Hydrogen peroxide generation and lignification by peroxidases from *Acacia eburnea* infected with *Ravenelia esculenta*

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ABSTRACT

Ravenelia esculenta Naras. and Thirum. is a rust fungus which infects *Acacia eburnea* Willd. producing hypertrophy in the infected organs. We analysed the changes in the reaction patterns of peroxidase from the tissues at progressive disease stages. Peroxidases (POX) from the tissues were analysed for oxidation of reduced nicotinamine adenine dinucleotide (NADH), NADH-dependent hydrogen peroxide (H_2O_2) generation and polymerization of cinnamyl alcohol, a lignin precursor in plants. The effect of cofactors like Mn^{2+} and dichlorophenol (DCP) was also studied. Effect of externally added H_2O_2 on NADH oxidation and cinnamyl alcohol polymerization was analyzed. NADH oxidation and H_2O_2 generation are accelerated by supplement of cofactors. The cofactor requirements of the two reactions are different. The amount of H_2O_2 generated varies with disease stages, but the rate of NADH oxidation is comparable. Externally added H_2O_2 has an accelerating effect on NADH oxidation. Cinnamyl alcohol polymerizing activity of POX increased with disease progression and was strongly inhibited by H_2O_2. Stimulation of these reactions by cofactors has a physiological significance in terms of susceptibility of the host.

Keywords: disease development; lignification; peroxidases; *Ravenelia*; rust

Ravenelia esculenta Naras. and Thirum. is a rust fungus which infects *Acacia eburnea* Willd. The infected organs show hypertrophy during various stages of disease development (Narasimhan and Thirumalachar 1961). The hard pointed thorns, inflorescence, pods change their morphology and become succulent and hypertrophied after the infection and the rust produces aeciospores on the hypertrophies structures. The hypertrophy is marked by the presence of brown coloured aecial cups (Figure 1). The hypertrophy in the infected organs is seen right at the time of their emergence. The pods, for example, emerge and are already infected and hypertrophied. Such type of disease development study in hypertrophied rust fungi is particularly challenging since the pathogen is an obligate parasite and cannot be cultured on the artificial culture media. In addition, the host in the present study is a forest tree which requires harsh environmental conditions for the optimum growth, and hence cannot be grown under controlled conditions in the greenhouse. Due to these reasons, the host–parasite interaction study in such cases heavily depends upon the collection of tissue directly from the field from the infected host. During the present study, the tissues were collected from the same host plant as and when required. The hypertrophied structures are edible and are consumed along with liquor, especially by tribals. This hypertrophy may be the outcome of altered host physiology and hormonal metabolism. Gandhe et al. (2004) screened the infected host to analyze the host–fungus interaction in *Ravenelia esculenta*. The infected parts accumulated enormous amounts of aluminium and hence consumption of these infected structures poses potential threat of neural diseases like Alzheimer's disease. Shaw and Samborski (1957) discussed the physiological changes in mildew and rust-infected wheat leaves and detected the initial increase in respiration rate as one of the early physiological responses to pathogen attack. It was observed that fungal, bacterial and viral diseases also lead to alterations in the biochemical constituents of hosts.

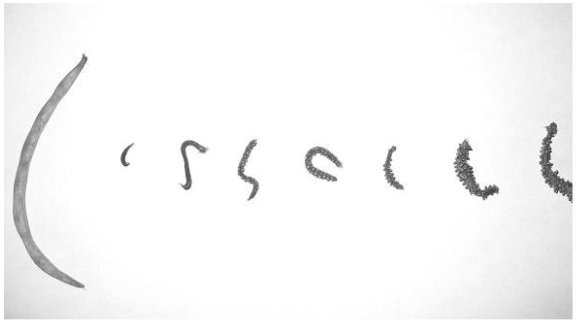

Figure 1. Pods of *Acacia eburnea* infected with *Ravenelia esculenta*. Note the hypertrophy and brown colored aecial cups on the infected pods. The initiation of infection is marked by hypertrophy in the infected tissues. The growth and maturation of the infected tissues lead to increase in the extent of hypertrophy. The progress in the disease can be monitored externally on the basis of the hypertrophy produced in the infected tissues

