Indian Res. J. Genet. & Biotech. 8(2) : 138-145 (2016)

Host-Pathogen Interaction in *Sesamum indicum*

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(Received : March, 2016 : Revised : April, 2016; Accepted : May, 2016)

Abstract

Differential activation of early defense responses like increased activity of phenylalanine ammonia-lyase (PAL), peroxidases (PO), and pathogenesis proteins (PR proteins) predisposes the host plant resistance against pathogens. Therefore, in the present study interaction between this defense related compounds and pathogen (Macrophomina phaseolina) was used as new strategy to enhance sesamum defense responses against root rot disease. There was a marked increase in these biochemical compounds in pathogen treated sesamum plants after pathogen attack. The investigation was carried after infection, which shows increase in activities of all defense related proteins to varying degrees of infection. Interestingly, there are significant changes in cytoplasmic proteins under pathogen inoculation. In the control plants, 8 major bands and 3 minor bands are obtained. In contrast, in the inoculated plants 8 major and 4 minor bands are obtained, which however, are of different Rf-values than the control plants. The differences in the protein are more pronounced in the molecular mass range of 15 to 36 kda and 40 to 59 kda. Individual protein bands obtained through SDS-PAGE were further quantified by scion image programme. This programme gives the peak area values of the corresponding band. The peak area denotes the densities of protein bands on the gel. A comparison of the densities of the protein bands in control and inoculated plants amply demonstrated that exposure to pathogen not only results in higher protein amounts but synthesis of a few new proteins, possibly PR- proteins. The biochemical approach described in this paper should provide the basis for further efforts concentrating on genetic regulation of sesamum in the form of transcript accumulation for expression of disease resistance proteins.

Keywords: *Sesamum indicum, Tiarosporella Phaseolina*, Semi-arid region, Pathogenesis -related (PR) proteins, Root rot disease

Introduction

Defense responses in plants are assumed to be trigger after specific recognition by plant cell of components from an incompatible pathogen. When pathogen attacks the plant shikimic acid pathway is activated which involves the activation of some plant defensive enzymes like phenylalanine-ammonia lyase and peroxidase. These all work independently or together with the same objective of protecting the plant against pathogen attack by inhibiting the pathogen.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is the main enzyme of plant phenolics production even under stress exposure (Jones 1984) leading to functionally diverse defense related products such as lignin, suberin, wall-bound phenolics, flavanoids etc (Chen *et al*., 2000). Studies with several different species of plants showed that PAL activity increases with the biotic and abiotic stresses, such as fungal infection (Hammerschmidt, 1999).

Peroxidase (PO, EC 1.11.1.7) is an oxido-reductive enzyme that belongs to Class-9 PR proteins with a wide array of functional and genetic diversity in plant (Scherer et al., 2005). Peroxidase contributes to resistance by participating in the cell wall polysaccharides processes such as oxidation of phenols, suberization, and lignification of host plant

Corresponding author's e-mail : singhsatendra57@gmail.com
Published by Indian Society of Genetics, Biotechnology Research and Development,
5, E Biotech Bhawan, Nikhil Estate, Mugalia Road, Shastripuram, Sikandra, Agra 282007
Online management by www.isgbrd.co.in

cells during the defense reaction against pathogenic agents (Lin and Kao, 2001).

Many of the reserve proteins accumulated in seeds and fruits take the constitutive defense function against microbial pathogens. PR Proteins have been classified into 12 major groups or families. Some of them show antifungal activity. The functions of most PR proteins remain a mystery but some of them are known to be -1, 3-glucanases (PR-2), chitinases (PR-3) or fungal membrane permeablizers (PR-5) (Honee 1999). Sesamum or gingelly (*Sesamum indicum L.*) commonly known as til is an ancient oilseed crop. It is usually rich in oil (50%) and protein (18-20%). Of the various factors responsible for the low productivity levels of sesamum, a major cause is the losses incurred due to root rot disease. One of the most important diseases is root rot caused by *Tiarosporella phaseolina* (syn. *Macrophomina phaseolina*).

