed with 10µg protein. In fact the blot assay could be performed with crude extract containing about 5µg of protein (Data not shown). A very interesting observation was that the order, of decreasing peroxidase activity, in the clonally propagated plants accurately represented the order in the CPT parent. This data supports the hypothesis that the expression of the peroxidase gene is not altered in any way by the procedure for the production of the clones.

It is clear from the aforementioned results that this protocol can be used for rapid large scale screening of Eucalyptus peroxidase activity. This data may be used for screening seedlings or tissue cultured clones vis a vis their potential as lignin producers as discussed earlier. The immunoblot technique offers the further advantages of a non-invasive and relatively non-complicated procedure. It may be mentioned that a non-invasive technique for the study of lignification in the mature cell walls of maize has been reported (Joseleau & Ruel, 1997). These workers investigated the synthesis of three types of lignin, p- hydroxyphenylpropane, guaiacyl, and syringyl in the longitudinal axis of developing internodes by using Fourier transform infrared spectroscopy and immunocytochemical electron microscopy.

Figure 3a refers to the peroxidase activity of the leaf discs floated on distilled water (control), 20µg ml⁻¹ (T₂₀) and 40µg ml⁻¹ (T₄₀) β-amino butyric acid (BABA) solution respectively. The T₂₀ and T₄₀ systems showed an increase (in peroxidase activity) of 356 % and 448 % respectively over 0-hour after 24 hours post flotation (pf). However on comparing the T₂₀ and T₄₀ systems at the same time point, the T₄₀ system showed only a 20 % increase in peroxidase activity over T₂₀. Similarly at 48 hours (pf) peroxidase activity was increased by 185% and 236% over control for the T₂₀ and T₄₀ systems respectively. However the difference between the T₂₀ and T₄₀ systems was only 17%. At 72 hours (pf) the increased
activity in the treated systems (over control) was similar to that of the 48 hours (pf). The difference between the BABA treated systems was also similar. In general, the BABA treated discs showed approximately 180-300% increases over discs floated in water only. However there was an insignificant (P = NS) dose dependent increase in the peroxidase activities between the T\textsubscript{20} and T\textsubscript{40} systems.

When the incremental changes in peroxidase activity on a 24-hour basis (ΔPx) were considered (Figure 3b) an interesting trend emerged. In all three systems ΔPx was high at 0-24 hour. For the control the ΔPx was 28% whereas for T\textsubscript{20} and T\textsubscript{40} it was 356% and 448% respectively. At 24-48 hours ΔPx for the control system (47%) was almost double the value for the 0-24 hour period. However, for the T\textsubscript{20} and T\textsubscript{40} systems ΔPx (24-48 hours) was 17% and 15% respectively, which was much lower than the ΔPx (0-24 hours). In all the three systems the ΔPx value was sharply decreased at 48-72 hours relative to the previous periods. In control this value was only 6% (as compared to the 0-24 hours) whereas for T\textsubscript{20} and T\textsubscript{40} the corresponding values were even lower. These changes were insignificant (P =NS). It was interesting to note that control showed almost 100% increase over 0-hour values (0.25±0.01 U/mg P) over the 72 hour period of the experiment.

In spite of the large increase in activity the Western blot pattern revealed only a single band at all three conditions (control, T\textsubscript{20}, T\textsubscript{40}) as well as the 0-hour system (Plate 3c).

These findings lead us to several conclusions. The post-wounding response in Eucalyptus leaves involves enhanced peroxidase activity. This was reflected in the almost 100% increase of peroxidase activity in leaf discs (control) as compared to the 0-hour system over a period of 72 hours. This response was increased 2-3 fold in the presence of BABA at both the concentrations used. The increase for the systems exposed to BABA did not show a linear dose response relationship. This may be indicative of "saturation" with respect to the
concentration of BABA used. Since the presence of a BABA receptor has not yet been indicated this finding may be of interest in this context.

