EFFICACY OF TRICHODERMA VIRIDE TO INDUCE DISEASE RESISTANCE AND ANTIOXIDANT RESPONSES IN LEGUME VIGNA MUNGO INFESTED BY FUSARIUM OXYSPORUM AND ALTERNARIA ALTERNATA

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ABSTRACT

This study was designed to evaluate the potential of Trichoderma viride spore suspension as a biocontrol agent against Fusarium oxysporum and Alternaria alternata in legume, black gram (Vigna mungo) under greenhouse conditions. Seeds and seven days old plants of black gram were artificially infested with F. oxysporum and A. alternata. Concurrently, they were treated with Trichoderma viride spores. It was observed that the seed germination (%), growth (shoot and root), vigour index and disease resistance in plant samples treated with Trichoderma increased than in controls. Experiments were also conducted to understand the role of T. viride in inducing the antioxidant system of black gram plants so as to create systemic resistance against F. oxysporum and A. alternata. Lipid peroxidation levels were found to be decreased in Trichoderma treated samples. Our findings concluded that triggering of antioxidant systems played an important role in mitigating pathogen-induced oxidative stress during systemic resistance by T. viride in black gram. This study holds important inferences for developing effective strategies in promoting Trichoderma spp. based biocontrol agents in mitigating the fungal infections in legume plants.

KEYWORDS: Trichoderma viride, Vigna mungo, Fusarium oxysporum, Alternaria alternata, Biocontrol, Antioxidant enzymes, Lipid peroxidation

INTRODUCTION

Black gram (Vigna mungo) is one of the important food legumes for human consumption across the globe because of its nutritional and medical properties. Black gram is also known to improve fertility of the soil where it is grown. Despite its crucial role in tropical agriculture, the productivity levels are of lower order in comparison to their cereal counterparts. The productivity of black gram gets severely hampered by pests and diseases caused by bacteria, fungi and viruses. The percent of yield loss of legume crops due to biotic factors is in the tune of 40-60%, out of which the fungal pathogens alone account for 10-25%. Fusarium oxysporum and Alternaria alternata are soil borne fungal pathogens which cause wilt and leaf blight disease in legumes including Vigna mungo.

Several methods for controlling such fungal borne diseases have been evaluated by studying the use of resistant varieties of crop (Brisa et al., 2007), chemical control of pathogens, novel agricultural practices (Punja et al., 1986), by applying various plant volatile compounds (El-Mougy et al., 2007), plant extracts (Kumar and Tripathi, 1991) and biological control, especially with species of Trichoderma (Ristaino et al., 1994). Many researchers demonstrated the
potential of \textit{Trichoderma spp.} in controlling damping-off and leaf blight disease of leguminous crops caused by \textit{Fusarium} and \textit{Alternaria spp.} (Dubey et al., 2007).

Wide spread application of various methods to control pathogens in promoting agricultural productivity and quality has raised concerns about techno-economy and environmental pollution. For instance, properties and persistence behavior of formulations of biocontrol agents used in pathogen control can enhance the development of resistance among the plant pathogens. Development of biocontrol agents based on plant extracts need to address issues such as easy preparation & application, stability, shelf life, viable propagules and low cost. Therefore, it is essential to promote an alternative strategy such as use of biological control agents (BCA) to minimize the adverse effects of chemical agents on environment with feasibility of techno-economy. BCA are microbial agents which can provide systemic resistance to plants against pathogens by producing a wide range of antibiotic substances and parasitizing other fungi. They can stimulate plant growth by suppressing plant disease. Mycorrhizal fungi, rhizobium bacteria, plant growth promoting rhizobacteria (PGPR) and fungi such as \textit{Trichoderma spp.} are some of the microbial agents employed in formulating various BCAs to control pathogenesis in various crops.

\textit{Trichoderma spp.} is one of the important BCAs which have revolutionized the field of biological control of soil borne plant pathogens (Radjaconnare et al. 2010). They bring about induced systemic resistance fortifying the physical and mechanical strength of cell wall and changing physiological and biochemical reactions of the host leading to synthesis of defense chemicals against pathogens (John Christopher, 2010). \textit{T. viride} is one of the extensively used BCAs in agriculture because of its broader spectrum activity in terms of disease control and yield. However, the biochemical basis of induction of resistance in plants by \textit{T. viride} has been poorly studied compared with the responses that are induced by rhizobacteria. Till date, \textit{Trichoderma} research has been focused on factors that are associated with direct effects on other fungi, especially mycoparasitism and antibiotics (Harman, 2004). Isolates of the fungi, \textit{Trichoderma} are widely used biocontrol agents and are generally applied as conidial spore preparations (Papavizas, 1985). Studies emphasizing the application of \textit{Trichoderma spp.} spores on seeds and foliage to reduce incidence of the diseases are scarce. Systemic resistance against pathogen infection in plants is due to the induction of various antioxidant defense systems which can reduce disease incidence is not well documented with respect to the direct application of spores of \textit{Trichoderma spp.} Studies divulging the role of antioxidant systems during the evaluation of efficacy of \textit{Trichoderma} spores against \textit{Fusarium} and \textit{Alternaria} in legumes are limited.

