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INDUCTION OF DEFENSE ENZYMES AND PHENOLIC CONTENT BY *TRICHODERMA VIRIDE* IN *VIGNA MUNGO* INFESTED WITH *FUSARIUM OXYSPORUM* AND *ALTERNARIA ALTERNATA*

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ABSTRACT

Trichoderma viride is one of the most important biocontrol agents (BCAs) that have been used in agriculture across the globe. They provide systemic resistance to plants infested by various fungal phytopathogens. Biocontrol activity of *Trichoderma* based BCAs inheres in their ability to orchestrate various biochemical pathways in plants parasitized by fungi. Although studies delineating biocontrol activity of *Trichoderma* against fungal pathogens are documented, there is need for divulging the biochemical basis of disease resistance being induced by *Trichoderma.* Therefore, investigations pertaining to induction of such systemic resistance and associated biochemical responses is essential to understand the mechanism of biological control by *Trichoderma viride*. In this regard, current study was designed to understand the role of *T. viride* in inducing defense enzymes (Peroxidase, Polyphenol Oxidase and Phenyl Alanine ammonia Lyase) and total phenolic content in black gram exposed to pathogens *Fusarium oxysporum* and *Alternaria alternata.* It was found that the biocontrol agent, *T. viride* induced higher levels of defense enzymes in black gram during pathogenesis by *F. oxysporum* and *A. alteranata*. Therefore, it was concluded that plant defense enzymes play a vital role in mitigating pathogen-induced stress in legume, *Vigna mungo* during the biological control by *T. viride.* Outcomes of the study will be useful in formulating *T. viride* based BCA formulations to control wilt and blight diseases caused by fungal phytopathogens.

KEYWORDS: *Trichoderma viride*, Biocontrol Agent, *Vigna mungo*, Peroxidase, Polyphenol Oxidase and Phenyl Alanine Ammonia Lyase

INTRODUCTION

Application of biological control agents (BCA) is a promising and ecofriendly tool in improving current levels of agricultural production. It assists in reducing use of chemical pesticides thereby controlling release of their residues into environment. One of the most efficient ways to achieve this objective is to develop BCAs for disease control alone, or to integrate it with reduced doses of chemicals in the control of phytopathogens resulting in minimal impact of chemicals on the environment (Harman and Kubicek, 1998). To date, a number of BCAs have been registered and are available as commercial products, including strains belonging to bacterial genera such as *Agrobacterium, Pseudomonas, Streptomyces and Bacillus*, and fungal genera such as *Gliocladium, Trichoderma, Ampelomyces, Candida* and *Coniothyrium.*

Fungus based BCAs have gained wide acceptance only next to bacteria, primarily because of their broader spectrum of activity in terms of disease control. Fungi of the genus *Trichoderma* gained prominent place in controlling soil

borne phytopathogens. *Trichoderma* is a secondary opportunistic invader, fast growing fungus, strong spore producer, source of cell wall degrading enzymes and an important antibiotic producer. Application of *Trichoderma* as BCA can bring substantial changes in plant metabolism to promote plant growth, increase nutrient availability, improve crop production and enhance disease resistance (Harman *et al*., 2004). *Trichoderma* utilizes mycoparasitism to mitigate phytopathogenesis (Harman and Kubicek, 1998) and antibiosis (Sivasithamparam and Ghisalberti, 1998). The efficacy of *Trichoderma* as BCA is believed to involve antibiotic production and secretion of hydrolytic enzymes (Vinale et al, 2008). Recent reports suggest that *Trichoderma* isolates can stimulate production of biochemical compounds of phenolic nature associated with the host defense. However, more knowledge about these biochemical responses induced by *Trichoderma* based BCAs is need of the hour to improve efficient formulation and promote their wide application in agriculture.

Therefore current study was aimed at delineating biochemical mechanisms in support of systemic resistance caused by *T. viride* in legume, *Vigna mungo* infested by *Fusarium oxysporum* & *Alternaria alternata*. Biochemical responses in terms of defense enzymes such as polyphenol oxidase (PPO), peroxidase (PO) that catalyzes the formation of lignin, and phenylalanine ammonia-lyase (PAL) that is involved in phytoalexins and phenolic synthesis was assessed in legume *Vigna mungo.*

MATERIALS AND METHODS

Plant Material

Seeds of *Vigna mungo* var LBG 623 were washed thoroughly with sterile distilled water and surface sterilized with 0.1% (w/v) $HgCl_2$ for 10min and used in experiments.

Fungal Suspension

Biocontrol agent, *Trichoderma viride* (NCIM 1053), and two virulent cultures of *Fusarium oxysporum* (NCIM 1072) and *Alternaria alternata* (NCIM 718) causing wilt and blight in legumes were obtained from National Collection of Industrial Microorganisms (NCIM), NCL, Pune, India. These fungi were cultured at 28±2°C in potato dextrose agar (PDA) medium. The cultures were maintained on PDA medium at 4^0C till their use in further experiments. Inoculum of *T. viride*, *F. oxysporum* and *A. alternata* were prepared by blending 2-week old PDA grown cultures with sterile distilled water by straining the suspension through cheese cloth.

