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Hypersensitive response of *Sesamum prostratum* Retz. elicitated by *Fusarium oxysporum f. sesame* (Schelt) Jacz Butler.

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Aim of this study was to investigate the intensity and timing of the ROS formation, lipid peroxidation and expression of antioxidant enzymes as initial responses of calli of *Sesamum prostratum* (SP) against *Fusarium oxysporum f. sesame* crude toxin metabolite of varying concentrations. 2,4 dichlorophenoxy acetic acid (2,4-D) / coconut milk combinations were found to be more efficient among different hormonal regimes (2,4 -D, 2,4-D/casein hydrosylate and 2,4-D/ coconut milk). The concentration of hydrogen peroxide and lipid peroxidation were higher (13.2 and 5.7-folds, respectively) after 6 h in the treated callus confirmed the oxidative stress. An increase in total phenolics was also detected in inoculated callus. Increased activity of antioxidative enzymes viz., NADPH oxidase and superoxide dismutase (SOD) corroborate with the high level of ROSs, such as $O_2^{\cdot-}$ and H_2O_2. The poor activity of catalase confirmed the oxidative burst in the callus leading to necrosis. Activity of peroxidase was at par with total phenolics. Similarly, phenylalanine ammonia lyase (PAL) also showed high activity revealing the active phase in the synthesis of secondary metabolites in the plant. The oxidative burst generated in the interaction between *Sesamum* and *F. oxysporum f. sesame* toxin might be the first line of defense by the host mounted against the invading necrotrophic pathogen. The results suggested that the rapid production of reactive oxygen species in the callus in response to fungal toxin had been proposed to orchestrate the establishment of different defensive barriers against the pathogens.

Keywords: Antioxidant enzymes, Callus, *Fusarium oxysporum f. sesame*, Hypersensitive response, Oxidative burst, Reactive oxygen species, *Sesamum prostratum*

Hypersensitive response (HR), a putative form of programmed cell death, is a nearly invariant marker of disease resistance in plants and is characterized by rapid localized pathogen-induced cell death and restriction of further pathogen growth. Pathogenesis induces biochemical and physiological changes in host plants that may include the production of antioxidant species (AOS). These common responses are part of the incompatible interaction involving a resistant plant and an avirulent pathogen and are under genetic control.

Plants possess a complex battery of antioxidants that can protect cells from reactive oxygen species (ROS)[1]. It has been reported that peroxidases catalyze the polymerization of phenolic compounds to produce a variety of products which may take part in the defense system of plants against pathogens[2]. Moreover, the oxidation of phenolic compounds generally leads to the production of quinones, which are highly toxic compounds responsible for the generation of reactive oxygen species[3]. It has been recently suggested that reduction in the activity/expression of enzymes responsible for AOS catabolism is also relevant for AOS increase[3]. Repression of the antioxidative defense could be another mechanism for accumulation of AOS. Both, catalase and peroxidase, enzymes responsible for H_2O_2 scavenging in plant cells, seem to be locally down-regulated during the HR[4].

Fusarium wilt is one of the most important diseases of *Sesamum* in India caused by *Fusarium oxysporum f. sesame*. Plant cell cultures have been extensively used to investigate defense signal transduction in several plant species, but no work has been carried out with *Sesamum prostratum* (SP) callus cultures in this regard. The present study reports the consequences in the callus of SP challenged with *F. oxysporum f. sesame*. i.e., formation of AOSs such as H_2O_2, $O_2^{\cdot-}$, lipid peroxidation, activity of phenylalanine ammonia lyase, (PAL), change in activity of antioxidative enzymes and total phenolics.

Materials and Methods

Plant material—Sesamum prostratum (SP) growing wild was used to initiate callus cultures. For

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callus induction, surface cleaned juvenile stem with an axillary bud was placed on MS medium[5], supplemented with varying concentrations and combinations of 2,4-dichlorophenoxy acetic acid (2,4-D), 6-benzyl aminopurine (BAP) and kinetin. They were then incubated at 24° ± 1° C with a 12/12 h photoperiod from cool-white fluorescent lights with an intensity of 2,000 lux. For each treatment, three replications with twenty tubes per replication were maintained. The cultured explants were scored for the appearance of callus formation and were expressed in weight.