Therefore, the study is designed to elucidate the efficacy of \textit{Trichoderma} spore suspension in inducing systemic resistance by \textit{T. viride} in legumes against \textit{Fusarium oxysporum} and \textit{Alternaria alternata}. Change in parameters such as decrease in disease incidence, vigour index and growth (shoot and root) were evaluated in black gram infested with \textit{F. oxysporum} and \textit{A. alternata}. The role of antioxidant defensive enzymes during systemic resistance is discussed. The levels of lipid peroxidation are evaluated to understand the role of antioxidant enzymes in mitigating pathogen mediated oxidative stress in black gram by spore suspension of \textit{Trichoderma}. Outcomes of the current study will provide insights to codify role of antioxidant systems in legumes during systemic resistance induced by \textit{T. viride} against \textit{F. oxysporum} and \textit{A. alternata}.

\section*{MATERIALS AND METHODS}

\subsection*{Plant Material}

Seeds of black gram (\textit{Vigna mungo} var LBG 623) were washed thoroughly with sterile distilled water and surface sterilized with 0.1\% (w/v) HgCl\textsubscript{2} for 10min and used in experiments.
Efficacy of *Trichoderma viride* to Induce Disease Resistance and Antioxidant Responses in Legume *Vigna mungo* Infested by *Fusarium oxysporum* and *Alternaria alternata*

**Fungal Suspension**

Biocontrol agent, *Trichoderma viride* (NCIM 1053), and 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). The cultures were maintained on Potato Dextrose Agar (PDA) at 4°C till use. Inoculum of *T. viride*, *F. oxysporum* and *A. alternata* were prepared by blending 2-week old PDA grown cultures with sterile distilled water sieving the suspension through cheese cloth.

**Experiments under Greenhouse Conditions**

Experiments were designed under greenhouse conditions using polythene bags containing reasonable weight of sterilized loamy clay soil. Fifty seeds of black gram were pre-treated with *Trichoderma* spore suspension (10^6 conidia/ml) for 60min. And one seed was sown per polythene bag. The experiment was conducted in five replicates. The control seeds were sown without pre-treatment with *Trichoderma*. One week old plants of black gram raised in pots were artificially infested with spores of *F. oxysporum* (10^6 conidia/ml) and *A. alternata* (10^6 conidia/ml) and day to day observations were recorded. Control plants without pretreatment with *Trichoderma* were labeled as ‘C’, plants treated with *Trichoderma* were labeled as ‘T’, plants (C & T) were exposed to *Fusarium* were labeled as ‘C+PI’, ‘T+PI’, and *Alternaria* were labeled as ‘C+PII’, ‘T+PII’. Twenty five pots constituted one replicate and there were three replicates per treatment.

**Determination of Disease Incidence and Vigour Index**

The efficacy of seeds pretreated with *T. viride* and plants exposed to pathogens were evaluated for the percent of germination, vigour index and disease incidence as recorded on 7th and 14th days of exposure to pathogens using following formulae.

Seed germination (%) = (Number of seeds germinated/Total number of seeds sown) x100  
Vigour index = shoot length + root length x germination percentage.  
Disease incidence (%) = (Number of diseased plants/ Total number of plants) x100

**Biochemical Estimations**

**Sample Preparation**

1g plant sample was ground in 10ml ice-cold 50mM potassium phosphate buffer (pH 7.8) in pre-chilled pestle and mortar. The homogenate was subjected for centrifugation at 10,000rpm for 10min at 4°C in a cooling centrifuge. Within 12h of extraction, the supernatant was used as enzyme source.

**Total Proteins**

The amount of total proteins was evaluated by Lowry’s (1951) method.

**Antioxidant Enzymes**

**Superoxide Dismutase (SOD)**

Superoxide dismutase (SOD) activity was carried out as per the method described by Beauchamp and Fridovich (1997). The enzyme activity was expressed as enzyme units/mg of protein.

**Catalase (CAT)**

The Catalase activity was assayed by titrimetric method described by Radhakrishna and Sarma (1963). Catalase
activity was expressed as ml of 0.1N Potassium permanganate equivalent of Hydrogen peroxide decomposed/min/mg of protein.

