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ORIGINAL ARTICLE

Aspergillus niger, a dominant phylloplane coloniser, influences the activity of defense enzymes in *Solanum lycopersicum*

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Abstract

Phylloplane microbes have been studied as strategic tools in management against plant pathogens. Non-pathogenic bacteria and fungi have been applied as crop protectants against various plant diseases. The present study aimed at evaluating the potentiality of *Aspergillus niger* spores in altering the activity of four key enzymes related to defense in tomato. The experiment was designed such that two groups of 50 tomato plants were considered: group 1 – sprayed with autoclaved distilled water (control) and group 2 – sprayed with *A. niger* spores. Spraying was carried out under aseptic conditions. The experimental parameters included analysis of the activity of peroxidase (POX), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) as well as expression of POX and PPO isoforms. The results demonstrated an inductive effect of *A. niger* on the activity of POX, PPO, PAL and TAL. Enhanced expression of POX and PPO isoforms was also observed. The results indicated that *A. niger* can be considered probiotic for the management of tomato against its phytopathogens.

Keywords: *Aspergillus niger,* phylloplane, peroxidase, phenylalanine ammonia lyase, polyphenol oxidase, *Solanum lycopersicum,* tyrosine ammonia lyase

Introduction

An array of microbes colonize plant surfaces. These have been reported to play an important role in plant growth and development, crop productivity, uptake and use of nutrients and resistance to abiotic stress (Windham *et al*. 1986). Phylloplane microorganisms are also known to play vital roles with respect to host plants as pathogens, natural antagonists to various detrimental organisms and as stimulating sources of plant growth (Blakeman 1991; Andrews 1992; Braga *et al*. 2009). A better wheat variety was seen to harbor better plant growth promoting phylloplane bacteria, thus playing a role in the superior yield of the host (Batool *et al*. 2016). Fungal endophytes and bacteria are important in facilitating foliar water uptake through stomata by decreasing leaf surface tension (Fernandez *et al*. 2017). A number of phylloplane microbes have been shown to profoundly impact the physiology of host plants. *Trichoderma* spp. have been found to induce the tolerance of plants against abiotic stress such as soil salinity, drought or flooding in plants (Zaidi *et al*. 2014). Epiphytic fungi have been reported to control foliar, root and fruit pathogens along with invertebrates like nematodes (Shoresh *et al*. 2010). The influence of phylloplane microbes on host plants defense has been very widely studied. Studies have revealed that phylloplane colonisers play significant roles in host defense thus inducing systemic acquired resistance. *Trichoderma harzianum* OTPB3 was found to be antagonistic against *Alternaria solani* and *Phytophthora infestans in vitro* and induced systemic resistance in tomato seedlings against early and late blight (Chowdappa *et al*. 2013). *Trichoderma viride* and *Pseudomonas fluorescens* could protect rose plants against *Diplocarpon rosae* due to enhanced activity of phenylalanine ammonia lyase,

peroxidase and polyphenol oxidase (Karthikeyan *et al.* 2007).

A large number of phylloplane microbes have been isolated from the leaf surface of tomato. However, the influence of these microbes on defense physiology of the host is not well elucidated. In the present study *Aspergillus niger* was chosen because it is a major coloniser of tomato leaves (Saleem and Paul 2016) and is not known to be pathogenic to *Solanum lycopersicum*. Tomato was selected because its cultivation occupies maximum acreage globally (Stewart and Shepherd 2013). This crop is infested by a number of microbial pathogens. Hence it would be important to understand if non-pathogenic natural phylloplane colonisers could be used to enhance host resistance. Defense enzymes were studied since defense is very important and is greatly influenced by microbe-plant association. The study focused on the possible impact of *A. niger* on the induction of defense enzymes and their isoenzymes in tomato. Studies on the interaction of non-pathogenic phylloplane microbes with their hosts and its effect on defense physiology is not well understood but it can be important in order to exploit such colonisers as enhanced, natural biocontrol agents.

Materials and Methods

Host plant

Surface sterilized and aseptically dried tomato seeds (variety: Pusa Ruby) were sown in plastic trays (35 cm × 25 cm × 6 cm; L × W × H) containing sterile soil rite. Plants were grown at 25 ± 1°C and at 70% relative humidity with a 12-hour (L/D) photoperiod under aseptic conditions. Trays were watered daily with sterilized distilled water and once a week with sterile Hoagland's solution.

Phylloplane microfungus

Phylloplane microfungus *A. niger*, used for the study, was isolated from field grown tomato plants, identified morphologically, cultured and maintained on PDA slants.