Biocontrol Assay

Legume seeds (LBG 623) were surface sterilized with 0.2% mercuric chloride solution for 5 min. They were washed thoroughly with sterile distilled water to remove traces of $HgCl_2$. Pots (15×10cm) were filled with optimum weight of sterilized loamy clay soil. Seeds of *Vigna mungo* (LBG 623) pre-treated with *Trichoderma* spore suspension (10^6 conidia/ml) for 1h were sown (one seed per polythene bag) and the experiments were conducted in five replicates. The seeds without pretreatment with *Trichoderma* were labeled as 'C'; seed treated with *Trichoderma* were labeled as 'T'. In a separate set of experiments, *F. oxysporum* (10^6conidia/ml) and *A. alternata* (10^6 conidia/ml) spores were used to treat one week old plants and observations were recorded daily. Control and treated plants (C & T) exposed to *Fusarium* were labeled as 'C+PI', 'T+PI', and *Alternaria* were labeled as 'C+PII', 'T+PII'. Pots were maintained in a greenhouse at 28±2^0C. All the pots were irrigated with water at one-day intervals. Leaves of these plants were used in estimating various biochemical parameters as described below.

BIOCHEMICAL ESTIMATION

Sample Preparation

0.5g leaf was homogenized with 2ml of 0.1M sodium phosphate buffer (pH 6.5) at 4°C. The homogenate was centrifuged at 10,000 rpm for 2min and the supernatant is used as an enzyme source for estimating plant defense enzymes-Peroxidase (PO), Polyphenol oxidase (PPO), Phenylalanine ammonia lyase (PAL) and total phenols (TP).

DEFENSE ENZYMES

Peroxidase (PO)

Peroxidase activity was assayed by measuring the oxidation of guaiacol in the presence of hydrogen peroxide into water at 470nm as described by Hammer Schmidt *et al*. (1982). Activity was expressed as the increase in absorbance at 470nm in $min^{-1}mg^{-1}$ of protein.

Polyphenol Oxidase (PPO)

Polyphenol oxidase (PPO) activity was determined as per the procedure given by Mayer *et al.* (1965). Oxidation of the substrate catechol to yellow color benzoquinone was measured at 495nm. The activity was expressed as change in absorbance at 495nm in $min^{-1}mg^{-1}$ of protein.

Phenylalanine Ammonia Lyase (PAL)

Phenylalanine ammonia lyase (PAL) activity was carried out as per the method described by Ross and Senderoff (1992). The enzyme activity was expressed as μ moles of cinnamic acid in min^{-1} mg^{-1} of protein.

Total Phenols

Total phenolic content of leaf extracts was determined using to the Folin-Ciocalteu method of Singleton *et al*. (1999). Reaction of phenols with phosphomolybdic acid in the presence of Folin-Ciocalteau reagent in alkaline medium was measured at 695nm.

Statistical Analysis

A minimum of three plants were evaluated for each replicate. The results were calculated taking control as 100% to find increase or decrease in activities of enzymes. The data was analyzed by one-way analysis of variance (ANOVA). The treatment means were compared by *F*-values with level of significance $P<0.05$.

RESULTS

To evaluate biochemical basis of systemic resistance induced by *Trichoderma* against pathogens *Alternaria* and *Fusarium,* the possible role of defense enzymes, viz., PO, PPO, PAL and TP were assessed in *Vigna mungo*. The results revealed that *T.viride* induced higher levels of defense enzymes in black gram treated with *T. viride.* Plants infested with pathogen alone showed decreased defense enzymes activity. The results are presented below

Peroxidase

Peroxidase activity was found to be increased in plants infested with *Fusarium* (28%) *and Alternaria* (27%). There was no significant difference in the activity of peroxidase between *T. viride* pretreated plants and their corresponding controls infested with *Fusarium* (27%) *and Alternaria* (26%).

The decrease in peroxidase activity (5%) was found in plants treated with only *T. viride.* A significant difference in peroxidase activity was not found in plants infested with *Fusarium* and *Alternaria* [Figure 1 (A)].

Polyphenol Oxidase

Polyphenol oxidase activity was high in all plants pretreated with *T. viride* alone (57%) and *T. viride* pretreated pathogen infested with *Fusarium* (66%) and *Alternaria* (61%) when compared to their corresponding controls [Figure 1 (B)].