One of the most marked changes in the physiology of the infected host is change in the activity of oxidizing enzymes which are involved in the defense mechanism against the infecting pathogen. Most marked changes are observed in the activity of peroxidase (EC 1.11.1.7) (POX) enzymes during and after the infection by a pathogen. Peroxidase is an ubiquitous, haeme containing glycoprotein that catalyses the oxidation of cellular components by either hydrogen peroxide (H_2O_2) or organic hydroperoxidases (Kvaratskhelia et al. 1997). Increases in total peroxidase activity are often found during infection of higher plants by pathogens. This increased activity of POX is supposed to inhibit the growth of pathogens through participation in biosynthesis of phenolic compounds (Seevers et al. 1971). The activity of peroxidase in diseased tissue is to metabolize the H_2O_2 generated during hypersensitive reaction of the host to the pathogen attack. The plant POX also catalyzes variety of oxidase reactions leading to H_2O_2 generation (Bestwick et al. 1998). These reactions include the oxidation of variety of phenolic substrates, certain thiols, reduced nicotinamine adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH) etc. (Bestwick et al. 1998). The H_2O_2 formed during these reactions is utilized for polymerization of monolignols into lignins (Mader and Amberg-Fisher 1982). Besides lignin formation, the production of hydrogen peroxide leads to peroxidase – mediated cross – linking of structural proteins in the cell wall thereby reinforcing it against the pathogen invasion (Odjakova and Hadjiivanova 2001). Induction of variety of peroxidase isozymes is shown following infection by a pathogen to a host plant or during ethylene-induced leaf senescence in cucurbitaceae (Abeles et al. 1989, Kerby and Somerville 1992, Jakupovic et al. 2006). Role of different isoforms of peroxidases in lignification and suberisation was documented earlier (Barcelo 1998, Bernards et al. 1999, Gabaldon et al. 2005). The increase in the activity of peroxidase in response to infection by *Puccinia recondita* f.sp. *tritici* in wheat was reported earlier and found to be higher in resistant as compared to the susceptible lines (Southerton and Deverall 1990). Peroxidase is also one of the key enzymes involved in oxidative burst which is a common plant response to pathogenic attack. The importance of the role of peroxidase in defense reactions is supported by the fact that the transgenic tobacco plants with suppressed expression of peroxidase are over responsive to pathogen attack (Mittler et al. 1999).

Ryals et al. (1996) stated that the inhibition of oxidoreductases like peroxidase is important for lesion development by the infecting pathogen and hence is important in the resistance response by the host. Peroxidase enzymes may eventually affect the pathogen growth by reinforcing the cell wall, by producing the toxic substances to the pathogen or by accelerating the necrotic response in the infected and nearby cells as in case of hypersensitive response. Therefore study of these enzymes, which have important roles during pathogenesis and resistance of the host, becomes an indispensable tool for dissecting host–pathogen interaction. The enzymatic studies in compatible interactions have special importance as they help in understanding the role of such enzymes in disease development and effect of their altered expression on pathogenesis.

We have analyzed the shifts in the activity of POX from various disease stages of the host with reference to changes in their substrate preference at various pH values, role of POX in the oxidation of NADH, cofactor requirement for NADH oxidation, role of POX in lignification process and changes in the pH optima of the enzymes with progression of the disease. In this paper we have described the activity of POX in the oxidation of NADH and polymerization of cinnamyl alcohol, a precursor of lignin synthesis in plant tissues, during progressive developmental stages of the disease.

MATERIAL AND METHODS

Collection of plant material. Plant tissues from healthy (healthy – H) and progressive disease stages (progressive disease stages I–VII) with infections on the pods were collected directly from the field (Figure 1) and immediately frozen in liquid N_2. The material was stored in liquid N_2 till further use.

Extraction of enzymes. Preweighed tissues from healthy and progressive disease stages were frozen in liquid nitrogen and were homogenized in 0.1M phosphate buffer pH 7.0 in a chilled mortar and pestle. The homogenate was filtered through four layered muslin cloth and the filtrate was centrifuged at 10 000 rpm for 10 min at 4°C. The supernatant was stored at –15°C until used as an enzyme source.

Assay. The extracted enzymes were analysed for their activity in NADH oxidation and cinnamyl alcohol polymerization, a precursor of lignin synthesis in plants.

The activity of the enzymes in NADH oxidation was assayed with cofactors like dichlorophenol (DCP) and Mn^{2+} individually and in combination. The reaction was detected by recording A340 nm. At the end of the reaction, guaiacol was added to a final concentration of 5mM to detect the release of H_2O_2 during NADH oxidation. Formation of brown-coloured product at the end of reaction (A470 nm) is a measure of H_2O_2 released during the reaction. Effect of externally added H_2O_2 on NADH oxidation with DCP as cofactor was also determined with addition of H_2O_2 to the final concentration of 0.01 mmol, 0.1 mmol and 1 mmol at the initiation of the reaction. The activity of enzymes in polymerization of cinnamyl alcohol was studied with DCP as a cofactor. Progress in the reaction was detected by recording A620 nm. Effect of externally added H_2O_2 on polymerization of cinnamyl alcohol was also studied by addition of H_2O_2 to the final concentration of 0.01 mmol. Following are the reaction mixtures for different reactions:

NADH oxidation: 0.1 mmol acetate buffer (pH 5.0) + 2.5 mmol DCP + 20 mmol $MnCl_2$ + 0.15 mmol NADH + 40 µl enzyme (reaction volume 3 ml).