Isolation and identification of phylloplane microfungus

Aspergillus niger was isolated from abaxial and adaxial leaf surfaces of field grown tomato plants by the leaf impression method. The identification of isolated fungus was based on morphological and microscopical features and pure culture maintained on PDA slants. The identification of the fungus was confirmed by a mycologist at the Department of Botany, University of Delhi, Delhi, India. Subsequently, the fungus was identified by molecular methods. The NCBI accession number was MK590413.

Preparation of fungal inoculum

Fungal inoculum was prepared by diluting spores in 0.85% saline containing 0.02 µl Tween 20. The spore suspension was adjusted to 10^6 spores · ml^{-1}.

Treatment and sampling of plants

8-week-old seedlings were considered for the experiment and were divided into two groups of 25 plants each:

- Group 1 – sprayed with autoclaved distilled water (control);
- Group 2 – inoculated with *A. niger* spore suspension.

Inoculum was sprayed uniformly using a sterile atomizer. Leaves were sampled at 0, 15, 30, 45, 60 min and 2, 3, 4, 5 and 24 h post inoculation for estimating the activities of cytoplasmic peroxidase (POX), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) and the isoenzyme profile and expression of POX and PPO. Three replicates were collected for each sample.

Estimation of POX and PPO in cytoplasm

Frozen leaf tissue (300 mg) was homogenized at 4°C in 1.2 ml of ice-cold cytoplasmic extraction buffer (0.1 M, pH 9.0) containing 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.001% triton X-100, 1 mM ethylene diamine tetraacetic acid (EDTA) and 10% (w/w) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 15,000 ×g for 20 min at 4°C. The supernatant was used as crude enzyme extract for the estimation of POX and PPO activities and their isoforms. Three replicates were taken for each sample.

POX activity was assayed according to the method described by de Azevedo Neto *et al.* (2006). The reaction mixture consisted of 0.05 ml of enzyme extract, 1.655 ml of distilled water, 0.245 ml of 1 M sodium phosphate buffer (pH 7), 0.25 ml of 100 mM guaiacol and 0.05 ml of hydrogen peroxide (100 mM).

The reaction mixture for PPO consisted of 0.5 ml of phosphate buffer (1 M, pH 9.0), 1.25 ml of catechol (0.2 M), 0.05 ml of enzyme extract and 0.2 ml of distilled water (Bhuvaneshwari and Paul 2012). For both the enzymes, the reaction mixture was incubated at 25 ± 1°C for 5 min and terminated by the addition of 0.5 ml of 10% (v/v) sulphuric acid. Absorbance was recorded at 470/420 nm for POX/PPO, respectively, using a UV-VIS spectrophotometer (Shimadzu 1650). The reaction mixture without enzyme extract served as

a blank. The experimentally considered molar extinction coefficient for guaiacol was 26.6 mM · cm^{-1} (Costa *et al.* 2005) and 24.9 cm · min^{-1} for catechol (Rao *et al.* 1989). Enzyme activity was expressed as units · min^{-1} · g^{-1} fresh weight.

Estimation of PAL and TAL in cytoplasm

Frozen leaf tissue (300 mg) was homogenized in 1.2 ml of ice cold 0.05 M borate buffer (pH 7.0) containing 10 mM β mercaptoethanol, 1 mM phenyl methyl sulphonyl fluoride (PMSF), 0.001% triton X-100, 1 mM ethylene diamine tetraacetic acid (EDTA) and 10% (w/w) polyvinylpyrrolidine (PVP) at 4°C. The homogenate was centrifuged at 15,000 ×g for 20 min at 4°C. The supernatant was used as enzyme extract for estimation of PAL and TAL activity. The reaction mixture for PAL consisted of 0.05 ml of enzyme extract, 0.95 ml of 0.05 M borate buffer (pH 7.0), 0.11 ml of 100 mM L-phenylalanine. For estimation of TAL activity phenylalanine in the reaction mixture was replaced with tyrosine. For both enzymes, the reaction mixture was incubated in a water bath at 40 ± 1°C for 30 min and terminated by adding 2% w/v trichloroacetic acid (TCA). Absorbance was recorded on UV-VIS spectrophotometer (Shimadzu, 1650) at 275 nm/310 nm, respectively, for PAL/TAL. The reaction mixture without enzyme extract served as a blank. The PAL/TAL activity was calculated using respective molar extinction coefficient $\varepsilon = 15.56 \times 10^{-3}$ μM · cm^{-1}/ $\varepsilon = 9.554 \times 10^{-3}$ μM · cm^{-1} respectively. Enzyme activity was expressed as μM · min^{-1} · g^{-1} fresh weight. For all the enzyme assays, three replicates were taken for each replicate sample.

