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BIOCHEMICAL CHARACTERIZATION OF OXIDATIVE BURST DURING INTERACTION BETWEEN SESAME *(SESAMUM INDICUM* L.*)* IN RESPONSE TO *ALTERNARIA SESAMI*

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ABSTRACT
Induction of plant defense against pathogen attack is regulated by a complex network of different signals. The oxidative burst or rapid and transient production of large amount of reactive oxygen species (ROS) belongs to the fastest and earliest active defense responses to microbial infection known in plants. The aim of this study was to investigate the intensity and timing of the ROS formation, lipid peroxidation and expression of antioxidant enzymes as initial response of sesame (*Sesamum indicum* L.) against the invading pathogen *Alternaria sesami*. The concentration of hydrogen peroxide (H_2O_2) was 384 times higher at 72 h post-inoculation and lipid peroxidation was 5.5 times higher at 72 h post-inoculation in the extracts of inoculated leaves than in the control. An increase in total phenolic content was also detected in inoculated leaves. The activities of the antioxidative enzymes, viz., superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (GPX, EC 1.11.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11), increased in response to pathogen inoculation. SOD activity at 72 h post-inoculation in leaves was 92 times than the control. CAT activity also showed a decrease after 24 hpi and the increase in activities of GPX and APX was insignificant after 24 h post-inoculation in the inoculated leaves. The oxidative burst generated in the interaction between sesame and *Alternaria sesami* may be an early first line of defense mounted against the invading pathogen. However, seemingly less efficient antioxidative system (particularly the decrease of CAT activity after 24 hour post-inoculation) leading to sustained accumulation of ROS and the observed higher rate of lipid peroxidation indicate that the biochemical events are largely in favour of the pathogen, thus making this host–pathogen interaction a compatible combination. It is discussed that the oxidative burst served as a weapon for the pathogen because the antioxidative system was not significant enough to impede the pathogen ingress in the host.

KEY WORDS: Antioxidative enzymes, post-inoculation, Lipid peroxidation, *Sesamum indicum*, Oxidative burst, Reactive oxygen species.

INTRODUCTION

Resistance in much plant-pathogen interaction is associated with multifaceted defense system. The individual components of such systems include ion fluxes across plant membranes, the generation of reactive oxygen species (ROS), phosphorylation of specific proteins, activation of cell wall strengthening enzymes, structural barriers like lignin, hydroxyl proline rich cell wall proteins, transcriptional activation of several defense related genes, induction of phytoalexins, localized cell death at infection sites - hypersensitive response (HR) and induction of systemic acquired resistance in distal plant organs (Garcia-Limones *et al*, 2002). Proper recognition and judicious regulation of defense responses is essential for host plants, as these responses often have measurable deleterious effects on plant growth and metabolism (Gupta *et al,* 2010). Fungal pathogens deploy different strategies to escape host surveillance and establish themselves within the host depending on their nutritional requirements.

The transient production of ROS, in an oxidative burst, is frequently an early plant response to pathogen attack (Glazebrook, 2005). ROS have been suggested to be involved in plant defence responses in several ways. H_2O_2 is the major ROS of the oxidative burst in plants, since it is the most long-lived and able to cross plant cell membranes and thereby act as a diffusible and relatively lasting signal. ROS production including H_2O_2 has been especially well established in several plant tissue (Apel and Hirt, 2004) and suspension-cultured cell systems associated with the expression of an HR and SAR. In contrast, very little is known about oxidative metabolism in plant resistance reactions to pathogens that do not induce HR, such as the necrotrophic fungi that invade the plant vascular system. Sesame (*Sesamum indicum* L.), a member of Pedaliaceae is perhaps the oldest oil seed known and used by human beings. It is an important annual crop in the tropics and warm subtropics, known as the "Queen of oil seeds" because of its nutrient qualities (Prasad, 2002). Sesame seed oil has long shelf life and has remarkable antioxidant function. India is among the largest vegetable oil economies in the world (Ashri, 1998). India ranks first in area and production among the sesame growing countries. In this work, the aim is to investigate biochemical responses in leaves of sesamum cultivar (Tilarani) after *Alternaria sesami* inoculation in order to find reliable biochemical parameters that could be correlated with pathogen resistance mechanisms.

