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ORIGINAL ARTICLE

Effect of *Drechslera graminea* on total soluble proteins and defense enzymes of barley

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Abstract

Barley phylloplane is seriously colonized by *Drechslera graminea*, the causal agent of leaf stripe disease in the hos. The present study involved the elucidation of alterations induced in the protein content of the host due to *Drechslera* infection. Naturally growing barley plants were obtained from fields and *Drechslera graminea* was isolated and identified from diseased plants' leaves. After identification and preparation of the pure culture, the pathogen was inoculated on plants grown under aseptic and controlled laboratory conditions. Changes in the total soluble cytoplasmic proteins and defense enzymes of the host such as polyphenol oxidase (PPO), peroxidase (POX), phenylalanine lyase (PAL) and tyrosine ammonia lyase (TAL) were observed up to 5 h after inoculation. The results demonstrated a significant effect of the pathogen on the cytoplasmic protein expression of the host as well as in its defense system.

Keywords: barley, defense enzymes, *Drechslera graminea*, peroxidase, phenylalanine lyase, polyphenol oxidase, total cytoplasmic proteins, tyrosine ammonia lyase

Introduction

Barley (*Hordeum vulgare* L.) is an economically important food crop around the globe used both for feed and in the malting industry. High protein barleys are generally valued for food and feed, and starchy barley for malting (Duke 1983; Kling 2004; Sheidai *et al.* 2009). During germination and the early developmental phases of barley plants there is a rapid synthesis of protoplasmic proteins, particularly in the young leaves and root systems of the seedlings. These proteins form an integral part of protoplasts of cells, and ultimately most of the nitrogen assimilated by the plant is incorporated into cytoplasmic and chloroplastic structures (Abdelkader *et al.* 2012). Cytoplasmic proteins play a vital role in the physiological development of the plant and some play a critical role in the defense of the host plant from a wide range of abiotic and biotic stresses (Goel and Paul 2015a).

Drechslera graminea is a prominent pathogen of barley causing leaf stripe in the infected plants which leads to reddish-brown decay of the crown, rhizome and root tissues. The plants lack vigor and wilt during mid-day are eventually destroyed. When fungal pathogens invade plant tissues, some new proteins appear and accumulate in the infected tissue. These proteins are called pathogenesis-related (PR) proteins because they appear during pathogenesis (Goel *et al.* 2014). Pathogen-induced expression of cytoplasmic proteins, some of which are defense-associated, has been observed in several plants. Eventual accumulation of previously unexpressed proteins which have an important role both in physiological functions as well as in imparting resistance within the host against a wide range of abiotic and biotic stresses has been documented (Woloshuk *et al.* 1991; Yoshimura *et al.* 1998; Dodds and Rathjen 2010).

Polyphenol oxidase (PPO), peroxidase (POX), phenylalanine lyase (PAL) and tyrosine ammonia lyase (TAL) are four important PR proteins generic to almost

all known plants. Polyphenol oxidases are cosmopolitan in nature and are present in almost all organisms. They are responsible for oxidative browning of plant parts leading to senescence. Peroxidases are haem containing defense proteins responsible for oxidizing several pathogenic proteins in the presence of hydrogen peroxide (H_2O_2). They are known to induce resistance in host plants mediated by redox reactions in plasma membranes and cell wall modifications (lignification and suberinization) (Goel *et al.* 2013, 2014). Alterations in isozyme profiles of PPO and POX are signs of defense response and/or resistance-induction in a host plant. Stress-induced induction of PAL and TAL with a role in plant resistance has been reported many times. Their enhanced expression during pathogen attack and adverse conditions is mediated by transcriptional and translational regulation (Bhuvaneshwari and Paul 2012; Ganapathy *et al.* 2016).

The present investigation was aimed at understanding the host-pathogen relationship between *D. graminea* and barley, focusing on its effects on the total cytoplasmic proteins and profiles of defense enzymes namely PPO, POX, PAL and TAL on the host plant.

Materials and Methods

Isolation and identification of *Drechslera graminea*

Naturally infected barley leaves were procured from Amity University campus premises. The leaves were physically examined for stripes and conidia were isolated for lactophenol cotton blue (LCB) staining of the fungus for identification and confirmation (Khare 1996). Five replicates of the isolated fungus were used for identification.