Native polyacrylamide gel electrophoresis (PAGE) and in-gel activity staining for the study of POX and PPO isoforms

The isozyme pattern of cytoplasmic POX and PPO was analysed by native basic PAGE (Laemmli 1970) without SDS (sodium dodecyl sulfate). On a basic PAGE, only the acidic isoforms' bands could be observed in the gel. The samples had equal concentrations of proteins. The protein concentration for each sample was estimated by Bradford's method (Bradford 1976). The native gel was prepared by separating and stacking gels containing 10% polyacrylamide at pH 8.8 and 5% polyacrylamide at pH 6.8, respectively. Electrophoresis was carried out at 70 mA/gel for 4 h at 4°C.

Guaiacol and catechol were used as substrates, respectively, for analysing POX and PPO isoenzymes (Bogdanovic *et al.* 2005). For POX, the gels were placed in a substrate solution of 10 mM guaiacol in 0.1 M sodium phosphate buffer (pH 7.0) at room temperature. Drops of 30% H_2O_2 were gradually added to the gel and shaken gently until orange coloured bands appeared. For PPO, the gels were equilibrated in a solution of 0.1% p-phenylenediamine in 0.1 M sodium phosphate buffer (pH 7.0) at room temperature for about 20 min. This was followed by the addition of 10 mM catechol to the same buffer. The gel was shaken gently until discrete dark brown bands appeared.

Isoforms of POX and PPO were distinguished by calculating the relative distance (*Rf* value) of each isozyme band from the respective zymogram using the following equation:

Rf value = distance migrated by the isoenzyme band from the origin/distance migrated by tracking dye from the origin.

Statistical analysis

The data were statistically analysed by applying the paired t-test using Graph Pad QuickCalcs software.

Results

POX activity and expression of isoforms

Peroxidase activity in *A. niger* inoculated plants was significantly higher than in the control although there was a decrease in the activity during the initial sampling (0–30 min). There was a significant increase in the activity of POX at 45 min post inoculation ($p \leq 0.01$) (Fig. 1A) which continued for 5 h after inoculation ($p \leq 0.01$) (Fig. 1B). The POX isoform profile in both control and treated plants included isoforms with *Rf* values 0.34, 0.52, 0.56, 0.58, and 0.60. Isoform (*Rf* = 0.52) expression was significantly enhanced at 45 min and again 5 h after *A. niger* inoculation. Isoforms (*Rf* = 0.56 and 0.58) had significantly higher expression at 45 min post inoculation than the control. A new isoform (*Rf* = 0.58) was detected only in treated samples 45 min after spore inoculation. Isoform (*Rf* = 0.60) which was present in control samples from the beginning of sampling but after treatment with *A. niger* its expression was inhibited upto 4 h post inoculation (Figs. 2–3).

PPO activity and expression of isoforms

Although the activity in the initial samples was found to be significantly suppressed in comparison to the control, PPO activity increased in *A. niger* treated plants. Polyphenol oxidase activity increased significantly 45 min after inoculation ($p \leq 0.05$) (Fig. 4A) and continued for 4 h. It again increased significantly at 5 h post inoculation ($p \leq 0.01$) (Fig. 4B).

Four PPO isoenzymes (*Rf* = 0.23, 0.44, 0.50, 0.57) in both control and *A. niger* treated samples were

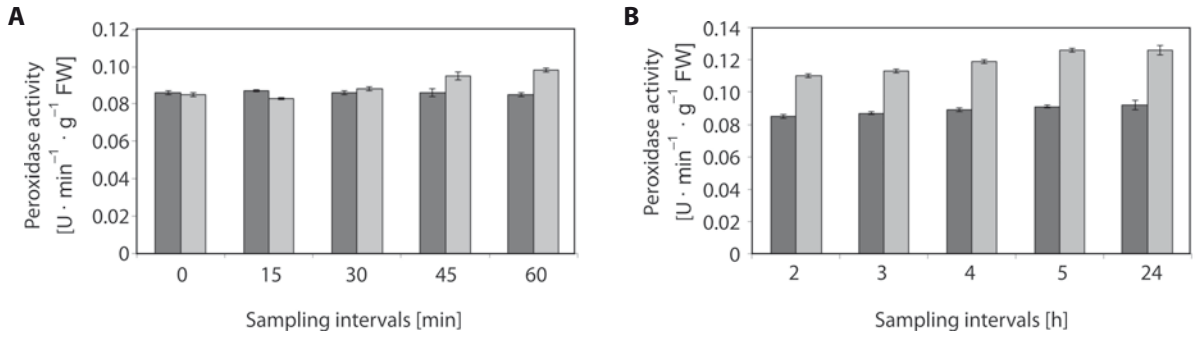

Fig. 1. Peroxidase (POX) activity in 8-week-old tomato plants in minutes (A) and hours (B) post inoculation; FW – fresh weight