Fungal culture—Lesion pieces (3-5 mm) cut from the diseased plants of *Sesamum* were plated on 2% water agar medium after surface cleaning with 1% sodium hypochlorite solution for 1 min. Fungal isolates obtained from the lesion pieces were transferred to potato dextrose agar (PDA) slants at 28ºC. Isolates of *Fusarium* spp. identified by morphological observation under a light microscope and the plates were maintained at room temperature (28°±2°C) for 24 h. For bulk production of culture filtrate, 200 ml of Czapek's liquid medium (*p*H 5.5) in an one Erlenmeyer flask was inoculated with three mycelial plugs taken from the age of 7 day old cultures grown on PDA. The flasks were incubated on a rotary shaker (150 rpm at 28ºC). At one week following the incubation, the toxic metabolite was obtained by culture filtration through Whatman filter paper no. 1 to remove the mycelial mass. The filtrate was centrifuged at 10,000 × *g* for 15 min and then the supernatant was filtered through a millipore filter (pore diameter of 0.2 µm) under vacuum, to obtain a cell free metabolite suspension. The metabolite suspensions were transferred to small sterile glass bottles and kept at 4ºC. The crude toxic metabolites produced from *F. oxysporum* were interacted *in vitro* with the callus of *Sesamum prostratum*. The lyophilized fungal toxic metabolite was made to a final concentration of 1 mg ml^{1}. The calli were inoculated with different concentrations of the toxic metabolites (0, 1, 2, 4, 6, 8 and 10 mg ml^{-1}). The inoculated calli were subjected to all the biochemical and analytical studies in a time dose of 1, 2, 3, 4, 5 and 6 h duration. All experiments were repeated six times and the mean value was taken for consideration.

Isolation and assay of phenylalanine ammonia lyase (PAL)—Isolation and quantification of PAL was made following the method of Morrison *et al*[6].

Quantification of O_2^- and H_2O_2—H_2O_2 concentration in the samples was measured spectrophotometrically by following the method of Grossimann *et al*[7]. Superoxide anion was estimated using nitro blue tetrazolium assay as per the method of Vasilieva *et al*[8].

Isolation and assay of antioxidant enzyme—Callus (0.5 g) was homogenized in 25 m*M* potassium (K) -phosphate buffer (*p*H 7.8) containing 0.4 m*M* EDTA, 1 m*M* ascorbate and 2% (w/v) of polyvinylpolypyrrolidone (PVPP). The homogenate was then centrifuged at 15,000 *g* for 20 min at 4°C. The filtered supernatant was used as the enzyme source for CAT. For SOD assay, the extract was dialyzed overnight with 10 mM potassium phosphate buffer (*p*H 7.8) at 4°C. The dialyzed extract was centrifuged at 15,000 × g for 20 min at 4°C. The supernatant was filtered, and the filtrate was used as the enzyme extract for SOD assay[9]. In superoxide dismutase assay, the reaction mixture consisted of 500 m*M*, potassium-phosphate buffer (*p*H 7.8) containing 0.1 m*M* EDTA (0.1 ml); 1 m*M* xanthine dissolved in 10 m*M* NaOH (0.1 ml), 0.1 m*M* cytochrome c (0.1 ml), distilled water (0.66 ml), xanthine oxidase diluted 30 times in 2 *M* $(NH_4)_2SO_4$ containing 1 m*M* EDTA (0.02 ml); and enzyme extract (0.02ml). The reaction was started by adding xanthine oxidase and the reduction rate of cytochrome c was measured by the initial rate of increase of absorbance at 550 nm. SOD assay activity was calculated following the formula − [Assay SOD units = (Vb/Vs)-1], where Vb is the reaction rate of the blank; and Vs is the reaction rate of the sample. In catalase assay, the reaction mixture consisted of 50 m*M* of K-phosphate buffer (*p*H 7.0) containing 10 m*M*, H_2O_2 (0.95 ml); and enzyme extract (0.05 ml). 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/m*M*/cm) was used to calculate the enzyme activity. Peroxidase was isolated and assayed following the method of Goliber[10] and Ingham[11]. NADPH oxidase was extracted and assayed as per the method of Mader and Fisher[12] by recording the optical density at 340 nm.