Raising of plants

Surface sterilized and aseptically dried barley seeds (Vijaya cultivar, developed at CSA, Kanpur, India) were sown in sterilized soilrite in plastic trays (35 × 25 × 6 cm; L × W × H). The plants were raised in a sterile culture room maintained at 25°C (±1°C) with a relative humidity of 70% and a photoperiod of 12 h of light and dark. Trays were watered daily with sterile distilled water supplemented with Hoagland's solution once a week. Ten-day old barley plants were used for analysis.

Inoculum preparation and spraying

Undiluted aqueous spore suspension was prepared aseptically in order to maintain the natural pathogen concentration (corresponding to 10^7 spores · ml^{-1} as counted using a hemocytometer) for inoculation. Leaves of healthy barley plants were sprayed with the inoculum under strict aseptic conditions in multiple replicates. The selection of test plants was done on a random basis. Five test replicates were chosen and surface sterilized with 0.9% sodium hypochlorite before further analysis. Sampling was performed at 1 h intervals up to 5 h post-inoculation. The control plants were sprayed with autoclaved distilled water.

Cytoplasmic protein isolation and estimation

The total cytoplasmic proteins from inoculated leaves were extracted using the method described by Goel and Paul (2015b). Leaf samples (0.3 g) were homogenized using a chilled mortar and pestle (in a cold room maintained at 4°C) in 1.2 ml sodium phosphate buffer (0.1 M, pH 9.0) containing 0.001% Triton X-100, 10 mM β-mercaptoethanol, 10% (w/w) polyvinyl pyrrolidone (PVP), 1 mM phenyl methylsulphonyl fluoride (PMSF) and 1 mM EDTA at 4°C. The homogenate was centrifuged at 10,000 rpm at 4°C for 20 min. The supernatant thus obtained was used as cytoplasmic protein extract. The concentration of total proteins was quantified using the total protein kit, micro (Sigma) as described by Dias *et al.* (2013) and analyzed via SDS PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), using the midivertical gel kit (X-1235-01, Medox Biotech. Pvt. Ltd., India) having 4% stacking gel and 10% resolving gel (Cohen and Karger 1987). The gels were run at 30 mA for about 4 h. The gels were stained with 0.2% Coomassie Brilliant Blue R-250 with gentle agitation for 40 min and destained with 40% methanol and 10% glacial acetic acid solution. The gels were observed under a UV-VIS transilluminator (Medox Biotech. Pvt. Ltd., India) for observation and analysis.

Enzyme assays

The activities of PPO, POX and PAL were estimated by the method of Goel *et al.* (2017). For PPO activity estimation, 0.05 ml of enzyme extract was added to 0.5 ml of sodium phosphate buffer (1 M, pH 9.0), 1.25 ml of catechol (0.2 M), and 0.2 ml of distilled water. The mixture was incubated at 25°C (±1°C) for 5 min and reaction was terminated by adding 0.5 ml 10% (v/v) sulfuric acid. Absorbance was recorded at 420 nm using a UV-VIS spectrophotometer (Shimadzu, 1650, New Delhi, India). The reaction mixture without enzyme extract served as the blank. Enzyme activity was expressed as units $g^{-1} \cdot min^{-1}$ fresh weight. One unit of enzyme activity was defined as the amount of enzyme required for a change in absorbance of 0.001 per minute.

The reaction mixture for POX consisted of sodium-phosphate buffer (0.245 ml, 1 M, pH 7.0), guaiacol

(0.25 ml, 0.1 M), H_2O_2 (0.05 ml), crude enzyme extract (0.05 ml) and distilled water (1.655 ml). Incubation of the reaction mixture was performed at 25°C (±1°C) for 5 min and the reaction was terminated by the addition of 0.5 ml 10% (v/v) sulphuric acid. Absorbance was recorded at 470 nm using a UV-VIS spectrophotometer (Shimadzu, 1650, New Delhi, India). Enzyme activity was expressed as mM $min^{-1} \cdot g^{-1}$ fresh weight. The reaction mixture without enzyme extract served as the blank. One unit of enzyme activity was defined as the amount of enzyme required for a change in absorbance of 0.001 per minute.

For PAL activity, 1 ml of enzyme extract was allowed to react with 0.2 ml of 1 M-phenylalanine in 0.5 ml of borate buffer (pH 8.7) and 1.3 ml of distilled water. Changes in absorbance at 290 nm were observed at 30 s intervals for 10 min on a Biochrom UV–VIS spectrophotometer. The reaction mixture without substrate served as the blank. One unit of enzyme activity was defined as the amount of enzyme required to produce 3.37 nm of cinnamic acid per hour. Results were expressed as units of activity g^{-1} fresh weight.