The pattern of the response i.e. increased peroxidase activity was remarkably different for control and BABA treated discs. For the latter systems, the increase appeared to be “quantal” rather than “progressive”. A very large increase during the first 24 hours (pf) was followed by relatively small or insignificant increases at later periods. For the control the incremental increase was somewhat similar for the 0-24 and 24-48 hour periods. Both the control and treated systems showed insignificant changes during the last phase (48-72 hours) of the experiment. This may be of advantage under conditions that require rapid and large bursts of peroxidase activity. Enhanced peroxidase may protect against tissue damage in plants (Hildebrand et al., 1986) or generate quinone compounds that are toxic to insects or interfere with their development (Stout et al., 1996; Felton et al., 1994). It has recently been reported (Heng-Moss et al., 2004) that buffalo grass with higher peroxidase activity was more resistant to cinch bug attack.

We further observed that even under conditions where peroxidase activity was increased several fold there did not appear to be any induction of multiple isozymes. The results indicate an enhanced expression of a single peroxidase gene following wounding and potentiation by BABA.

Our findings indicate that BABA acts to strongly potentiate the post-wounding activity of peroxidase in Eucalyptus leaves. The rapidity and magnitude of the response at the low BABA concentrations strongly suggest the use of BABA as a chemical elicitor for conferring resistance to woody plants in general and forest trees in particular. Whether this resistance would be equally effective against both biotic and abiotic challenges is currently being investigated in our laboratory.
The purification of peroxidase from crude extract using specific antibody coupled cyanogen bromide activated Sepharose 4B is shown in Figure 3c, 3d & Table 3c. It was evident that binding of the peroxidase with the antibody was very high. A single passage through the column resulted in high purification (99.86 fold). However, the recovery of the enzyme activity was very low (4.2%). This may have been a result of the denaturing conditions utilized for the elution process.

To verify the cross reactivity of our immuno reagents we used the antibody developed against Eucalyptus peroxidase on jute plants. Plate 3d shows the results of the immunoblotting experiments with jute plant samples. Panels 1, 2 and 3 show the reactivity of polyclonal antibodies against EP with 10 µg of protein sample from the bark of TAN/X/112C X THA/YA/064C, JRO -620, TAN/SM/040C respectively while panels 4, 5 and 6 represent varieties CMU-012 XJRC-JRC-321, CMU011 X JRO-524 and CMU013 X JRC-321. The analysis of the intensity of the spots in Easy Gel is shown in Table 3d. On the basis of intensity value the varieties were arranged in following order


The colorimetric analysis of peroxidase activity was further carried out from these varieties (Table 3e). Arrangement of the plant varieties (higher to lower peroxidase activity) on the basis of colorimetric analysis and dot blot analysis showed exactly similar sequence.

On analyzing the lignin content of the varieties a close similarity was found to exist between lignin content and peroxidase activity. This data has now been mathematically plotted and expressed (Annual Research progress Report, 2003) where a biphasic linear relationship has been established.
These results highlight a variety of uses for the antibody to purified Eucalyptus peroxidase. On the one hand the antibody may be utilized for detecting and screening of Eucalyptus varieties with potential for high or low lignin formation. This has enormous implications while selecting for mass grown seedlings. On the other hand the antibody may be used to verify the purity of clonally propagated plants. The expression of peroxidase in general, which is linked to various phenomena including systemic acquired resistance, may be suitably monitored with the antibody. The antibody showed a high degree of sensitivity and cross reactivity with peroxidase from other plant species (Goswami et al, 2003) and this trait has been used by us to screen jute cultivars for their lignin content. Finally, the use of the immobilized antibody to purify peroxidase from Eucalyptus has provided a tool for recovery of a small amount of the enzyme from fairly crude sources. This method offers a means of separating peroxidase from a variety of plant sources, where more conventional methods of separation may be difficult.
Tables, Figures & Plates
Table 3a. Quantitation of leaf dot blots (peroxidase) from clonally propagated Eucalyptus

<table>
<thead>
<tr>
<th>Blot</th>
<th>Plant Sample</th>
<th>Intensity</th>
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<tbody>
<tr>
<td></td>
<td>J5</td>
<td>34.22</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>26.34</td>
</tr>
<tr>
<td></td>
<td>S5</td>
<td>28.40</td>
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<td></td>
<td>S1</td>
<td>14.26</td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td>10.21</td>
</tr>
<tr>
<td></td>
<td>A4</td>
<td>10.21</td>
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Table 3b. Leaf peroxidase activity of clonally propagated Eucalyptus plants