Cinnamyl alcohol polymerization: 0.1 mmol acetate buffer (pH 5.0) + 2.5 mmol DCP + 0.15 mmol NADH + 10 mmol cinnamyl alcohol + 40 µl enzyme (reaction volume 3 ml).

The reaction rate for NADH oxidation was calculated as a ratio of absorbance at 340 nm (NADH oxidation) to absorbance at 470 nm (H_2O_2 generation). From the reaction rates thus calculated, the increase or decrease factor in the reaction rate with or without supplement of cofactors were calculated.

All the reactions were carried out at 25°C and enzymes were stored on ice till assayed.

All experiments were done in triplicate and each replicate had three representative samples from each progressive stage of the disease.

All chemicals and reagents were from Sisco Research Laboratory Pvt. Ltd. (SRL), Mumbai, MS, India.

RESULTS

Time course of oxidation of NADH with and without cofactors and generation of H_2O_2 catalyzed by POX from various disease stages is shown in Figures 2 a–h. The reaction of NADH oxidation was much slower as compared to H_2O_2 generation, whether the cofactors were absent or present, singly or in combination. But the rate at which H_2O_2 generated, varied among the progressive disease stages and with presence of cofactors. The concentration of H_2O_2 generated by POX at initial stages was observed to reach at a plateau at the end of the reaction, even in the presence of cofactors. But the rate and amount of H_2O_2 generation was accelerated by presence of cofactors in advanced stages of the disease. Mn^{2+} alone and both Mn^{2+} and DCP in combination had an accelerating effect on the amount of H_2O_2 generated and the reaction was seen to progress logarithmically with time. Increase or decrease factor in the reaction rate are shown in Table 1.

Figure 3 shows the pattern of NADH oxidation with or without cofactors by these peroxidases. POX from healthy material showed a decrease in NADH oxidation with addition of Mn^{2+} but the reaction was accelerated by addition of DCP as cofactor. NADH oxidation was observed to be maximum with DCP as a single cofactor. Mn^{2+} and DCP in combination accelerated the reaction but to a lesser extent, the acceleration in NADH oxidation was more than that for Mn^{2+} as cofactor alone but less than DCP as a cofactor. The same pattern was observed for POX from stage I. Stage II POX showed no effect of Mn^{2+} or DCP as cofactors, but the oxidation reaction was inhibited by both cofactors in combination. Maximum acceleration with Mn^{2+} as cofactor was observed for stage III POX, although the reaction was accelerated to a lesser extent by DCP and both cofactors in combination, respectively. Stage IV POX exhibited preference to DCP as cofactor. Mn^{2+} alone inhibited the reaction rate of POX in stage V, whereas DCP accelerated the rate of the