Lipid peroxidation—The level of lipid peroxidation in the cells was measured in terms of malondialdehyde content determined by the thiobarbituric acid reaction as described by Zhang and Kirkham[13]. The cells (0.4 *g*) were homogenized in 4 ml of 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 10,000 x *g* for 10 min. One ml of the supernatant was diluted 1:5 (v/v) with

20% (w/v) trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid. The mixture was heated at 95°C for 30 min and cooled in an ice bath, after which it was centrifuged at 10,000 *g* for 10 min, and the absorbance of the supernatant was read at 532 nm. The value for the nonspecific absorption at 600 nm was subtracted from the value read at 532 nm. The concentration of malondialdehyde was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Quantification of phenol—Total Phenol was extracted and estimated as per the method of Mayr *et al*[14].

Results

Callus induction—Nodal explants were cultured in the MS culture medium fortified with various combinations of growth regulators such as 2, 4-D, BAP and kinetin. Callus mass found to increase with increase in the concentration of 2, 4-D up to 3.5 mg l^{-1}. 2,4-D along with casein hydrosylate (3 mg l^{-1} + 0.1 g l^{-1}) recorded 3.1 g of callus growth. The highest mass (3.94g) of callus was recorded in 2,4-D (3 mgl^{-1}) and coconut milk ($0.1g^{-1}$) combination. The data was statistically significant at 1% level.

Estimation of reactive oxygen species, superoxide anion ($O_2^{\cdot-}$) and H_2O_2—The production rate of $O_2^{\cdot-}$ and H_2O_2 content in the challenged SP cells increased at 2 to 6 h after elicitation, and then becomes steady (Fig. 1). Production rate of $O_2^{\cdot-}$ and H_2O_2 content in the calli accelerated at 6 h was 14.6 and 13.2 times respectively, compared to control.

Lipid peroxidation (LPX)—The levels of LPX were determined by TBA test, which quantifies MDA as an end product of LPX. Lipid peroxidation immediately followed ROS production during the oxidative burst, indicating them as the origin of the increased levels of oxidative damage suffered by the cells. The amount of LPX in control after 6 h of elicitation was found to be negligible (46 nmole /g fresh wt), in contrast with the experimental (274 nmole /g fresh wt). The physiological correlation of LPX with ROS content was further studied by quantifying the antioxidant enzymes.

Total phenolics—Phenolic compounds are secondary metabolites and play many roles including lignin synthesis[19]. A positive correlation was observed between total phenolics and enzyme activities such as phenylalanine ammonia lyase and peroxidase suggesting its involvement in secondary metabolite synthesis or lignin (Fig. 2).

Antioxidant enzyme activities—Different ROS implicated in the blast-induced calli necrosis might be generated by oxidative enzymes, like NADPH oxidase (NOX), SOD and peroxidase (POX). As shown in Table 1, the enzymes NOX, SOD and POX induced elicitor-dependent ROS reactions of callus cultures, implying the participation of POX, SOD and NOX in scavenging reaction. Catalase (CAT) was found not to be significantly different in any of the treatments in contrast to the control (Fig. 3).

Phenylalanine ammonia lyase activity (PAL)—PAL represents the first enzyme of the phenylpropanoid pathway and was evaluated by quantification of transcinnamic acid at 290 nm. The results revealed the changes in the production of secondary metabolites, as the levels of PAL activity

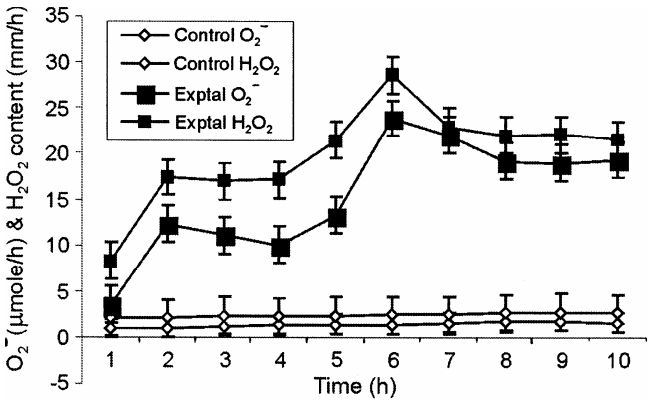

Fig 1—Changes in $O_2^{\cdot-}$ and H_2O_2 level in callus cultures of *Sesamum prostratum* after inoculation with fungal toxic metabolite of *Fusarium oxysporum* f. *sesame*