MATERIALS AND METHODS

Sesamum indium cultivar was raised from seeds in healthy conditions in a glasshouse. For *in vitro* fungal inoculation studies, mature plants were inoculated with 20 µl of *Alternaria sesami* conidial suspension (1×10^3 conidia ml^{-1}) or 20 µl of water (mock inoculation). The inoculated plants, along with their respective healthy controls, were then maintained at 30ºC in a temperature controlled glasshouse under a photoperiod of 12/12 h (light/dark) and 60% RH. After the development of symptoms in infected plants the experiment was terminated and the leaves harvested regularly from 24 to 120 h for analysis.

Estimation of $O_2^{\bullet-}$ and H_2O_2

H_2O_2 concentration was quantified using the protocol of Bellincampi *et al,* 2000. Superoxide anion was quantified following the method of Doke, (1983).

Quantification of lipid peroxidation (LPX)

The level of lipid peroxidation in the cells were measured as malondialdehyde (MDA) content by the thiobarbituric acid (TBA) reaction (Zhang and Kirkham, 1996).

Superoxide dismutase (SOD)

The SOD enzymatic samples were prepared from biological tissues and assayed as per the method of Elstner *et al,* (1995).

Ascorbate peroxidase (APX)

APX was isolated from the experimental samples and the activity determination was carried out according to the modified method of Yanagida *et al,* (1999).

Catalase (CAT)

In CAT assay, the reaction mixture consisted of 50 mM K-phosphate buffer (pH 7.0) containing 10 mM H_2O_2 (0.95 ml) and 0.05 ml enzyme extract. Immediately after adding the enzyme to the buffer, the initial rate of absorbance at 240 nm was determined. The molar absorption coefficient of H_2O_2 (0.04 mM cm−1) was used to calculate the enzyme activity (Shanker *et al,* 2004).

Peroxidase

Peroxidase was isolated following the method of Goliber, (1989). POX activity was assayed using the method of Ingham *et al*, (1998).

Phenylalanine ammonia lyase (PAL)

Isolation of PAL was made following the method of Morrison *et al,* (1994), and the activity was estimated by the method of Whetten and Sederoff , (1992).

Quantification of total phenol

Total phenol was isolated and quantified by the method of Mayr *et al*, (1995).

Reverse phase high performance liquid chromatography (RP-HPLC) of phenols

Quantitative fractionation of various phenolic acids in the samples were studied by RP-HPLC analysis. Phenolic acids extracted from fresh tissues in aqueous methanol were used for the study (Beta *et al,* 1999).

RESULTS AND DISCUSSION

Changes in Hydrogen Peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet-}$)

The production of reactive oxygen species (ROS) is one of the earliest cellular responses following successful pathogen recognition (Grant *et al,* 2000b). As shown in Fig 1, infection by *A .sesami* significantly increased the levels of H_2O_2 and $O_2^{\bullet-}$ production in sesamum leaves, and its levels increased with increasing the time of infection (H_2O_2 and $O_2^{\bullet-}$ levels increased by 384 and 86 fold, respectively, after 72 h from infection). ROS was considered as the first defense line against pathogen and may act as direct antimicrobial agent against phyto pathogen attack (Shetty *et al,* 2007).