For TAL assay, 100 μl of cytoplasmic extract was incubated with 0.9 ml 0.02 M L-tyrosine at 30°C for 60 min. After incubation, 0.5 ml 10% trichloroacetic acid (TCA) was added to stop the reaction. Absorbance was measured at 310 nm. For enzyme activity calculation, the calibration curve was prepared using p-coumaric acid solutions at concentrations of 1 to 100 $\mu g \cdot ml^{-1}$. One unit was defined as the amount of enzyme that produced 1.0 μg p-coumaric acid per min under the conditions of the assay. The results were expressed as U per mg of protein (Świeca 2016). The reaction mixture without enzyme extract served as the blank.

Polyphenol oxidase (PPO), peroxidase (POX) in-gel-isozyme staining

Fifty μg proteins were loaded onto the native basic polyacrylamide gel (10% resolving gel and 4% stacking gel) for isoform analysis. Electrophoresis was carried out at 70 $mA \cdot gel^{-1}$ for 3 h at 4°C. After electrophoresis, the gels were stained for PPO isozymes by equilibrating the gel in 0.1% p-phenylene diamine followed by adding 50 mM catechol in 0.1 M sodium phosphate buffer (pH 7.0). Iso-POXs were stained by incubating the gel in 0.1 M sodium-phosphate buffer (pH 7.0) containing 10 mM guaiacol and 0.75% H_2O_2 (Goel *et al.* 2017). The isozymes were distinguished on the basis of the Rf value of each band.

Statistical analysis

The experiments were performed with at least five random replicates for each test. The data were statistically analyzed for analysis of variance (ANOVA) using the general linear model procedure and the least squares means test of the statistical software SAS (version 9.2 developed by SAS Institute Inc., Cary, NC, USA). Multiple pairwise comparison tests using least-square means were performed for post-hoc comparisons after two-way with treatment and time as the two factors with replications. The corrections used for multiple comparisons were Tukey's honest significantly differences test (HSD) procedure.

Results

The LCB staining of the isolated fungus from naturally-infected barley plants resulted in a dark brown wood like structure, thus confirming the identity of the barley leaf stripe pathogen *D. graminea* (Fig. 1).

The total protein estimation in the test samples demonstrated an increase in protein concentration and expression in the inoculated host when compared to the control. A significant rise in total cytoplasmic proteins was observed 1 h post-inoculation which gradually increased up to 3 h ($p = 0.03$; $p = 0.05$; $p = 0.03$) after treatment and decreased later (although it remained higher than that in control up to the end of the sampling period) (Fig. 2). The number of peptide bands increased in the test samples within 1 h post-inoculation. This was not observed in the controls. Peptide bands of 205 kDa and 166 kDa appeared in test samples after 1 h of inoculation. In the samples collected 2, 3, 4 and 5 h after inoculation, another peptide band corresponding to about 60 kDa was observed unlike that of the control. There was a gradual appearance and significant increase in the thickness of lighter bands (ranging from 15 to 40 kDa) in test samples with the passage of time after pathogen treatment (Fig. 3).

Enzyme assay for PPO demonstrated an immediate, sharp increase in its activity within the first sampling hour in test samples. In the samples collected after 1 and 2 h of pathogen treatment, significant enhancement in PPO activity ($p = 0.05$ and $p = 0.03$ respectively) was observed. However, the activity returned to normal in the 3, 4 and 5 h samples (Fig. 4). A similar pattern was observed in the expression of PPO isoforms in the test samples while additional PPO isoforms were induced by the pathogen infection. Two additional isoforms (Rf = 0.59 and 0.63) were induced by *Drechslera* as observed in 1 h samples which were not expressed in the controls. This expression of additional isozymes was maintained for 2 h post-inoculation after which the expression of PPO isoforms returned to normal except for the isoform having Rf = 0.59 which continued being expressed till the end of the sampling period (Fig. 5).

Peroxidase had a deviated behavior in comparison to that of PPO in response to *Drechslera* infection.