**Ascorbic Acid Oxidase (AOX)**

The rate of oxygen consumption during ascorbic acid oxidation is proportional to the amount of enzyme present. Ascorbic acid has an absorption maximum at 265nm. 3 ml of substrate solution of ascorbic acid was added to each sample and reference cuvettes of a spectrophotometer. Cuvette containing 0.1ml of enzyme extract was used as reference cuvette. The decrease in absorption peak of the substrate due to the oxidation by ascorbic acid oxidase was measured at 265nm in 30sec intervals for 5min in spectrophotometer.

**Lipid Peroxidation**

The level of the lipid peroxidation was measured in terms of Malondialdehyde (MDA) contents as given by Heath and Packer (1968).

**STATISTICAL ANALYSIS**

Sampling of the plants was done in three independent experiments with three replicates in time and space. A minimum of three plants were evaluated for each replicate. The results were calculated taking control as 100% to find increase or decrease of various activities. The data were analyzed by one-way analysis of variance (ANOVA). The treatment means were compared by F-values, with level of significance $P<0.005$.

**RESULTS**

**Disease Incidence and Vigour index**

Germination percentage of seeds, shoot length, root length, vigour index and disease incidence were calculated according to the formulae given in section 2.4 and are tabulated in Table 1. Germination was more in plants pretreated with *Trichoderma* when compared to plants without treatment and the increase was found to be 11-16%.

**Table 1: Effect of *T. viride* on Seed Germination, Vigour Index and Disease Incidence in *Vigna mungo* Infested with *Fusarium* and *Alternaria***

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Seeds/Plants without <em>Trichoderma</em> Treatment</th>
<th>Seeds/Plants with <em>Trichoderma</em> Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (C)</td>
<td>Fusarium</td>
</tr>
<tr>
<td></td>
<td><em>Fusarium</em></td>
<td>Alternaria</td>
</tr>
<tr>
<td>Germination (%)</td>
<td>74.6±1.45</td>
<td>71.6±2.92</td>
</tr>
<tr>
<td></td>
<td>65.33±2.18</td>
<td>83±0.76</td>
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<tr>
<td></td>
<td>79.3±2.6</td>
<td>76±1.52</td>
</tr>
<tr>
<td>Shoot length (cm)</td>
<td>4.1±0.04</td>
<td>4.1±0.5</td>
</tr>
<tr>
<td></td>
<td>3.13±0.04</td>
<td>6.03±0.04</td>
</tr>
<tr>
<td></td>
<td>5.1±0.08</td>
<td>4.1±0.04</td>
</tr>
<tr>
<td>Root length (cm)</td>
<td>2.56±0.06</td>
<td>1.4±0.05</td>
</tr>
<tr>
<td></td>
<td>2.03±0.04</td>
<td>3.53±0.08</td>
</tr>
<tr>
<td></td>
<td>2.5±0.02</td>
<td>2.2±0.02</td>
</tr>
<tr>
<td>Vigour Index</td>
<td>496.8±3.32</td>
<td>436.76±2.36</td>
</tr>
<tr>
<td></td>
<td>410.713±6.38</td>
<td>793.66±7.38</td>
</tr>
<tr>
<td></td>
<td>602.91±6.80</td>
<td>463.6±7.38</td>
</tr>
<tr>
<td>Disease Incidence (%)</td>
<td>18.41±0.11</td>
<td>17.63±0.16</td>
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<tr>
<td></td>
<td>21.76±0.19</td>
<td>8.92±0.01</td>
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<tr>
<td></td>
<td>16.05±0.06</td>
<td>17.63±0.16</td>
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<tr>
<td>7th day</td>
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<tr>
<td>14th day</td>
<td>14.5±0.14</td>
<td>11.21±0.08</td>
</tr>
<tr>
<td></td>
<td>10±0.05</td>
<td>6±0.02</td>
</tr>
<tr>
<td></td>
<td>10 ± 0.05</td>
<td>8.48±0.07</td>
</tr>
</tbody>
</table>

*C= Control, T= Treated*

Vigour index was high in all plants pretreated with *Trichoderma* when compared to controls. *Fusarium* infested plants pretreated with *Trichoderma* showed more increase in vigour index when compared to plants infested with *Alternaria* treated with *Trichoderma*. Disease incidence was recorded on 7th day and 14th day after artificial infestation by pathogens. It was found that disease incidence was decreased in plants pretreated with *Trichoderma* when compared to controls. Plants pretreated with *Trichoderma* and infested with *Alternaria* showed more decrease in disease incidence when compared to *Fusarium* infested plants pretreated with *Trichoderma*. 
Antioxidant Enzymes

Our results indicated that the activity of antioxidant enzymes played a crucial role in *Trichoderma* mediated systemic resistance. Change in the levels of the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and Ascorbic Acid Oxidase (AOX) are shown in Figure 1 and Figure 2. The data from experiments were analyzed by one way analysis of variance (ANOVA), the treatments were compared by *F*-values, with levels of significance *P*<0.005.