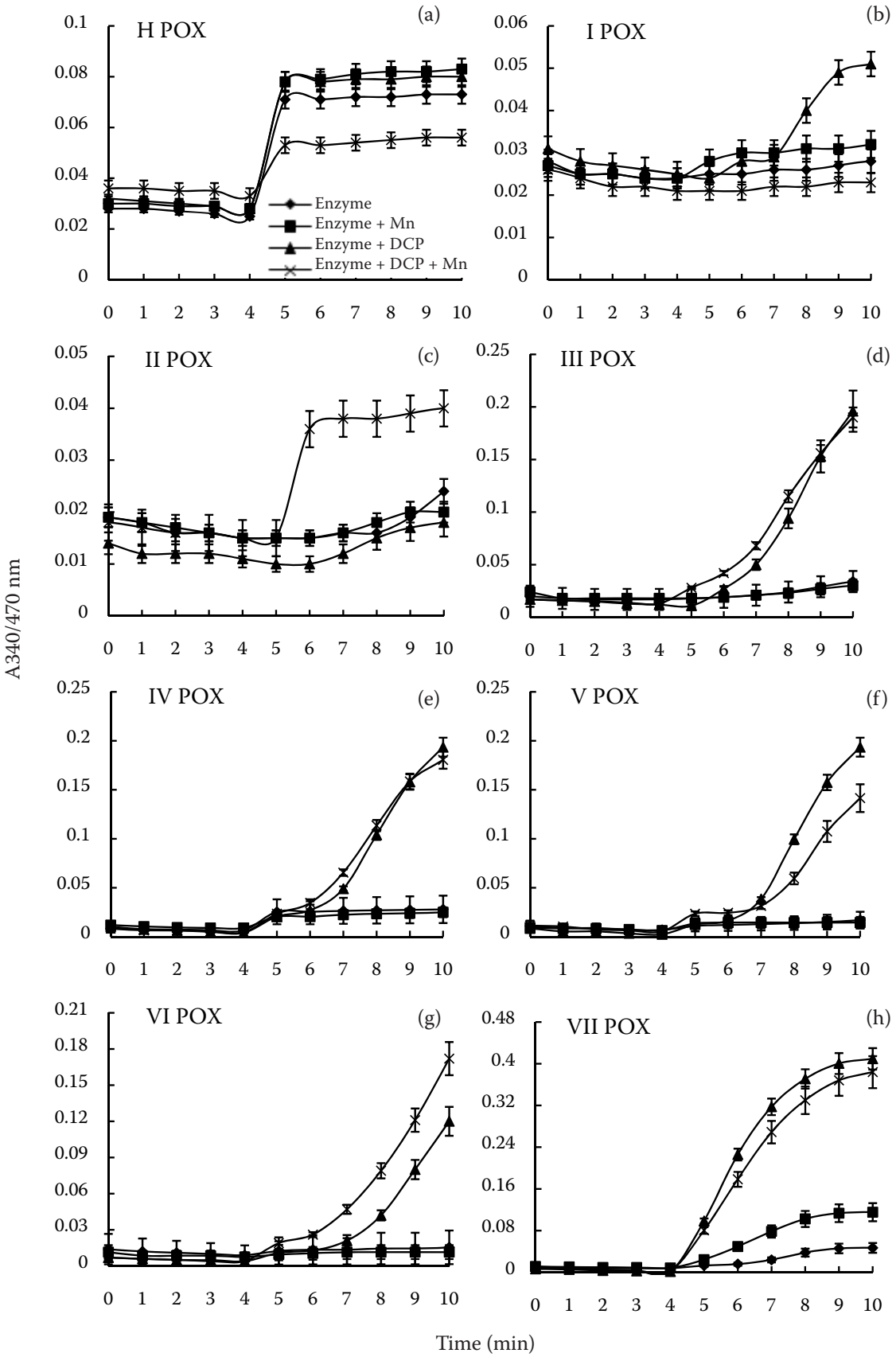

Figure 2. NADH oxidation and H_2O_2 generation by peroxidases at progressive disease stages. The rate of reaction was calculated by dividing A340 nm by A470 nm. The rate of NADH oxidation is comparable at different disease stages but the rate of H_2O_2 generation increases with progression in the disease stages. Supplement of cofactors (Mn^{2+} and DCP) in combination and even singly have stimulatory effect on the rate of the H_2O_2 generation, (a) healthy; (b) Stage I; (c) Stage II; (d) Stage III; (e) Stage IV; (f) Stage V; (g) Stage VI; (h) Stage VII

Table 1. Increase or decrease factor in the reaction rate with cofactors. The cofactors were supplied singly or in combination as indicated in the Table

Disease stage	Enz	Enz + Mn^{2+}	Enz + DCP	Enz + Mn^{2+} + DCP
H	1	2.6×10^{-1}	1.3	6.6×10^{-1}
I	1	5.6×10^{-1}	1.6×10^{-1}	1.8
II	1	1.8	1.1	4.05
III	1	2.6	1.4×10^{-1}	1.3×10^{-1}
IV	1	8.3×10^{-1}	4.6×10^{-2}	3.1×10^{-2}
V	1	2	1.1×10^{-1}	7.6×10^{-2}
VI	1	1.2	1.1×10^{-2}	3.0×10^{-2}
VII	1	3	1.5	1.6

reaction. Both cofactors in combination did not have any effect on the reaction rate as compared to that of DCP as a single cofactor. Stage VI POX showed increasing inhibition of the reaction with Mn^{2+} and DCP. The combination of the cofactors at this stage also had an inhibiting effect on NADH oxidation. The POX from stage VII showed a different behavior with cofactors. The rate of reaction went on increasing with supplement of Mn^{2+}, DCP and both cofactors in combination, in the same order.

In most cases, addition of cofactors accelerated the oxidation of NADH by these peroxidases. The stimulation of the reaction occurred either in presence of DCP or both cofactors in combination. Exceptions to this were the enzymes from stage III and VI where the reaction of NADH oxidation required Mn^{2+} or no cofactors, respectively.

The reactions of NADH oxidation and H_2O_2 generation are reported to be interconnected but activity of the enzymes in catalyzing these two reactions was found to be different (Figures 3–4). The cofactor requirement of the enzymes at various disease stages for these two reactions is tabulated in Table 2.

Figure 4 show the effect of cofactors on H_2O_2 generation. All the reactions except for stage H, II and VI were accelerated by DCP as a single cofactor.