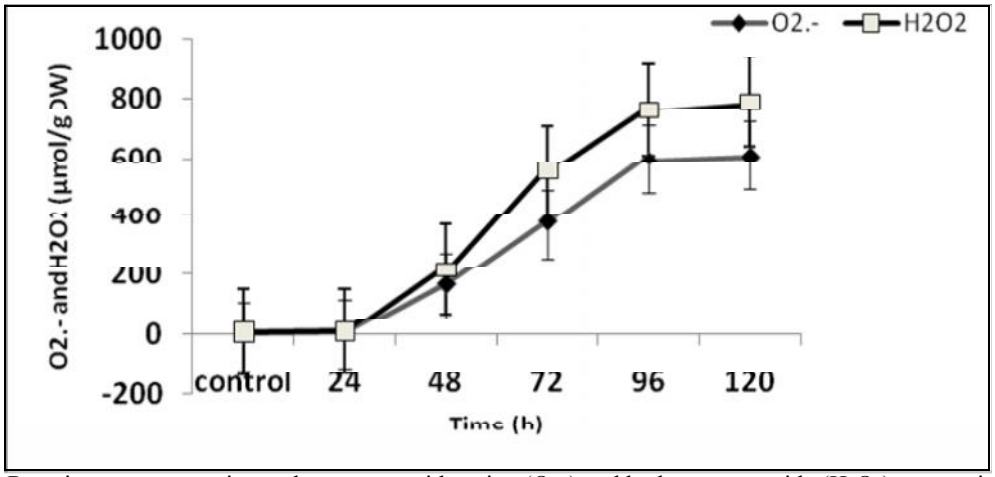

FIGURE 1. Reactive oxygen species such as super oxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) content in control and infected sesamum leaves from 24 h to 120 h. Values are means of three individual experiments with duplicates.

Therefore, high production of H_2O_2 and $O_2^{\bullet-}$ in sesamum leaves is an important element of disease resistance mechanism which are involved directly or indirectly in restricting pathogen growth and giving the time for plants to mobilize further defense reactions. Accumulation of H_2O_2 levels may leads to the accelerated senescence and decreased photosynthesis in infected tomato leaves as observed by El- Khallal, (2007). Induction or suppression of ROS generation in leaves of these treatments could be related to the activity of antioxidant enzymes which decrease or increase the levels of H_2O_2 (either by direct decomposition or oxidation or by its dismutation). ROS was proposed to act synergistically in a signal amplification loop with Shikimic acid-dependent pathways to drive the HR and the establishment of systemic defence. Similarly, Jasmonic acid induced H_2O_2 production in tomato plants (Orozcon-Cardenas *et al,* 2001) and pepper (Ueeda *et al,* 2006), which triggers expression of plant

resistant and HR – related genes are also supports our findings. Thus, present results suggests that the high production of ROS (H_2O_2 and $O_2^{\bullet-}$) and the capacity of plants to control its concentrations might contribute to increased resistance against *A.sesami* attack and giving the time for plants to mobilize further defense reactions. ROS have been involved in plant defense responses in multifold ways: a) reinforcing plant cell-wall through cross-linking reactions of lignin and proteins, b) acting as toxic agents against either the host plant cells, with development of HR and SAR, or against the pathogen through killing them or stopping their growth, and c) participating as a second messengers in signalling routes leading to the activation of plant defense related genes (Shimizu *et al,* 2006).

Changes in Lipid Peroxidation

ROS (H_2O_2 and $O_2^{\bullet-}$) activity frequently cause membrane damage through peroxidation of fatty acids, which may be coincided with the activity of lipoxygenase in plants as a consequence of infection (Fig 2). Levels of MDA gradually increased in leaves of infected sesamum plants with increasing the time of infection. When plants are subjected to pathogen attack, the equilibrium between production and scavenging of ROS is broken, resulting in oxidative damage of proteins, DNA and lipids. MDA is the marker for lipid peroxidation released from cellular membranes of tissues and are formed by the reaction of ROS (H_2O_2 or/and $O_2^{\bullet-}$) with lipid molecules (Shimizu *et al,* 2006). Thus, lipid peroxidation in sesamum plants has been proved to be induced by pathogens, and the subsequent products have been shown to passes antimicrobial properties and signalling function (Melan *et al,* 1993).