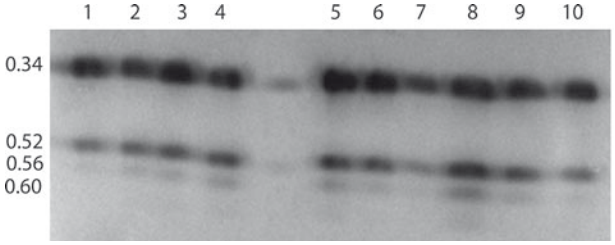

Fig. 2. Native PAGE of cytoplasmic peroxidase (POX) (sampling at 0–60 min): Lane 1 – control at 0 min; Lane 2 – *Aspergillus niger* treated at 0 min; L3 control at 15 min; L4 – treated at 15 min; L5 – control at 30 min; L6 – treated at 30 min; L7 – control at 45 min; L8 – treated at 45 min; L9 – control at 60 min; L10 – treated at 60 min. Numerical values represent the respective *Rf* value of each band

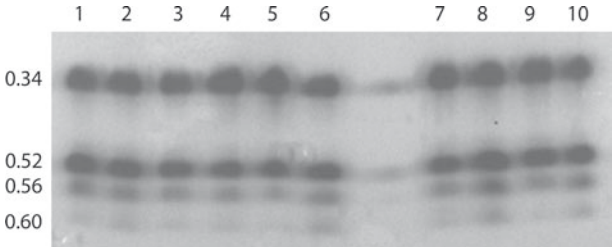

Fig. 3. Native PAGE of cytoplasmic peroxidase (POX) (sampling at 2–24 h): Lane 1 – control at 2 h; Lane 2 – *Aspergillus niger* treated at 2 h; L3 control at 3 h; L4 – treated at 3 h; L5 – control at 4 h; L6 – treated at 4 h; L – control at 5 h; L8 – treated at 5 h; L9 – control at 24 h; L10 – treated at 24 h. Numerical values represent the respective *Rf* value of each band

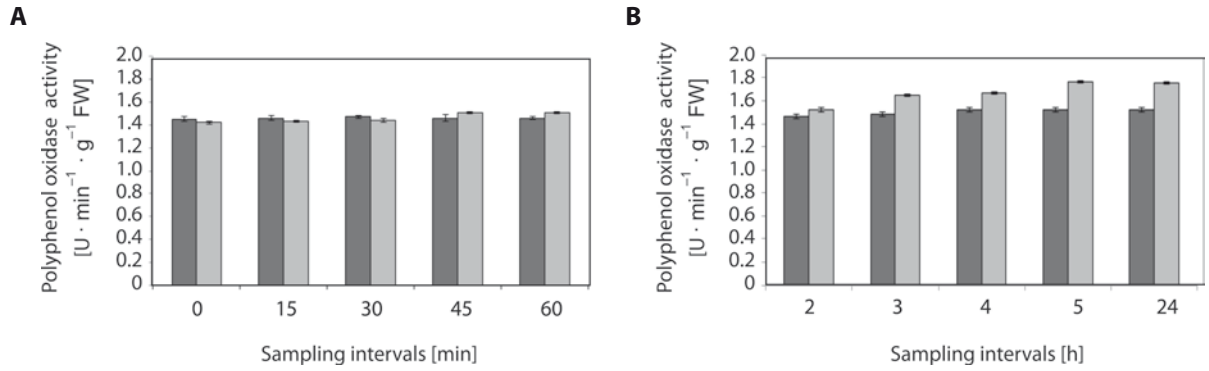

Fig. 4. Polyphenol oxidase (PPO) activity in 8-week-old tomato plants in minutes (A) and hours (B) post inoculation; FW – fresh weight

observed in the zymogram. However, enhanced expression of isoenzyme (*Rf* = 0.50 and 0.57) was observed in inoculated leaves sampled at 45 min as well as 3 and 5 hours post inoculation (Figs. 5–6).