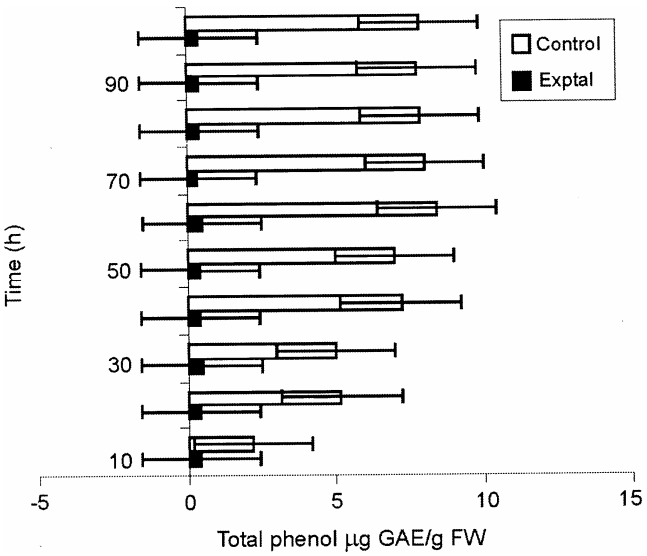

Fig. 2—Total phenolics in the callus of *Sesamum prostratum,* units are expressed as µg gallic acid equivalent (GAE)/g fresh wt.

Table 1—Change in activity of NADPH oxidase (NOX), superoxide dismutase (SOD) and peroxidase (POX) enzyme in callus culture of *Sesamum prostratum* treated with crude fungal elicitor from *Fusarium oxysporum f. sesame*

[Values are mean ±SE from combining six samples]

Time (min)	AOX enzymes					
	NOX (U/mg protein)		SOD (U/mg protein)		POX (U/mg protein)	
	Control	Expt.	Control	Expt.	Control	Expt.
60	4.2 ±0.1	10± 0.4	2.7 ±0.09	4.9± 0.24	2.6 ±0.31	4.8± 0.44
120	5.6±0.43	15 ± 0.21	4.6±0.3	10.2 ± 0.1	3.3±0.3	9.5 ± 0.21
180	4.8 ±0.11	12± 0.34	3.5 ±0.4	9± 0.24	3.2 ±0.21	8.2± 04.4
240	4.7±0.3	14 ± 0.1	3.6±0.23	6.7 ± 0.2	3.5±0.51	8 ± 0.11
300	4.5 ±0.6	16± 0.5	3.5 ±0.48	8.3± 0.64	3.1 ±0.21	7.8± 0.6
360	6.4±0.3	20.7 ± 0.1	5.9±0.3	19.65 ± 0.2	6±0.43	17.5 ± 0.7
420	5 ±0.1	19± 0.5	2.5 ±0.6	14± 0.5	4 ±0.1	16± 014
480	5.2±0.4	18.6 ± 0.45	3±0.4	15 ± 0.2	4.1±0.43	15.7 ± 0.3
540	5.3 ±0.56	18.2± 0.43	3.1 ±0.7	14± 0.54	4.1 ±0.1	15.6± 0.2
600	5.3 ±0.13	18.2± 0.24	3±0.3	14 ± 0.51	4.2±0.8	14.9 ± 0.4

(*P* values: < 0.00001. F ratio: NOX-165.3; SOD-172.6; POX-159.6)

dramatically increased in the calli in a time dependent manner (Fig. 4).

Discussion

Susceptibility to oxidative stress is a function of the overall balance between the factors that increase oxidant generation and those substances that exhibit antioxidant capability[1]. To determine the possible association of the oxidative processes accompanying the interaction with the ability to regulate ROS, the activities of CAT, POX, SOD and NOX in the calli were monitored and changes in the enzyme activities may be regarded as a consequence of pathogen-induced oxidative stress. Oxidative stress was confirmed by a significant increment of H_2O_2 levels in the cells and this molecule function as a signalling cascade as well as a toxic by product of cellular stress metabolism[15]. SOD activity directly modulates the amount of ROS and higher SOD activity contributes to dismutation of $O_2^{\cdot-}$ to H_2O_2 under oxidative stress. Similarly the activity of NOX may be corroborated with the $O_2^{\cdot-}$ content in the cells. The low profile of CAT activity may be justified by the more accumulation of ROS coupled with the oxidative burst leading to necrotic lesions in the calli.