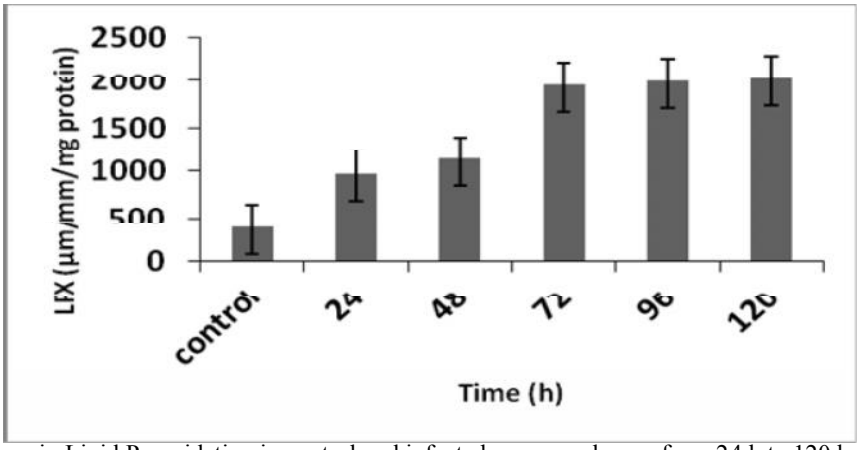

FIGURE 2. Changes in Lipid Peroxidation in control and infected sesamum leaves from 24 h to 120 h. Values are means of three individual experiments with duplicates.

Changes in the Activity of Antioxidant Enzymes

Organisms protect themselves against oxidative stress by the synthesis of various antioxidant enzymes. Results in Table 1 showed that infection by *A. sesami* significantly increased SOD, APX and CAT activities in leaves of sesamum plants at different stages of infection as compared with non-infected control. In banana plants, Subramaniam *et al,* (2006) reported that levels of H_2O_2 and other enzyme activities were increased to the levels of tolerance or susceptibility to Fusarium wilt disease. On the other hand, accumulation of H_2O_2 (Fig.1) in plants of infected compared to control plants could be related to the increase in SOD activity and not coordinate with the same increase in H_2O_2 scavenging enzyme (CAT and APX). Accordingly that increase in CAT and APX activity in pathogen infected plants did not seem to be high and/or enough to avoid the development of disease symptoms. Durner and Klessing, (1996) have presented evidence suggesting that Salicylic acid mediated inhibition of CAT and APX probably results from peroxidative reactions. It appears that Shikimic acid-dependent pathways require H_2O_2 to potentate lipid peroxidation as translocation signal that invokes PR genes and establish SAR (Rao *et al,* 1997). Finally, results in Figures 1 and 2 suggested that increased in levels of ROS, built up by either enhanced production and decreased scavenging potential, may contribute to the resistance reaction in sesamum against *Alternaria* infection. Finally, imbalance between H_2O_2 generation and scavenging enzymes in leaves may reflect a defense mechanism in sesamum or a pathogenicity strategy of the fungus.

TABLE 1. Activities of antioxidant enzymes in control and infected sesamum leave. Results are means of three individual experiments with duplicates.

	control	24	48	72	96	120
SOD (U/mg protein)	6.48±0.2	17.7±0.3	354±0.2	597.6±0.1	713.4±0.5	788.5±0.8
APX (U/mg protein)	22.4±0.5	52.6±0.5	214±0.3	319.4±0.3	343.6±0.5	369.8±0.5
CAT (U/mg protein)	22.8±0.1	18.4±0.6	39.6±0.2	42.3±0.8	43.4±0.1	42.8±0.3
POX (U/mg protein)	4.5±0.3	11.9±0.5	84.5±0.1	112.7±0.7	126.6±0.2	128.4±0.3
PAL (U/mg protein)	2.4±0.4	5.4±0.1	42.3±0.1	89.8±0.6	96.5±0.7	99.4±0.5