PAL activity

Phenylalanine ammonia lyase activity was significantly higher in treated samples than in the control. PAL activity was significantly enhanced after 1 hour of inoculation ($p \leq 0.01$) (Fig. 7A) and gradually increased. It was highest at 24 h ($p \leq 0.001$) (Fig. 7B).

TAL activity

Compared to the control there was an enhanced activity of tyrosine ammonia lyase in *Aspergillus niger* treated plants as compared to control. A significant increase in the activity of TAL was observed at 60 min post inoculation which continued and was significantly the highest at 24 h ($p \leq 0.001$) (Fig. 8A–B).

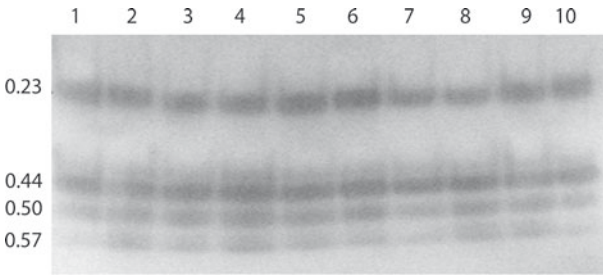

Fig. 5. Native PAGE of cytoplasmic polyphenol oxidase (PPO) PPO (sampling at 0–60 min): Lane 1 – control at 0 min; Lane 2 – *Aspergillus niger* treated at 0 min; L3 control at 15 min; L4 – treated at 15 min; L5 – control at 30 min; L6 – treated at 30 min; L7 – control at 45 min; L8 – treated at 45 min; L9 – control at 60 min; L10 – treated at 60 min. Numerical values represent the respective *Rf* value of each band

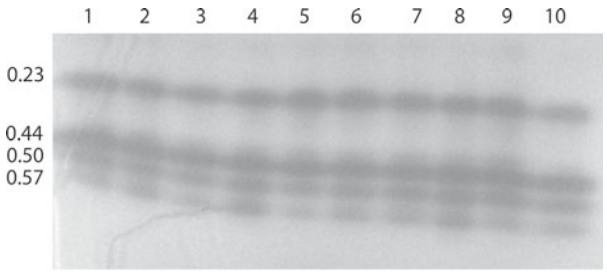

Fig. 6. Native PAGE of cytoplasmic polyphenol oxidase (PPO) (sampling at 2–24 h): Lane 1 – control at 2 h; Lane 2 – *Aspergillus niger* treated at 2 h; L3 control at 3 h; L4 – treated at 3 h; L5 – control at 4 h; L6 – treated at 4 h; L7 – control at 5 h; L8 – treated at 5 h; L9 – control at 24 h; L10 – treated at 24 h. Numerical values represent the respective *Rf* value of each band

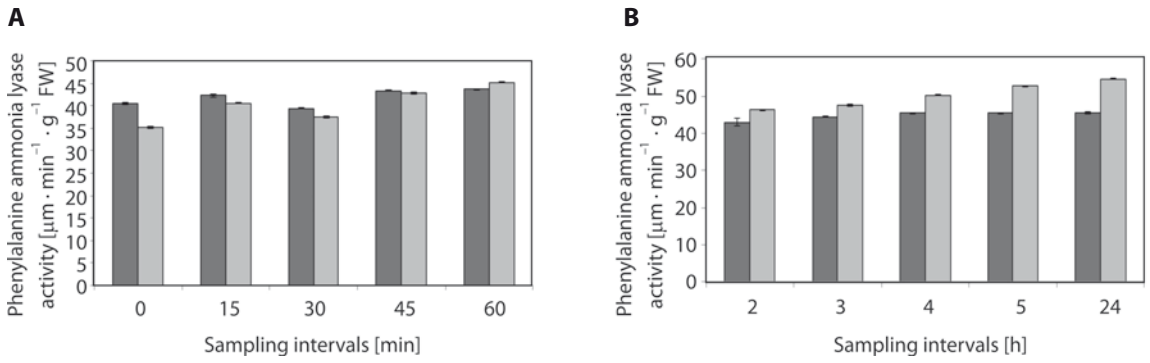

Fig. 7. Phenylalanine ammonia lyase activity (PAL) in 8-week-old tomato plants in minutes (A) and hours (B) post inoculation; FW – fresh weight