Fig. 1. *Drechslera graminea* spore after lactophenol cotton blue (LCB) staining at 40X resolution

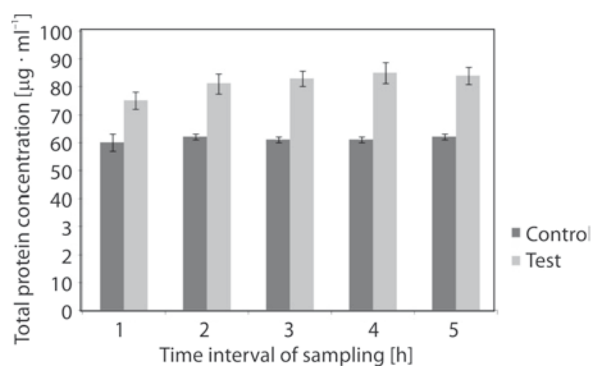

Fig. 2. Comparison of total protein concentration in control and test samples up to 5 h post-inoculation

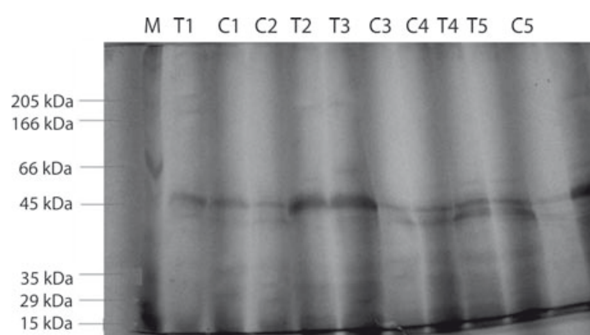

Fig. 3. SDS PAGE of samples after inoculation with *Drechslera graminea*. M – molecular marker (broad range protein molecular marker, MX-0211-01, Medox Biotech. Pvt. Ltd., India), C1 – control sample at 1 h, T1 – test sample at 1 h, C2 – control sample at 2 h, T2 – test sample at 2 h, C3 – control sample at 3 h, T3 – test sample at 3 h, C4 – control sample at 4 h, T4 – test sample at 4 h, C5 – control sample at 5 h, T5 – test sample at 5 h

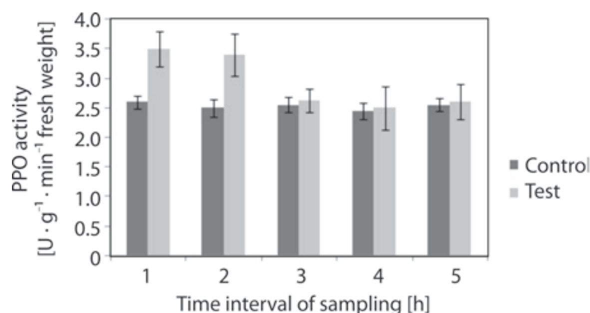

Fig. 4. Polyphenol oxidase (PPO) activity in inoculated plants. The bars represent the average PPO activity ±SE (n = 5)

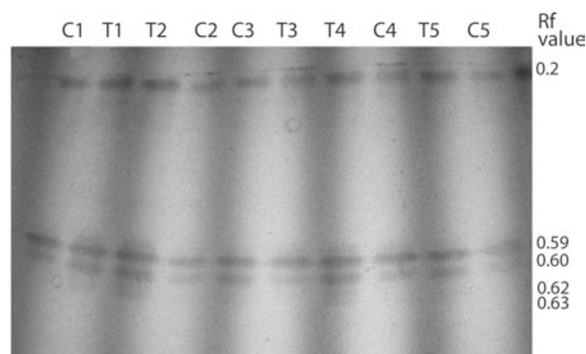

Fig. 5. In-gel-activity staining of polyphenol oxidase (PPO). C1 – control sample at 1 h, T1 – test sample at 1 h, C2 – control sample at 2 h, T2 – test sample at 2 h, C3 – control sample at 3 h, T3 – test sample at 3 h, C4 – control sample at 4 h, T4 – test sample at 4 h, C5 – control sample at 5 h, T5 – test sample at 5 h

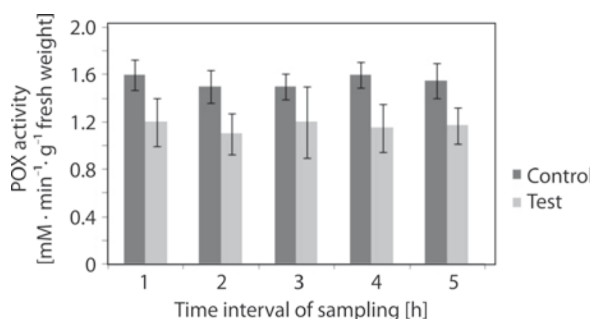

Fig. 6. Peroxidase (POX) activity in inoculated plants. The bars represent the average POX activity ±SE (n = 5)

Significant ($p = 0.04$) reduction in POX activity within an hour of infection was observed which was maintained till the end of the sampling period (Fig. 6). The isozyme analysis also demonstrated an absence in expression of a POX isoform (Rf = 0.4) in the test samples in comparison to the control within an hour of pathogen inoculation and did not appear in any of the *Drechslera* infected samples (Fig. 7).