<table>
<thead>
<tr>
<th>Plant</th>
<th>Peroxidase activity (μ-mol o-dianisidine oxidized/min/gfw)</th>
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<tbody>
<tr>
<td>J5</td>
<td>4.21 ± 0.29</td>
</tr>
<tr>
<td>A2</td>
<td>4.10 ± 0.24</td>
</tr>
<tr>
<td>S5</td>
<td>3.82 ± 0.22</td>
</tr>
<tr>
<td>S1</td>
<td>0.87 ± 0.03</td>
</tr>
<tr>
<td>A3</td>
<td>0.66 ± 0.03</td>
</tr>
<tr>
<td>S4</td>
<td>0.60 ± 0.04</td>
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Table 3c. Purification profile of Eucalyptus peroxidase using Immunoaffinity chromatography

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Activity (units/mg)</th>
<th>Activity (Total units)</th>
<th>Fold purity</th>
<th>Recovery (%) Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>2.21</td>
<td>1.10</td>
<td>1.44</td>
<td>15.9</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Eluted fraction</td>
<td>0.048</td>
<td>0.0048</td>
<td>143.80</td>
<td>0.67</td>
<td>99.86</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Activity (μ-mol o-dianisidine oxidized/min/mg of protein)
### Table 3d. Quantitation of leaf dot blots (peroxidase) from different jute cultivars

<table>
<thead>
<tr>
<th>Blot</th>
<th>Plant Sample</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Blot" /></td>
<td>TAN/X/112C X THA/YA/064C</td>
<td>34.22</td>
</tr>
<tr>
<td><img src="image2" alt="Blot" /></td>
<td>JRO 620</td>
<td>26.34</td>
</tr>
<tr>
<td><img src="image3" alt="Blot" /></td>
<td>TAN/SM/040C</td>
<td>28.40</td>
</tr>
<tr>
<td><img src="image4" alt="Blot" /></td>
<td>CMU-012 X JRC 321</td>
<td>14.26</td>
</tr>
<tr>
<td><img src="image5" alt="Blot" /></td>
<td>CMU-011 X JRO 524</td>
<td>10.21</td>
</tr>
<tr>
<td><img src="image6" alt="Blot" /></td>
<td>CMU-013 X JRC 312</td>
<td>10.21</td>
</tr>
</tbody>
</table>

### Table 3e. Peroxidase and lignin content of jute cultivars

<table>
<thead>
<tr>
<th>Variety</th>
<th>Peroxidase activity</th>
<th>Lignin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAN/X/112C X THA/YA/064C</td>
<td>6.81 ± 0.40</td>
<td>15%</td>
</tr>
<tr>
<td>JRO 620</td>
<td>6 ± 0.42</td>
<td>14.5%</td>
</tr>
<tr>
<td>TAN/SM/040C</td>
<td>5.8 ± 0.29</td>
<td>12%</td>
</tr>
<tr>
<td>CMU-012 X JRC 321</td>
<td>4.30 ± 0.25</td>
<td>8%</td>
</tr>
<tr>
<td>CMU-011 X JRO 524</td>
<td>4 ± 0.32</td>
<td>6%</td>
</tr>
<tr>
<td>CMU-013 X JRC 312</td>
<td>3.20 ± 0.17</td>
<td>4.8%</td>
</tr>
</tbody>
</table>
Activity -μ-mol o-dianisidine oxidized/min/ mg of protein
Fig. 3c. Elution profile of purified Eucalyptus peroxidase using Immunoaffinity chromatography

Fig. 3d. Activity profile of purified Eucalyptus peroxidase using Immuno affinity column
Plate 3a. Immune & preimmune antibody of Eucalyptus peroxidase

Plate 3b. Quantitation of Eucalyptus peroxidase by Dot blot

1 (J5) 2 (A2) 3 (S5) 4 (A3) 5 (S1) 6 (A4)
Plate 3c. Western blot of Eucalyptus peroxidase

Legend:
Lane 1: Control
Lane 2 & 3: T20 & T40

Plate 3d. Quantitation of Jute peroxidase by Dot blot technique

Legend:
1 — TAN/X/112C X THA/YA/064C
2 — JRO 620
3 — TAN/SM/040C
4 — CMU-012 X JRC 321
5 — CMU-013 X JRC 312
6 — CMU-011 X JRO 524