Total Phenols

Total phenols are accumulated in high concentrations in plants pretreated with *T. viride* alone (150%) and *T. viride* pretreated pathogen infested with *Fusarium* (113%) and *Alternaria* (81%) when compared to their corresponding controls. All plants pretreated with *Trichoderma* alone showed more accumulation of total phenol than controls and plants infested with pathogens [Figure 1 (C)].

Phenylalanine Ammonia Lyase

Phenylalanine ammonia lyase activity was found to be high in plants pretreated with *T. viride* alone (97%) and *T. viride* pretreated pathogen infested with *Fusarium* (101%) and *Alternaria* (115%) when compared to their corresponding controls. All plants pretreated with *Trichoderma* infested with *Alternaria* showed highest activity of PAL [Figure 1 (D)].

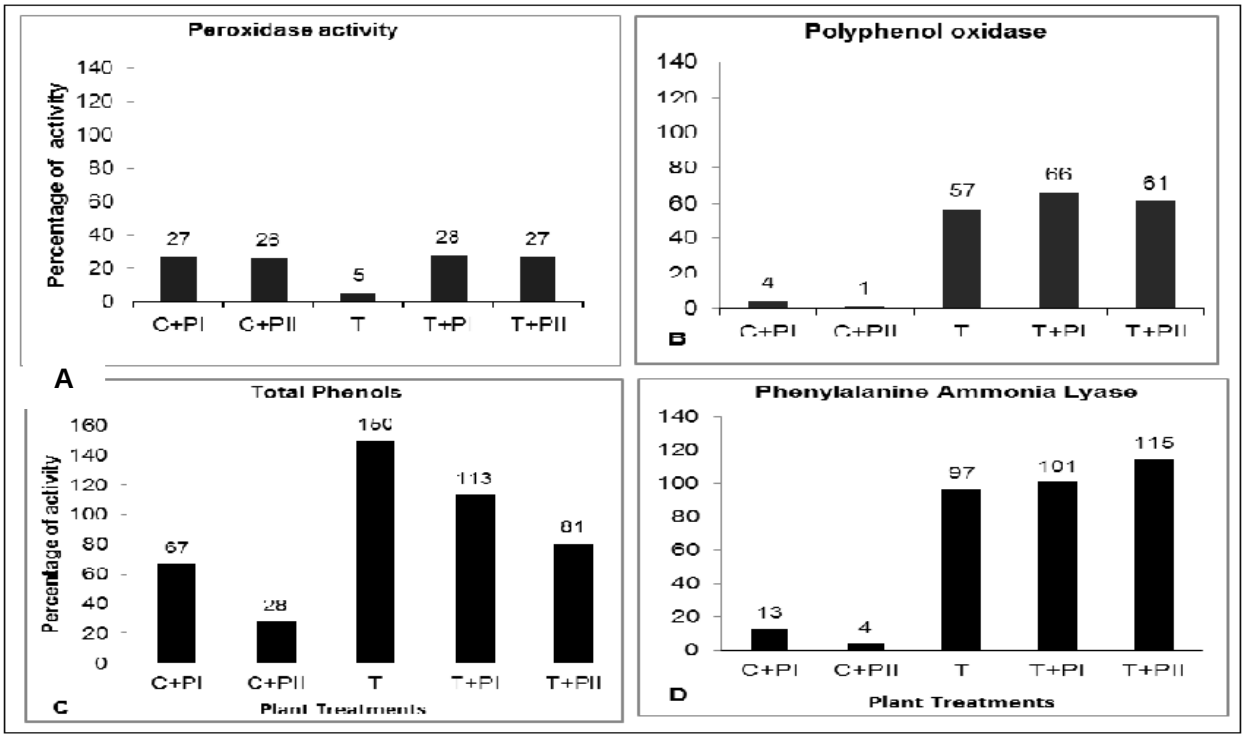

Figure 1 Role of *T.viride* in Inducing Defense Enzymes and Total Phenols in *Vigna mungo* Infested with *Fusarium oxysporum* and *Alternaria alternata*. (A) Peroxidase Activity (B) Polyphenol Oxidase Activity (C) Total Phenols and (D) Phenylalanine Ammonia Lyase Activity (Plant Treatments on y Coordinate: C+PI= Control Plant Infested with *Fusarium*, C+PII= Control Plant Infested with *Alternaria*, T= Plants Treated with *T.viride*, T+PI= Plants Treated with *T.viride* and Infested with *Fusarium*, T+PII= Plants Treated with *T.viride* and Infested with *Alternaria*). All Results are Significant at P≤0.05