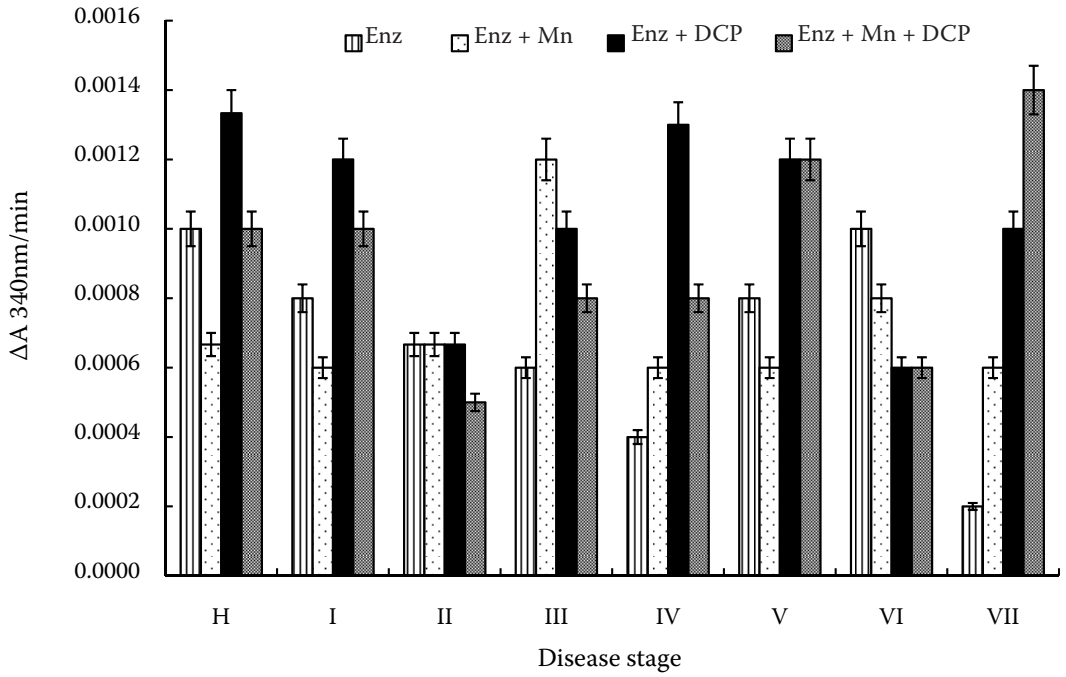

Figure 3. The reaction rate of NADH oxidation by peroxidases at progressive disease stages. The reaction rate was measured as the rate of change of A340 nm/min. The requirement for the optimum oxidation of NADH at same disease stage varies with the supplement of cofactors. In most of the disease stages, only DCP or Mn^{2+} and DCP in combination have stimulatory effect on the rate of the reaction

Table 2. Variation in the cofactor requirement for NADH oxidation and H_2O_2 generation by peroxidases at progressive disease stages

Disease stage	NADH oxidation	H_2O_2 generation
H	DCP	Mn^{2+}
I	DCP	DCP
II	–	DCP
III	Mn^{2+}	DCP
IV	DCP	DCP
V	DCP, DCP and Mn^{2+} in combination	DCP
VI	–	DCP and Mn^{2+} in combination
VII	DCP and Mn^{2+} in combination	DCP

Stage H showed preference to Mn^{2+} as a cofactor for the reaction. The reaction of H_2O_2 generation by POX from stage II was instead inhibited by supplement of cofactors. The control reaction in stage II showed maximum production of H_2O_2. Stage VI POX produced maximum H_2O_2 when both cofactors were supplemented in combination.

Figure 5 depict the effect of externally added H_2O_2 on NADH oxidation in presence of DCP as a single cofactor. In general, there was an increase in the rate of reaction by addition of external H_2O_2, but in some cases the effect was found to be inhibitory. Stage I POX showed an inhibitory effect of H_2O_2 at a concentration of 0.01 mmol, but H_2O_2 at a concentration of 0.1 mmol accelerated the reaction. The reaction was accelerated at a concentration of 1 mmol H_2O_2 but to a lesser extent as compared to that at 0.1 mmol. Stage II POX showed acceleration of the reaction by H_2O_2, the maximum acceleration was observed at a concentration of 0.01 mmol followed by 0.1 and 1 mmol. The stage III POX showed acceleration in the reaction at lower concentrations of H_2O_2 (0.01 mmol and 0.1 mmol) but the reaction was inhibited in presence of 1 mmol H_2O_2. Stage IV POX showed maximum acceleration at a concentration of 0.01 mmol H_2O_2 and inhibition at 1 mmol H_2O_2. H_2O_2 at a concentration of 0.1 mmol had an accelerating effect on the oxidation reaction by POX from stage V but the reaction was inhibited at other concentrations of H_2O_2. Stage VI and VII reactions were accelerated by H_2O_2, the acceleration was maximum at 1mM concentration of H_2O_2 for stage VI and at 0.01mM for stage VII. In stage