PAL activity was not affected significantly by pathogenic attack. Although an initial rise in the activity was observed, it was not significant ($p = 0.13$) enough to be considered a result of *Drechslera* treatment (Fig. 8). Throughout the sampling period the PAL activity in test samples remained stable and almost similar to that in control plants.

The assay of TAL indicated a delayed enhancement in its activity. There was no significant rise in TAL

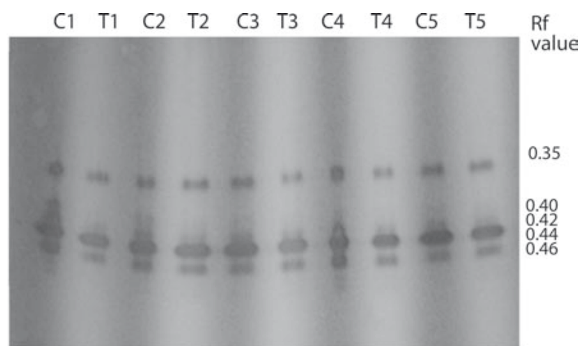

Fig. 7. In-gel-activity staining of peroxidase (POX). C1 – control sample at 1 h, T1 – test sample at 1 h, C2 – control sample at 2 h, T2 – test sample at 2 h, C3 – control sample at 3 h, T3 – test sample at 3 h, C4 – control sample at 4 h, T4 – test sample at 4 h, C5 – control sample at 5 h, T5 – test sample at 5 h

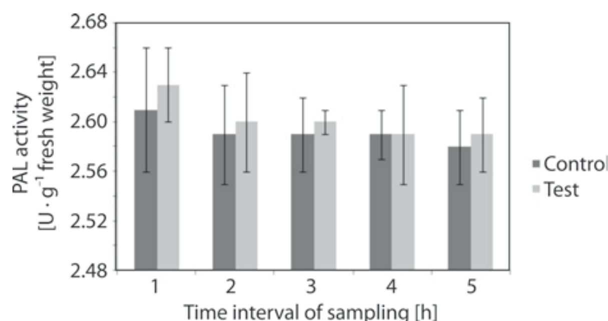

Fig. 8. Phenylalanine lyase (PAL) activity in inoculated plants. The bars represent the average PAL activity ±SE (n = 5)

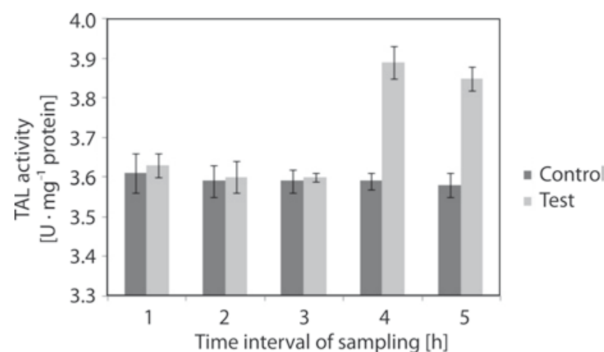

Fig. 9. Tyrosine ammonia lyase (TAL) activity in inoculated plants. The bars represent the average TAL activity ±SE (n = 5)

activity up to 3 h of infection. However, in the 4 and 5 h samples of infected plants, TAL activity suddenly rose to significantly (p = 0.05 and 0.04 respectively) high levels as compared to the controls (Fig. 9).

Discussion

The identity of the fungus isolated from naturally-diseased barley plants was confirmed to be *D. graminea* by LCB staining as reported earlier (Tsukiboshi 2003). The inoculation of *Dreschlera* on aseptically (previously unexposed to pathogen) raised barley plants had significant effects on the total soluble cytoplasmic proteins as well as the defense-related PR proteins of the host. An immediate response from the host upon pathogen-challenge resulted in enhanced expression of several proteins which were not expressed in control plants, and some other previously existing proteins were suppressed as evident by the total cytoplasmic protein content analysis, SDS PAGE and NATIVE PAGE of the protein isolates. The increase of protein concentration at 5 h post infection may suggest the presence of pathogenic proteins secreted by *D. graminea*. This implies that the leaf stripe pathogen has a direct impact on the physiology of barley, thereby instigating changes in the synthesis of a number of proteins in it. Dodds and Rathjen (2010) reviewed the role of pathogens in the manipulation of host proteins through secretion of virulence effector molecules. Pathogen-associated alterations in expression of proteins have been noted in several plants (van Loon *et al.* 2006). Several theories concerning protein synthesis under normal and stressed conditions in a variety of plants have been the focus of recent research on plant-pathogen interactions. One of the specialized mechanisms (such as Type III secretion system) which allow a pathogen to interact with the host machinery without invading their cytoplasms (mediated by effector molecules) has raised eyebrows among plant pathologists (Hogenhout *et al.* 2009). Differential expression in the cytoplasmic protein content of host plant upon pathogenic infestation has been reported every now and then in a variety of plants (Bais *et al.* 2004; Torres *et al.* 2006; Büttner 2012).