Changes in Phenolic Compounds and its Related Enzymes

Many plant phenolic compounds are known to be antimicrobial, function as precursors to structural

polymers such as lignin, or serve as signal molecules (Hammerschmidt, 2005). Results in Fig.3 showed that total phenolic compounds markedly increased in leaves of all treated sesamum plants at 72 h from pathogen inoculation. Similarly, *Hypericum perforatum* showed a 6-fold increase in phenolic compounds was observed in cells suspension after jasmonic acid elicitation (Gadzouska *et al,* 2007). However, levels of certain phenolic acids greatly changed in leaves of sesamum plants in response to pathogen inoculation as compared with control (Fig 4 a and b). Sinapic, coumaric, vanillic, chlorogenic, ferullic, and hydroxyl benzoate contents markedly increased in all treatments under pathogen infection. However, contents of gallic acids in sesamum plants decreased marginally as compared with non-infected control. Accordingly, the induction of total phenol and changes in the contents of various phenolic acids in leaves of treatments which play an important role in plant resistance and defense against *A.seami* infection, which are intimately connected with ROS (Fig 1). These results agree with the general speculation that when plant cells are recruited into infection, switch from normal primary metabolism to a multitude of secondary metabolism defense pathway and activation of novel defense enzymes and genes takes place (Tan *et al,* 2004). Also, rapid esterification of phenolic compounds as cinnamic and ferullic acids into plant cell walls are a common and early response to fungal attack, resulting in cell wall strengthening and then enhances resistance to pathogen penetration (Stadnik and Buchenouer, 2000). High accumulation of coumaric acid (Table 2), might be related to the activation of phenyl propanoid pathway through increased PAL activity (Table 1) which reflect a component of a defense response of the plant against *A. sesami* penetration. However, induction in ferullic and chlorogenic acids in infected leaves indicated that both acids are highly antifungal (Agrios, 1997), supported its role in reducing disease through the formation of defense barriers and activation of defense responses. Although hydroxy benzoic acid was more effective and greatly phytotoxic against soil borne root infecting fungi, its content increased in leaves of infection treatments of sesamum plants and this might be because HBA converted to both bound salicylic acid and gentisic acid (Belles *et al,* 1999). In parallel, due to the induction of various phenolic acids in sesamum leaves showed that activities of POX and PAL significantly increased Table 1 in sesamum plants in response to *A. sesami.*

TABLE 2. Amount of phenolic acid by RP-HPLC from control and *A.sesami* infected sesamum leaves (µg/g tissue).

	Sinapic acid	Coumaric acid	cinnamic acid	Vanillate	Gallate	Chlorogenate	Ferullate	Phloroglucinol	Catechol	HBA
control	1765.01	2302.6	-	240.4	222.3	-	-	-	285.5	43.9
infected	2039.89	2661.2	-	245.07	220.7	235.6	737.6	967.08	291.04	164.7

Further investigations are needed to determine whether our results support the induced susceptibility theory of host-biotrophic pathogen interactions. Summarizing, results from this present work showed the induction of the antioxidant enzyme system and other oxidative stress markers during *A. sesami* in sesamum, suggesting that changes in oxidative metabolism may be a quite general plant defense response not only restricted to foliar pathogens causing resistant reaction through HR and necrotic processes. Our results also suggest that increased levels of ROS, built up by either enhanced production and decreased scavenging potential, may contribute to the resistance reaction. Studies now in progress about direct ROS estimation and induction of ROS-forming enzymes, induction of non-enzyme antioxidants and compartmentation of antioxidant responses at the apoplast level, will probably provide more conclusive insights about the production of an oxidative burst and related responses and their role in the pathogenesis of *A. sesami* on sesamum.

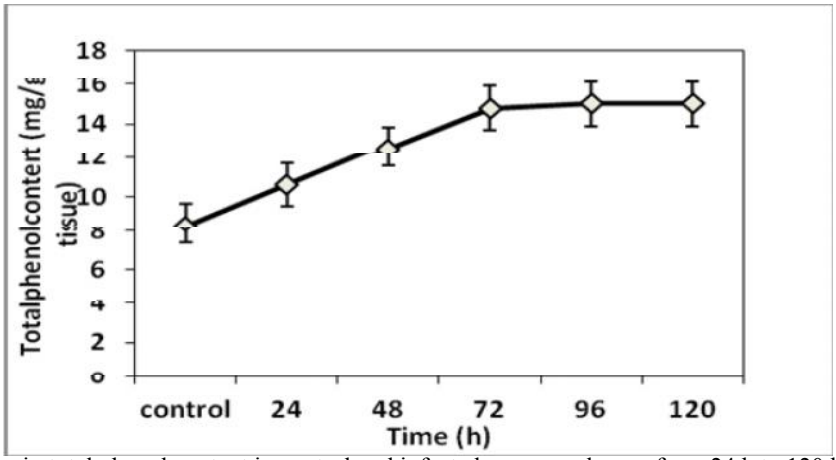

FIGURE 3. Changes in total phenol content in control and infected sesamum leaves from 24 h to 120 h. Values are means of three individual experiments with duplicates.