On the whole, phenylpropanoid pathway enzymes & their quantification and qualitative evaluation of PR-protein profile in pathogen inoculated and control plants were conducted to find out the role of these proteins in host pathogen interaction in *Sesamum indicum* after *Tiarosporella phaseolina* attack. The availability of a large assortment of well characterized stress related PR genes (For biotic or abiotic stress), further understanding of stress signals and transduction mechanisms, and identification of additional defense genes will provide attractive opportunities for enhancing crop protection (Datta *et al.*, 1999).

Materials and Methods

Plant material

Seeds of til (*Sesamum indicum* L. var. RT 46) were surface sterilized with 0.1% $HgCl_2$ and washed several times in distilled water. The plants were maintained in green house at 25± 2°C temperature and 75% humidity.

Culture and preparation of spore suspension and mode of infection

The fungal strain, *Tiarosporella phaseolina* (MTCC No.166) was obtained from IMTECH Chandigarh. The fungal strains were grown on potato dextrose agar (PDA) medium (37 gml^{-1}) at pH 7.1. The activation of the lyophilized fungal strain was done by inoculating in PDA broth. Periodic subculturing was done at intervals of 30 days.

For the purpose of soil inoculation, the fungus was multiplied on sorghum grains. The seeds of *Sorghum* grains were first surface sterilized with 0.1 % $HgCl_2$. Then, the seeds were soaked in water overnight, and sterilized at 20-lbs. pressure for 30 min. The seeds were inoculated with *Tiarosporella phaseolina* and incubated at 28 2°C for 10 days. The flasks were shaken daily.

Soil was sterilized at in an oven at 180°C for 96 h. The inoculated sorghum grains (20 gkg^{-1} soil) were mixed in the pot containing plants grown in sterilized soil. Seven and 15 day old plants after inoculation were taken for the experiments. Those plants where no pathogen inoculation was done, served as controls.

Preparation of enzyme extract

The enzyme activity was determined using the method of Camm and Towers (1973). In brief, 1 g plant tissue was homogenized in 15 ml, 0.05 M borate buffer (pH 8.8) and filtered through two layers of cheesecloth. The homogenate was centrifuged in a high speed refrigerated centrifuge (Beckman Avanti TM_{30}) using an angle rotor (RF 0650) at 10,000 *g* for 15 min. The pellet containing cell wall and other debris was discarded and the supernatant was made to a definite volume. This constituted the crude enzyme extract.

Quantitative determination of PAL & Peroxidase

PAL activity. The PAL activity was determined by using the method of Camm & Towers (1973). Thereafter 0.1ml of purified enzyme extract was mixed with 0.3 ml of 50 mM L-phenylalanine and the total volume was adjusted to 3 ml with 0.05M borate buffer (pH 8.8). The reaction mixture was incubated at room temperature for 15 min. The absorbance was recorded at 290 nm. The PAL activity was calculated by using the standard curve of cinnamic acid.

Peroxidase. Peroxidase (EC 1.11.1.7) activity was assayed using guaiacol as substrate as described by

Lin and Kao (2001). The fresh plant tissue of normal and infected plants was homogenized in 0.1 M phosphate buffer (pH 7). The reaction mixture was prepared by mixing 0.1M phosphate buffer (pH 7) with guaiacol solution in crude enzyme and hydrogen peroxide. The change in absorbance was recorded at 436 nm after every 30 second upto 3 min.

Quantitative determination of PR- proteins:

The protein content was determined by the method of Lowry *et al*. (1951) using crystalline bovine serum albumin (BSA) as standard.

Qualitative determination of proteins by SDS-PAGE

Protein profile in the normal and infected plant samples was analyzed by SDS-PAGE following the method of Lammeli, 1976**.** The samples were run at 100 V, 25 mA for at least 7 h at 4ºC using discontinuous SDS-PAGE (12.5% resolving gel) to obtain proper resolution or until the tracking dye covered most of the column.

After the run was completed, the gel was removed from the gel mould. The gel was then stained for 5 h in the staining solution containing 0.1% Coomassie Blue R-250 prepared in methanol: water: acetic acid (5:5:1).