DISCUSSIONS

Plants are equipped with well-organized and coordinated defense network of biochemical reactions, which are inducible in response to appropriate stimuli/ signals (Jones and Dangl, 2006). Inducing innate biochemical defense mechanisms in plants by treating them with BCAs such as fungi are thought to be novel plant protection strategies in agriculture (Van Loon *et al.*, 2008; Kashyap and Dhiman, 2009). Fungi belonging to *Trichoderma* are the well-known BCAs which reduce disease incidence in plants infested by fungal phytopathogens (Howell, 2003; Freeman *et al*., 2004; Harman *et al.*, 2004; Ashrafizadeh *et al*., 2005; Dubey *et al*., 2007; Vinale et al., 2008; Jyotsana *et al*., 2008; Shoresh et al., 2010). There are several mechanisms involved in *Trichoderma* antagonism, like antibiosis- whereby the antagonistic fungus produces antibiotics, competes for nutrients and mycoparasitism, whereas *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as chitinases, β-1, 3 glucanases and proteases (Almeida *et al*., 2007; Radjacommare *et al*., 2010). Recent reports suggested that *Trichoderma* isolates might stimulate the production of biochemical compounds of phenolic nature associated with the host defense (Kavino *et al*., 2008; Radjacommare *et al*., 2010). The activity of defense-related enzymes can substantiate the host resistance against plant pathogens. Increase in activity and accumulation of these enzymes also depends on the plant genotype, physiological conditions and the type of pathogen. Synthesis of defense chemicals against pathogens is triggered by a series of morphological and biochemical changes initiated by specific strains of fungi (Siva Prasad et al., 2013). Therefore, Treatment of legume seeds of *V. mungo* with *T. viride* spores induced PO, PPO & PAL and total phenolic contents in plants infested with *Fusarium* and *Alternaria*.

PO is a useful marker of plant development, physiology, infection and stress (Welinder, 1992). Interestingly, increased peroxidase activity in leaf extracts of black gram infested with pathogens might be due to its utilization in cell wall lignification. Increased activity of PO contributes to disease resistance in infected plants (Vidhyasekaran, 1997). Therefore, induction of PO by *T. viride* in black gram during wilt and blight can be considered as one of the marker of disease resistance during fungal phytopathogenesis in legumes. Our results corroborate with earlier studies (Van Wees *et al.*, 2008; Nawar and Kuti, 2003; Hassan et al, 2007) which delineated the induction of PO in plants infected by pathogens or insects, resulting in faster and stronger resistance against them.

Current study showed induction of PPO by *T. viride* in legume leaves during wilt and blight. Increased PPO activity contributed to disease resistance due to its property to oxidize phenolic compounds to more toxic quinines which invade pathogenic micro-organisms (Vinale et al, 2008). It substantiated the role of PPO in disease resistance during fungal phytopathogenesis. Our results are in affirmation with the study that reported a gradual increase in polyphenol content in red pepper (Sriram et al., 2009) and Tomato (Nawrocka et al., 2011) treated with *Trichoderma*.

T. viride activated PAL enzyme activity in legume leaves. Induction of PAL might have led to increased levels of signaling molecule salicylic acid in the leaves thereby contributing to disease resistance (Harman et al, 2004). In our study, increase in the level of PAL in the treated leaves of legume is in agreement with earlier reports (Yedidia et al. 2003; Verma et al. 2007). *T. viride* treatment might have prevented the fungal invasion, and thus, the activity maintained at higher levels during the experimental period.

It was observed that resistant plants contain more phenols or produce polyphenols more rapidly than susceptible ones. A multifold increase in phenol content was observed in *T. viride* treated legumes plants along with pathogen inoculation compared with the infected control plants. Accumulation of phenols might be due to excess production of H_2O_2 in infected plants through increased respiration (Farkas and Kiraly, 1962) or due to the activation of

hexose-monophosphate shunt pathway, acetate pathway and release of bound phenols by hydrolytic enzymes (Goodman *et al*., 1967). These biochemical reactions might have mediated antimicrobial activity followed by increased esterification of phenylpropanoids of the cell wall (Mandal and Mitra 2007). Similarly, synthesis of high amount of phenols by *Trichoderma viride*, compared to controls, suggests their role in inducing resistance against wilt and blight in legumes. *Trichoderma viride* might have elicited the production of sensing molecules in legume during disease resistance against wilt and blight. In the current study, enhanced phenol content had strong influence on induction of PO and PPO and PAL content in legumes.

CONCLUSIONS

In conclusion, current study contributes to biochemical characterization of induced resistance by *Trichoderma viride* against *Fusarium* and *Alternaria* in *Vigna mungo.* Outcomes of the study are useful in understanding the role of plant defense enzymes in developing systemic disease resistance in legumes by *Trichoderma viride.* Our results demonstrate the role of *Trichoderma viride* as BCA in eliciting a series of defense responses such as accumulation of phenols, induction of (PO, PPO and PAL) enzymes involved in phenylpropanoid pathway, deposition of lignin against *Fusarium* and *Alternaria*. In this regard, it is recommended to use *Trichoderma viride* based BCA as a promising alternate to chemical fungicides to minimize the adverse impact on the environment and ensuring plant disease management.

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