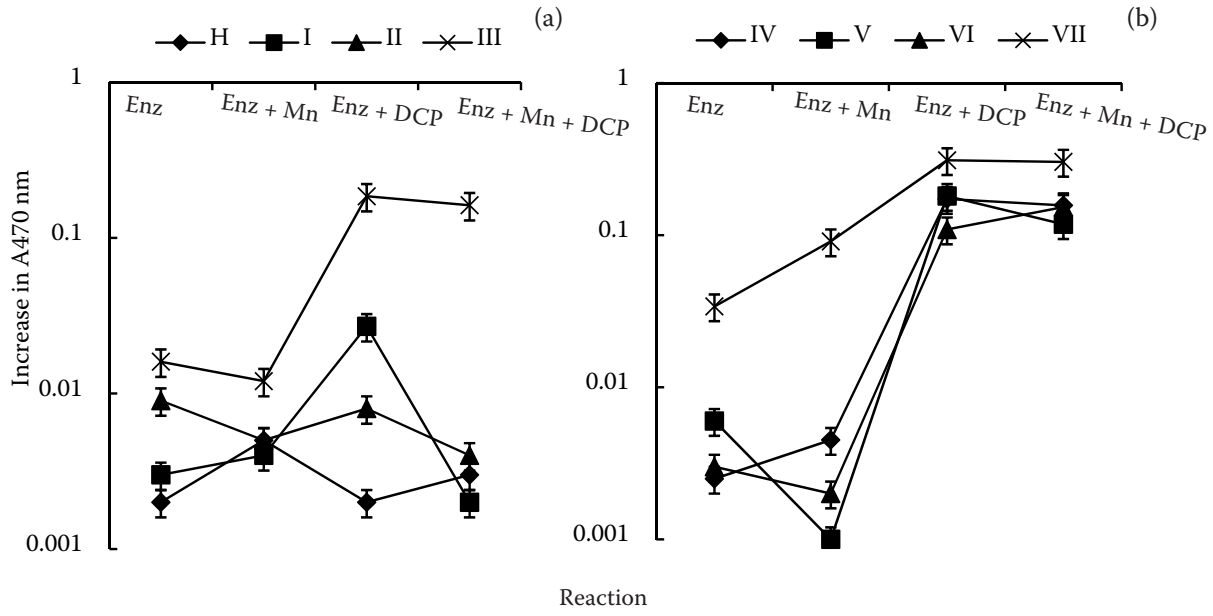

Figure 4. NADH – dependent H_2O_2 generation by peroxidases at progressive disease stages. Initial disease stages have lower production of H_2O_2 (a) as compared to the advanced stages of the disease (b) Supplement of cofactors, principally DCP, has a stimulatory effect on H_2O_2 production

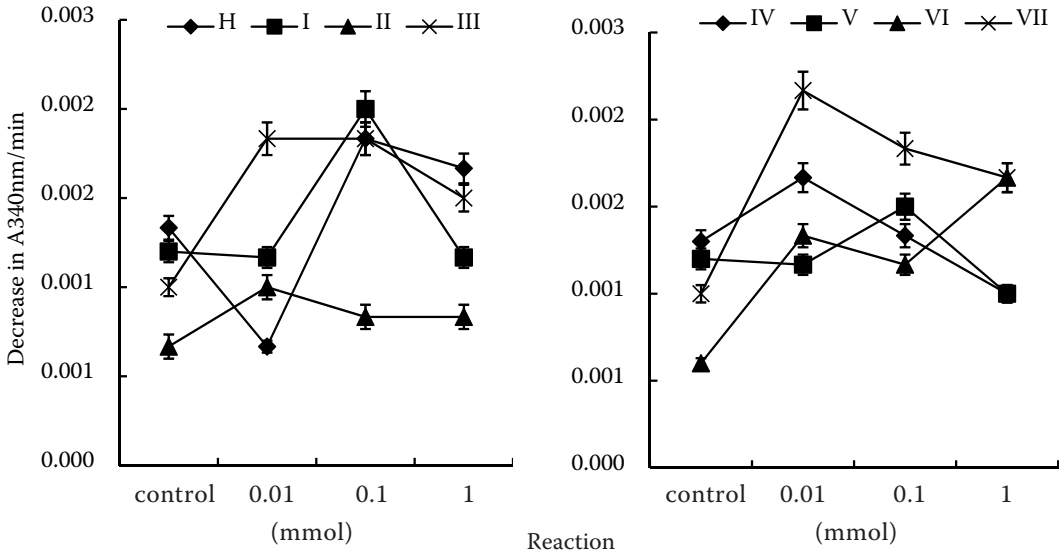

Figure 5. Effect of externally added H_2O_2 on NADH oxidation by the peroxidases from progressive disease stages (a–b). Externally added H_2O_2 has a stimulatory effect at least at one of the concentrations studied. The reaction was carried out only in the presence of DCP as a cofactor

VII, the accelerating effect of externally added H_2O_2 decreased with increasing concentration.