Molecular weight of different protein bands was determined by constructing a regression curve of known molecular weights of the standard versus their relative mobility (R_f). Relative mobilities of different bands were calculated by using following formula:

$$\text{Relative mobility } (R_f) = \frac{\text{Distance travelled by protein band}}{\text{Total distance travelled by tracking dye}}$$

Statistical analysis. All the experiments were repeated twice with similar results. The data were stastically analysed and treatment means were compared by three way ANOVA.

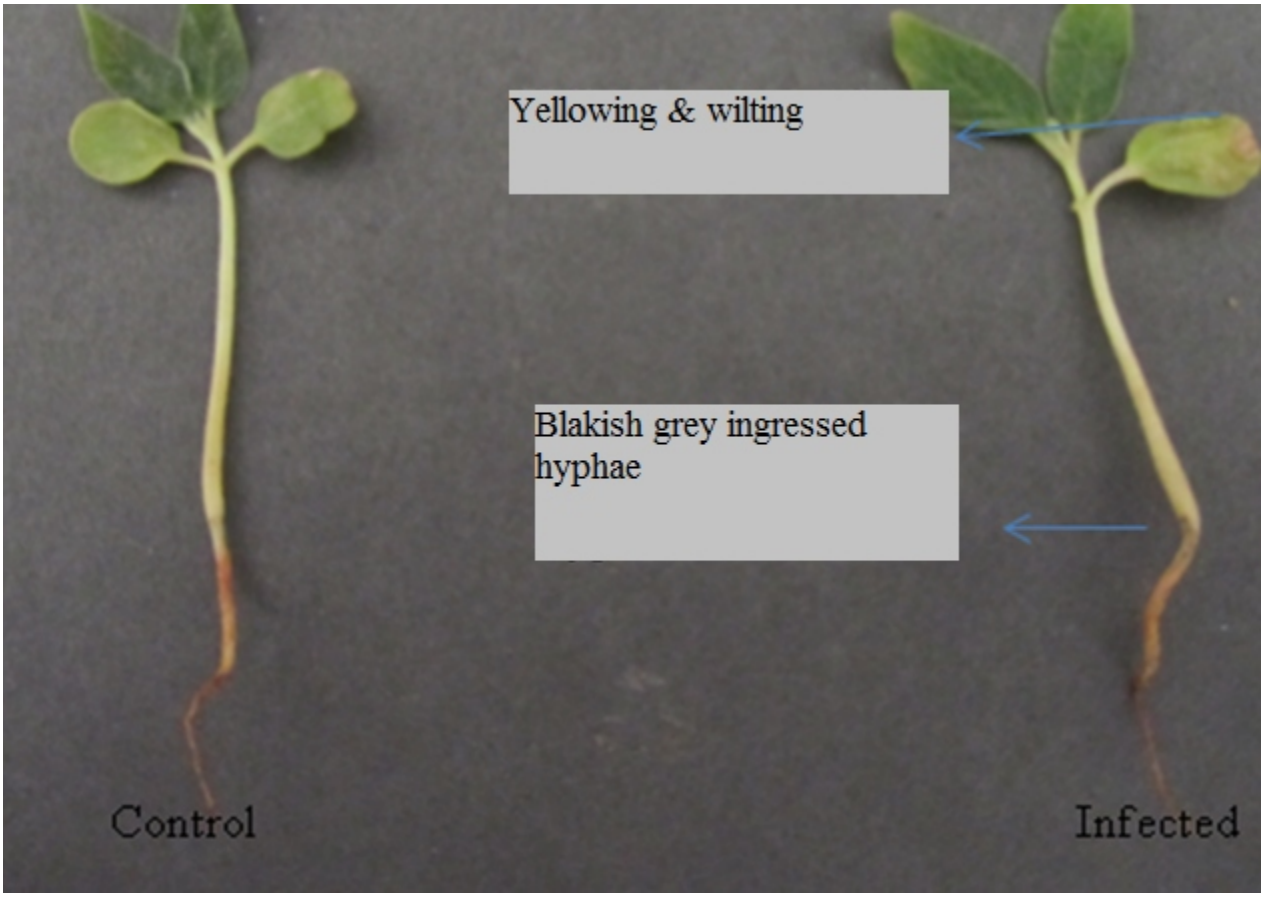


Fig 1: 7 days old control and infected four sesame cultivars RT-46 after infection