A correlation between POX activity and phenol levels has been proposed for various crops[16]. POX is capable of oxidizing different phenols and it would seem plausible that this enzyme may be involved in the insolubilisation of phenylpropanoids into quinines, melanin and suberin. The observation that POX activity increased in callus at 6 h after inoculation (Table 1.) and phenols reached the maximum levels (Fig. 3)

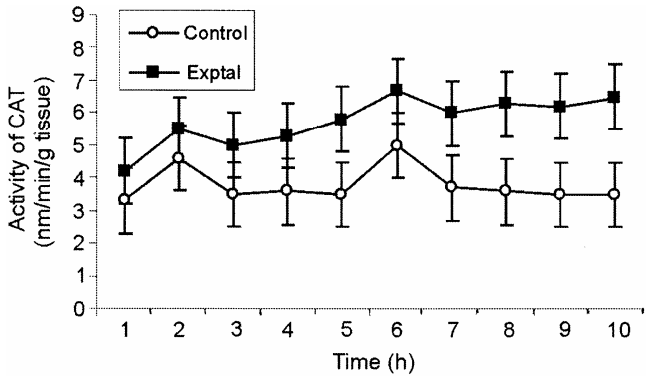

Fig. 3—Changes in catalase (CAT) activity in response to *Fusarium oxysporum* f. *sesame* toxic metabolite in *Sesamum prostratum* callus cultures

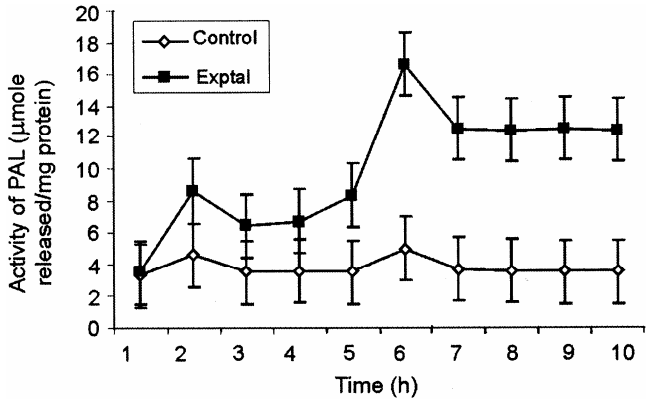

Fig. 4—Time course analysis of phenylalanine ammonialyase (PAL) activity in *Sesamum prostratum* callus cultures after inoculation with crude fungal toxic metabolite *Fusarium oxysporum* f. *sesame*

suggests that in *Sesamum*, this enzyme might be involved in the defense response. However, the role played by POX enhancement in the
resistance to plant pathogens has not been established unequivocally and it is still not clear whether it is a cause or a consequence of this phenomenon[16]. Auh and Murphy[17] have shown that inhibitors of the oxidative burst do not affect extracellular POX activity in rose cells and their data suggest that increased POX activity is not a major source of ROS during the HR. It has also been suggested that phenolics accumulation in some plants play a potential role in disease resistance[18].

PAL activity dramatically increased in challenged cells, as shown in Figure 4. This prominent difference between control and challenged cells indicated that the latter indeed activate certain defense responses, demonstrated by the increased activity of the first enzyme of a metabolic pathway that led to the production of compounds with an important role in the resistance mechanisms.

Collectively, these results provided evidence that the *Sesamum—Fusarium* culture exudate interaction was accompanied by a substantial increase in oxidative stress, probably as a direct consequence of a progressive decline in the enzymatic systems responsible for catabolism of active oxygen species. On the basis of quantitative data, among the enzymes tested, changes in NOX, SOD, POX and CAT activity seemed to be related with the interaction of *Sesamum* × *Fusarium*. Since all of the measurements reported here was made with crude extracts and, the possibility that there could be differential changes in antioxidant enzymes cannot be excluded. Further studies are needed to address the changes that occur at the purified level during the interaction, which would lead to a better understanding of this complex, but a highly regulated process.

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