Figure 6 shows the effect of externally added H_2O_2 on cinnamyl alcohol polymerization by POX. H_2O_2 had a strong inhibitory effect on cinnamyl alcohol polymerization even at a very low concentration of 0.01 mmol H_2O_2. The efficiency of POX in cinnamyl alcohol polymerization was observed to be increasing with disease progression except for the initial stage I. Stage II showed a sudden increase in the activity of POX in cinnamyl alcohol polymerization. Stage III to stage VII POX showed an increase in the reaction rate with disease progression.

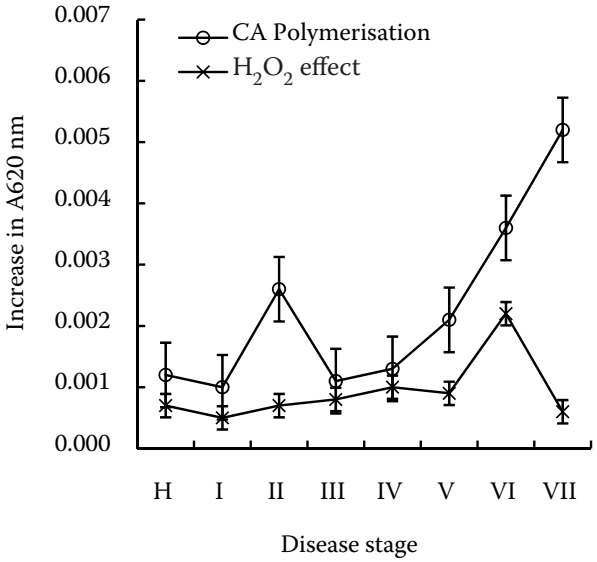

Figure 6. The polymerization of cinnamyl alcohol (lignin monomer) by peroxidases from progressive disease stages and effect of externally added H_2O_2 on the reaction. The ability of cinnamyl alcohol polymerization goes on increasing with progression in the disease. Externally added H_2O_2 at a concentration of 0.01 mmol has inhibitory effect on the polymerization of cinnamyl alcohol

DISCUSSION

The POX from *Acacia eburnea* infected with *Ravenelia esculenta* have a role in oxidation of NADH, H_2O_2 generation through NADH oxidation and utilisation of generated H_2O_2 in polymerization of cinnamyl alcohol. These studies are carried out on progressive disease stages to detect the changes in the activity of POX during disease progression. However, Akazawa and Conn (1958), Yokota and Yamazaki (1965), Elstner and Heupel (1976) and Miller (1985) obtained similar type of results in commercially available purified horseradish peroxidase.

The activity of the peroxidase enzymes from *Acacia eburnea* infected with *Ravenelia esculenta*, in NADH oxidation and H_2O_2 generation varies with the progression in the disease. The role of the H_2O_2 generated during the reaction *in vivo*, can be in host resistance during pathogenesis, through oxidative burst or through participation of released H_2O_2 in the process of lignification which raises the mechanical barrier for invading pathogen. The reaction is stimulated by metallic and phenolic cofactors but the reactions in which

both cofactors have an accelerating effect on the progress of the reaction, are physiologically more important than those with single or without cofactor. Studies on the POX from tobacco leaves revealed the role of POX in the oxidation of NADH, with concomitant release of H_2O_2, the amount of which is independent of the rate at which NADH is oxidized (Mader and Amberg-Fisher 1982). Purified horseradish peroxidase is also found to oxidize NADH (Mader and Fussl 1982).

The oxidation of NADH by POX proceeds through the elaborate sequence of complex reactions (Halliwel 1978). During the reaction, various complexes of compounds are formed with peroxidase, where inactive intermediate compound III is generated under aerobic conditions in presence of NADH. The compound III accumulates during the reaction and slows down the rate of NADH oxidation. Phenols as cofactors have a key role in breakdown of the compound III and accelerating the reaction (Halliwel 1978, Mader and Amberg-Fisher 1982). Cofactor requirement studies for tobacco peroxidase in NADH oxidation indicated the sole role of DCP as a cofactor for maximum stimulation of reaction (Mader and Amberg-Fisher 1982).

In the present studies, DCP stimulates NADH oxidation in the progressive disease stages with or without Mn^{2+}. Exception to this are stages III, VI and VII which show varying requirement of cofactors as mentioned previously.