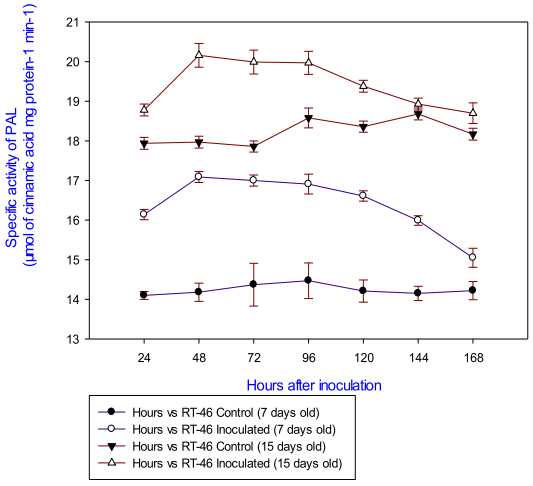


Fig 2(a): Specific activity of PAL

Fig 2(b): Specific activity of PO

Results & Discussion

The synthesis of biochemicals involved in defense response are under the control of defense related enzymes. Since the synthesis of these enzymes are regulated by specific genes, so any change in the activity of an enzyme would reflect the pattern of gene expression and corresponding metabolic events in the cell (Senthil *et al*., 2010). Besides these enzymes, oxidative enzyme peroxidase is also involved in defense of plants by oxidation of phenolic compounds. Therefore, in the present investigation, changes in the activities of PAL and PO along with pathogenesis related proteins (PRPs) have been studied in sesamum plants to understand the role of existing biochemical compounds in these plants upon infection by *Tiarosporella phaseolina*.

PAL being the first enzyme of the phenylpropanoid pathway, plays a key role by controlling the metabolic flux entering the pathway under the stress condition like fungal attack (Ramanathan *et al*. 2000). The increase in PAL activity has frequently been mentioned as a defense reaction of plants to pathogen attack, showing significant increases after infection by pathogen or wounding in forms of resistance to fungi (Zheng *et al*. 2005). The time required to activate the defense mechanisms is important for the suppression of the invading pathogen. Earlier and higher levels of expression of defense enzymes and accumulation of inhibitory compounds at the infection site certainly prevent the fungal mycelial colonization (Karthikeyan *et al.,* 2006). Similarly sesamum plants were observed for development of symptoms of disease in 7 days old plants. In Sesamum, the stem, leaves and collar region appeared dark brown. In addition the leaves also showed extensive yellowing. In contrast the control plants without inoculation but grown under similar condition showed no symptoms (Fig 1).

Inoculated plants also show increased PAL activity than the control plants. In this experiment, the enzyme activity started to increase at first day and then reached maximum at 120 hrs in (Fig 2 (a)). The Specific activity of PAL is found lowest at 24 hours of infection but after that it gradually increases upto 72 hours and thereafter turns down significantly. In 7 days old pathogen inoculated RT-46 cultivar, specific activity is noticed as 16.04±0.25 µmol of cinnamic acid mg-1 protein min-1. The percentage increase in specific activity of PAL in infected RT-46 cultivar in comparison to control ones after different time interval is 14%, 23%, 17%, 9% and 7%. This early increase in PAL activity was prerequisite for lignification process (Sharma et al., 2011). Several studies have shown that PAL activity is induced in banana plants after treatment with *Fusarium oxysporum* (Thakker *et al*., 2007).