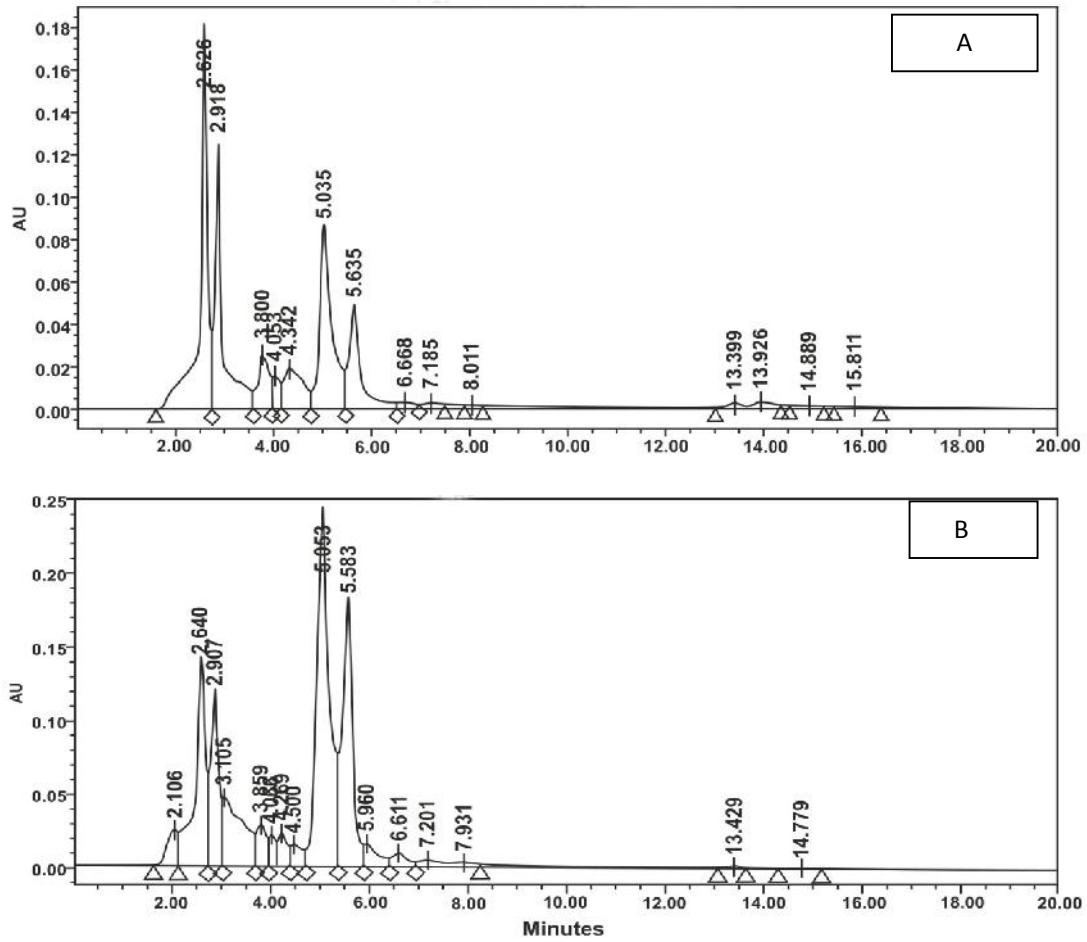

FIGURE 4. RP-HPLC chromatogram showing the peaks of phenolic acids in control (A) and infected (B) sesamum leaves.

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