Thus it seems that the ability of POX in catalyzing the reaction of NADH oxidation and H_2O_2 generation during disease progression is quite different. In all disease stages studied, H_2O_2 formation is either catalyzed by DCP or both cofactors in combination, whereas the POX from healthy material show the requirement of only Mn^{2+} as cofactor for maximum production of H_2O_2. Preferences for requirement of cofactors for optimum NADH oxidation by these enzymes in the same disease stages are also different (Figure 3). Comparison of Tables 1 and 2, and Figures 2–4 help to reveal the difference in the cofactor requirement for the complete reaction studied as a time course and when two reactions of NADH oxidation and H_2O_2 generation are considered separately.

Halliwel (1978) and Mader and Amberg-Fisher (1982) detected inhibitory effect of addition of H_2O_2 on NADH oxidation by purified peroxidase in the presence of Mn^{2+} as cofactor. Later, the authors showed the concentration-dependent accelerating effect of externally added H_2O_2 on NADH oxidation in presence of DCP. However, we did not detect the sole accelerating effect of H_2O_2, but some concentrations have little or inhibitory effect on the reaction (Figures 5a–b). The accelerating effect of external H_2O_2 can be explained on the basis of formation of compound I through the reaction of H_2O_2 and peroxidase. Compound I oxidizes NADH leading to formation of compound II which again reacts with NADH forming active peroxidase (Halliwel 1978).

We analyzed the role of the POX in polymerization of cinnamyl alcohol, a precursor for lignin synthesis in plants. The efficiency of the POX to polymerize cinnamyl alcohol goes on increasing with disease progression (Figure 6), with initial decrease in the activity followed by sudden increase at stage II. Increase in polymerization activity of the enzymes can be taken as a response of the host plant to pathogenic challenge. But addition of external H_2O_2 at a final concentration of 0.01 mmol has a strong inhibitory effect on the reaction. This is in contrast with the results of Mader and Amberg-Fisher (1982) who studied the reaction of NADH oxidation and polymerization of coniferyl alcohol. The two reactions proceed simultaneously, but there is a strong competitive interaction between the two substrates with no accumulation of H_2O_2 in the test sample despite the rapid oxidation of NADH. H_2O_2 generated during the reaction was supposed to bind to large quantities of enzymes present leading to formation of compound I which further accelerates NADH oxidation. However, we detected strong inhibition of otherwise rapid polymerization reaction by the POX at progressive disease stages. The reaction mixture in our studies contained both NADH and cinnamyl alcohol with DCP. Inhibition by externally added H_2O_2 can be attributed to its role in converting active POX to compound I, production of which might divert the reaction of polymerization to NADH oxidation thereby showing a strong inhibitory effect of external H_2O_2.

The results obtained in this study are also significant in understanding the shifts in the enzyme properties with disease progression. Activation of large number of POX isozymes during disease development is one of the responses of the host plant to the infection. Correlation of disease resistance and expression pattern of peroxidase has indicated its role in disease resistance. Seevers et al. (1971), Kar and Mishra (1976), Kristensen et al. (1999), Song and Goodman (2001), Guimil et al. (2005) and Jakupovic et al. (2006) showed an increase in the peroxidase activities in different host-pathogen systems. Though we detected the increase in activities of peroxidases, the host is highly susceptible to

fungal infection. Activity of peroxidase enzymes in NADH oxidation, H_2O_2 generation and cinnamyl alcohol polymerization is maximum at severe infection stage (stage VII). These reactions ultimately lead to formation of H_2O_2 or in lignification of the cells, both responses typical of the host response to pathogenic attack. Activation of these defenses by host at a very late stage of disease development when pathogen has been established in the host, might render the host susceptible to disease leading to typical pathogenic symptoms in terms of enzyme activities. Activation of POX indicates the biochemical hurdle posed by the host to the growth of the pathogen. But the pathogen successfully establishes in the host and sporulates profusely at severe infection stage (Stage VII), even when there is maximal activity of peroxidase in terms of all the parameters studied previously by the authors (Gandhe and Kuvalekar 2007). We also analyzed these POX from various disease stages for their preference of substrates (guaiacol, chlorogenic acid, caffeic acid and tetramethyl benzidine) at different pH values (5.0 to 8.0) (data not in context with this paper). The preferences of the enzymes for the substrates and their pH optima change substantially with disease progression.

There are no previous reports of study of POX during disease progression with the parameters that are studied in this paper. These studies help to understand the behaviour of POX during disease progression, changes in their activities, their role in NADH oxidation, H_2O_2 generation and lignification through polymerization of cinnamyl alcohols. Differential behaviour of POX with two cofactors suggests the existence of isoforms of the enzymes, and stimulation of reaction by these cofactors has physiological significance in terms of susceptibility of the host to the disease. However, the results presented in this paper exclusively illustrate the role of POX from various disease stages in different reactions and changes in their preferences to cofactors. But caution is needed in interpreting these results as side reactions may occur if high concentration of enzyme and low concentration of substrates are used.

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