In the present study, a significant increase in PO activity was detected in sesamum plants after infection with *Macrophomina phaseolina* in comparison to control plants at different time intervals. The activity of PO reached the highest level within 120 hours after

treatment in 7 days old plants and then slowly declined. Higher peak is observed at 72 hours of inoculation which is noticed 12.79±0.55 µmol mg^{-1} protein min^{-1}. Increased activity of peroxidase has been elicited in different plants such as cucumber (Chen et al., 2000), sunflower (Anjana et al., 2007) and wheat (Mohammadi and Homayoon 2002) due to pathogen infection. The percentage increase in PO specific activity in 7 days old inoculated RT-46 cultivar is 15%, 17%, 22%, 19%, 10%, 4% and 2% correspondingly (Fig 2 (b)). The activated PO may have a dual role in establishing resistance responses, which includes an antioxidative response mainly to detoxify the reactive oxygen species (ROS) produced at the site of infection and a secondary activity, which might trigger the activation of systemic acquired response against further pathogen attack (Sharma et al., 2012). Localized cell death due to the increase in ROS during the early stage of infection inhibits the spread of pathogen.

The present studies revealed the results of total protein profile and qualitative changes in PR-Proteins of *Seasamum* plants on elicitation with *Tiarosporella phaseolina* (*Macrophomina phaseolina*).

Quantitative Analysis on Protein Content versus Pathogen Inoculation

The total protein content was determined in sesamum plants using in vivo system. It is evident from fig 3 that while compared to the controls, the protein content is marginally higher at different time intervals after inoculation with the pathogen. The protein content is observed as 1.78, 1.79, 1.80, 1.78, 1.77, 1.77 and 1.78 mg gfw^{-1} at 24, 48, 72, 96, 120, 144 and 168 h respectively in contrast to the control plants. The highest values of protein content are recorded at 72 h after pathogen inoculation which is 1.80 mg gfw^{-1} for inoculated plants. It is further observed that the protein content in control plants is slightly lower than the inoculated plants.

Qualitative Analysis of Proteins using SDS-PAGE versus Pathogen Inoculation

In order to obtain a qualitative pattern of the proteins, SDS- PAGE of the protein extract obtained from

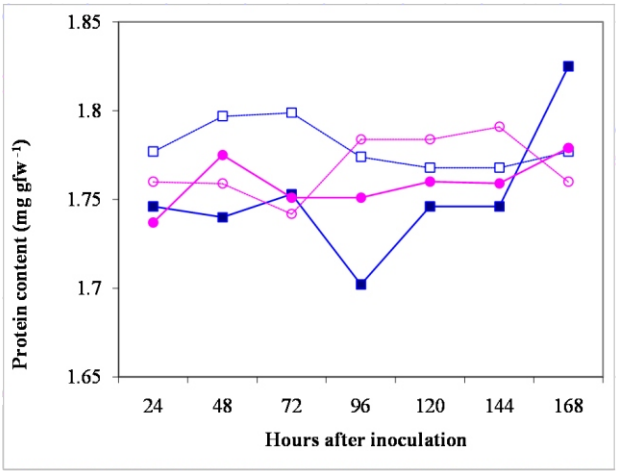


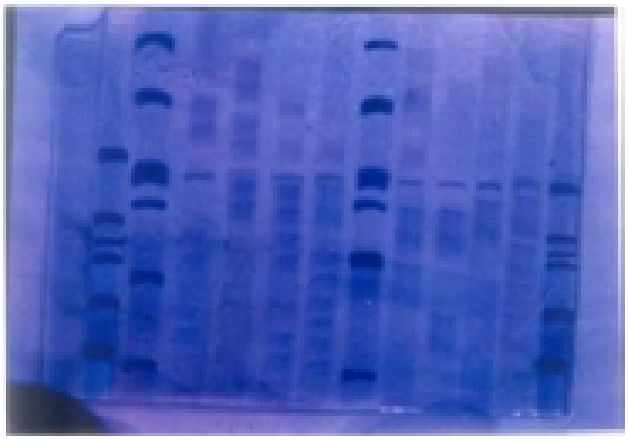
Fig. 3: Protein content in control and inoculated plants of *Sesamum indicum* inoculated with *Tiarosporella phaseolina* using i*n vivo* system.