The activity of PPO was observed to increase substantially upon infection by *Dreschlera*. Expression of additional PPO isoforms in the test plants, which were absent in the control samples, was also noted within the first hour of pathogen infection which continued till the end of the sampling period. Although the PPO activity was reduced in test samples 2 h post-infection, expression of the aforementioned additional isoforms suggests that apparently these were in an inactive state and were probably synthesized by the host in anticipation of a subsequent pathogenic encounter. The effect of *Dreschlera* on the activity and isoform expression of POX varied from that on PPO. Significant reduction in its activity and expression of its isoforms in test samples were noted throughout the sampling period. Considering the importance of POX in imparting resistance to the hosts to all type of stresses, it can be assumed that the pathogen directly targeted its expression, thereby reducing it's expression and aiming at successfully manifesting itself on barley. This symbolizes direct interference of *Dreschlera* on the physiological activities of barley plants. Successful recognition of a pathogen

via the action of disease resistance (*R*) gene products in the plant immune system elicits a biphasic reactive oxygen species (ROS) accumulation with a low-amplitude, transient first phase, followed by a sustained phase of much higher magnitude which correlates with disease resistance (Torres *et al.* 2006). Post-transcriptional suppression of peroxidase in virus-induced programmed cell death has been reported by Mittler *et al.* (1998). Hemetsberger *et al.* (2012) demonstrated suppression in immunity of maize plants due to the reduction of peroxidase activity upon successful interaction with *Ustilago*.

Phenylalanine lyase (PAL) activity in test samples showed no signs of deviation from the controls. *Dreschlera* infection didnot have any significant effect on its expression in barley. This indicates that either the pathogen did not target PAL or failed to make an impact on it. This could probably be because the pathways targeted by *Dreschlera* might not be those which could influence PAL expression or its activity. Strikingly similar to PAL, TAL activity was also not affected by pathogenic intrusion initially. However, a late burst in its expression was evident by a sudden increase in its activity in the samples collected 3 h post-infection. This delayed activation of TAL could have been the result of an overall defensive strategy of the host to combat any unsuitable situation after recovering from the *Dreschlera* attack. Pathogenic-induction of PAL and TAL enzymes has been considered to be a symbol of the defensive response of host plants. Ramesh Sundar and Vidhyasekharan (2005) reported that *Colletotrichum falcatum* could successfully induce production of PAL and TAL in sugarcane, thus increasing its resistance. Yeast-derived elicitor treated seedlings showed critical activation and over expression of PAL and PPO in pearl millet leading to elicitation in its resistance against diseases caused by a wide range of pathogens (Hindumathy 2012). PR proteins including PAL were induced in groundnut by *Trichoderma viridae* which eventually resulted in augmenting its capacity to cope with the pathogen attack and prevention of disease (Gajera *et al.* 2015).

All cytoplasmic enzymes have one or more particular functions in normal growth, physiology, development and sustenance of the host plant. Interruption of their synthesis during pathogen attack or exposure to other unfavorable or altered conditions may prove to be lethal for the plant because these functions cannot be carried out by the proteins. The enzymes PPO, POX, PAL and TAL are well known for their defense-related functions. The direct effect on overexpression or suppression of one or more of these enzymes by *Dreschlera* indicates that the pathogen has the ability to intrude the host DNA machinery and mold it according to its suitability. However, the host defense also becomes proactive upon successful interaction with the incoming pathogen and hence change in its overall total cytoplasmic proteins and defense protein expression was observed. Further analysis is required to understand the molecular mechanism of the pathogenic attack and the defense response of the host plant. This might include unraveling the pathways targeted by the pathogen for its manifestation and the defense pathways activated in response to it.

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