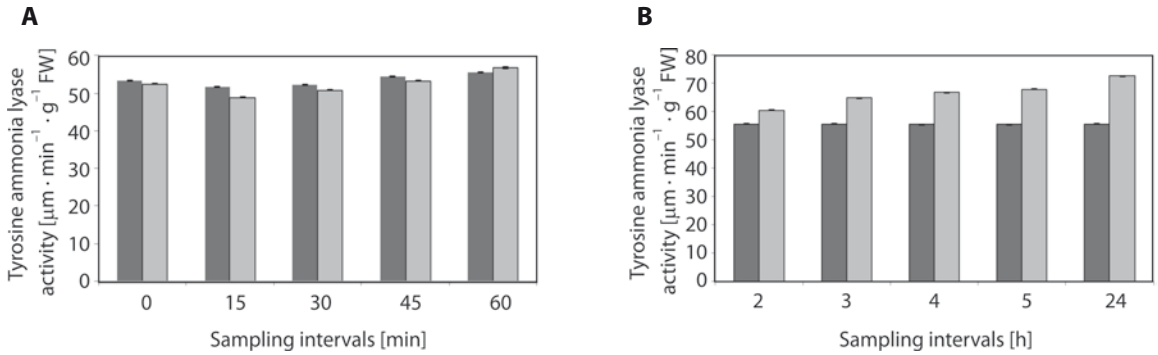

Fig. 8. Tyrosine ammonia lyase (TAL) activity in 8-week-old tomato plants in minutes (A) and hours (B) post inoculation; FW – fresh weight

Discussion

This study reveals that *A. niger* could enhance the activity of POX, PPO, PAL and TAL in inoculated plants. These four enzymes are well known for their defense related functions. The initial suppression of the enzyme could be due to the time lag of the *A. niger* spores to germinate and form a threshold colony which could initiate its interaction with the host plant through some specific elicitors. The enhanced expression of some isoenzymes seems to indicate the expression of some specific genes pertaining to these isoenzymes. This observation indicates that fungal

inoculation results in possible induction of a signalling pathway which initiates expression of selected genes. It is very likely that the elicitors from *A. niger* interact with the leaf tissue and elicit enhanced activity of these defense enzymes and expression of some isoenzymes. Previous studies have shown that certain beneficial plant associated microbes have synergistic effects on the host plants thus improving immunity and growth. Phylloplane fungi are reported to be biocontrol agents against *Colletotrichum* leaf disease of rubber (Evueh and Ogbebor 2008). *Trichoderma viride* was also found to be an important biocontrol agent against *Glomerella cingulata* in tea (Kuberan *et al.* 2012). Fungal metabolites have been found to enhance POX activity (Mathivanan *et al.* 2008). It has been postulated earlier that phylloplane microfungal metabolites influence the expression and activity of POX (Mitra *et al.* 2013). Thakur and Sohal (2013) demonstrated that various biosynthetic pathways can be activated by various natural and synthetic elicitors. *Pseudozyma aphidis* has been seen to sensitize *Arabidopsis thaliana* plants' defense machinery through induction of local and systemic resistance against *Botrytis cinerea* (Buxdorf *et al.* 2013). *Trichoderma harzianum* could suppress seedling blight of sunflower through elevation of PPO activity (Singh *et al.* 2014). Elevated PPO activity was also observed in *T. harzianum* treated tomato plants infected with *F. oxysporum* f.sp *lycopersici* (Ojha and Chatterjee 2012). *Trichoderma viride* JAU60 could effectively enhance the activity of PPO and PAL by many fold against *A. niger* Van Tieghem challenged groundnut seedlings (Gajera *et al.* 2015).

It can thus be postulated that *A. niger* interacts with host plant to significantly enhance the activity of POX, PPO, PAL and TAL and expression of some isoenzymes of POX and PPO which could be significant in aiding the host plant in its defense against a wide array of pathogens. Therefore, it can be concluded that phylloplane microfungal colonisers possibly interact actively with the host plant and enhance its biochemical defense against invading pathogens. The overall defense of the host against natural pathogens could possibly be a function of host defense modulated by the activity of phylloplane colonisers.

However, the molecular aspects of how *A. niger* influences the activity of defense enzymes and expression of their isoforms need to be studied in order to understand the nature and mechanism of interaction between phylloplane colonisers and the host plant. Further studies should include isolation and characterisation of elicitor molecules from these microbes, their interaction with cell wall bound proteins (receptors), the induced signalling pathway and subsequent gene expression which would lead to enhanced expression of some selected genes. Further, studies should also include the effect of multiple microbial consortium inoculations to unravel the impact of such consortia on the activity and expression of defense enzymes.

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