Sesamum indicum after 48 hours inoculation with *Tiarosporella phaseolina* using in vivo system was carried out. In SDS- PAGE, the proteins separate into bands according to their molecular weight as these run on the gel. The gels were stained using both silver staining and coomassie blue staining methods. For calculation of molecular weight of the protein bands in the samples, both LMW and HMW (low and high molecular weight) markers were run simultaneously on different lanes of the gel.

Protein profile and molecular weights

In the control plants, 8 major bands and 3 minor bands are obtained. In contrast, in the inoculated plants 8 major and 4 minor bands are obtained, which interestingly are of different R_f values than the control plants (table 1, fig. 4).

It can be seen from the table 1 that there are significant changes in cytoplasmic proteins under pathogen inoculation. Results show that protein level increases with pathogen inoculation. Inoculated plants show protein bands of molecular weights (58.66 kda, 54.00 kda, 47.33 kda) at 0.812, 0.753, 0.658 R_f value. On the contrary, the control plants shows bands of higher molecular weights at 0.753, 0.658 R_f values. The protein bands of molecular weight of 2.00, 7.30, 12.67, 35.33, 40.00, 47.33 and 54.00 kda are similar both in control as well as inoculated plants. However an extra



1 2 3 4 5 6 7 8 9 10 11 12

Fig 4: Protein gel (lane 1 & 12: protein High molecular weight & Low molecular weight marker, lane 3-11 plant samples for 7 days)

protein band of molecular weight 15.33 (R_f value 0.218) is observed in the inoculated plants. In addition, in both inoculated as well as control plants, there are a few minor bands that also exhibit variations.

By other workers, increase in cytoplasmic protein content after pathogen attack is explained by increased enzyme activity after elicitation (Purkayastha 1998) as well as due to induction of genes for a group of proteins called pathogenesis related (PR) proteins (Van Loon and Van Strien 1999). A group of small proteins were also easily identifiable in 13 to 22 kda range, termed PR1 proteins were located previously (Somssich *etal*., 1986). Thus the results in this thesis clearly indicate not only increased amount of proteins some of which seem to be PR proteins, but also synthesis of new PR proteins; which obviously is due to activation of genes of such proteins. Increased cytoplasmic protein content after pathogen attack may be explained by increased enzyme activity after elicitation (Purkayastha 1998) as well as due to induction of genes for a group of proteins called pathogenesis related (PR) proteins (Bera and Purkayastha 1997). These workers have shown that PR proteins accumulate in greater quantity in infected plants and in lesser amount in control plants.

Analysis of coomassie blue stained gels by several other scientists (Van Loon 1997) reveals that the levels of enzymes and proteins are low or not detected

Table 1: SDS-PAGE based protein profile of *Sesamum indicum* plants

CONTROL PLANTS			INOCULATED PLANTS		
Distance Travelled(cm)	**R_f**	**Molecular weight (Kda)**	**Distance Travelled(cm)**	**R_f**	**Molecular weight (Kda)**
0.20	0.0235	2.00	0.20	0.0235	2.00
0.95	0.112	7.30	0.95	0.112	7.30
1.50	0.176	12.67	1.50	0.176	12.67
2.20	0.258	18.67	1.85	0.218	15.33
3.20	0.376	26.67	2.30	0.217	19.83
3.70	0.435	31.00	2.80	0.329	23.33
4.20	0.434	35.33	3.30	0.388	28.00
4.70	0.533	40.00	4.20	0.434	35.33
4.95	0.582	41.33	4.70	0.553	40.00
5.60	0.658	47.33	5.00	0.588	42.66
6.40	0.753	54.00	5.60	0.658	47.33
			6.40	0.753	54.00
			6.90	0.812	58.66

in untreated plants, but increase rapidly following treatment with elicitors. Thus our results are in agreement of the available reports in literature. Similarly increase in activity of PAL & PO is frequently observed under stress conditions, for example upon infection with pathogen (Knogge *et al*., 1986). Thus the results of this work clearly indicate not only increased amount of proteins some of which seem to be PR proteins, but also synthesis of new PR proteins; which obviously is due to activation of genes of such proteins.

Acknowledgment

Thanks are due to Dept. of Bio-Technology, Banasthali vidyapith for providing necessary help to conduct research.

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