**Superoxide Dismutase (SOD)**

Superoxide dismutase activity was high in all pretreated plants when compared to controls. Plants pretreated with *Trichoderma* exposed to pathogen I and pathogen II showed more increase in superoxide dismutase activity when compared to unexposed plants treated with *Fusarium* and *Alternaria* in all treatments [Figure 1 (A)].

**Catalase (CAT)**

Catalase activity decreased in all pretreated plants when compared to controls. Plants pretreated with *Trichoderma* exposed to pathogens showed more decrease in Catalase activity when compared to unexposed plants. Decreased percentage of catalase was observed in *Trichoderma* pretreated plants [Figure 1 (B)].

![Graphs showing Superoxide Dismutase Activity and Catalase Activity](image)

(C+PI= Control treated with *Fusarium*, C+PII= Control treated with *Alternaria*, T= *Trichoderma* treated, T+PI= *Trichoderma* treated and infested with *Fusarium*, T+PII= *Trichoderma* treated and infested with *Alternaria*)

**Ascorbic Acid Oxidase (AOX)**

It was observed that control plants when exposed to pathogens showed less AOX activity and pretreated plants showed increase in AOX activity but not prominent. Plants pretreated with *Trichoderma* exposed to *Fusarium* and *Alternaria* showed more increase in AOX activity when compared to their respective controls without *Trichoderma* treatment [Figure 2 (A)].

**Lipid Peroxidation**

Initially, lipid peroxidation activity decreased in all pretreated plants when compared to controls. But was much higher in untreated plants and control plants exposed to pathogens. Lipid peroxidation decreased in all pretreated plants but the percentage of activity was less during pathogen attack [Figure 2 (B)].
DISCUSSION

Plants have a very well organized and coordinated defense network, which is inducible in response to appropriate stimuli or signals during pathogenesis (Jones and dangl, 2006). Inducing the plant’s own defense mechanisms by prior application of BCAs such as fungi and bacteria are thought to be novel plant protection strategy (Van Loon et al., 2008). Fungi belonging to *Trichoderma* genera are well-known BCAs which induce systemic resistance in plants during pathogen infestation. They can be isolated from soil, decaying wood and other forms of plant organic matter (Howell, 2003) and they are antagonistic towards a number of plant pathogenic fungi in reducing the incidence of disease (Dubey et al., 2007; Freeman et al., 2004; Vinale et al., 2008). There is little information available on application of *Trichoderma* spores as BCAs in enhancing germination, vigour index, disease incidence and defense mechanisms against pathogen infestation by modulating antioxidant metabolites in the plants. Therefore, current study was designed to assess efficacy of spores of *Trichoderma viride* on these parameters in *Vigna mungo* infested with *F. oxysporum* and *A. alternata*.

Our results revealed that *Trichoderma viride* can act as the best antagonist against *A. alternata* than *F. oxysporum* in the legume plant, black gram. Our study also showed that seeds coated with *T. viride* spores were effective in reducing disease incidence and severity of *Fusarium* wilt and *Alternaria* blight in legumes under greenhouse conditions. *T. viride* alone reduced disease incidence by 59 & 62% by 7th day and 14th days respectively, whereas Sivan *et al.* (1986) showed 80% decrease in disease incidence in legumes seeds coated with *Trichoderma* after 75 days in various hydroponic media. Therefore, it was concluded that *Trichoderma* spores used in the current study significantly reduced disease incidence against *A. alternata* and *F. oxysporum*. Results also indicated that *T. viride* could enhance the seed germination, plant growth and vigour index in black gram under greenhouse conditions. These results are in agreement with the reports on *T. viride* mediated protection of germinating bean seedlings against *Fusarium spp.* (Abou-Zeid et al. 2003). Our results demonstrated that *Trichoderma* treatment could reduce disease incidence under greenhouse conditions when compared to the experiments conducted with soil treatment. Similar kind of result was reported by Pieta and Pastucha (2004). Our findings are also in fine tune with reports of Malik *et al.* (2005) in beans treated with *Trichoderma spp.*

*Trichoderma spp.* can induce systemic resistance in plants by increasing the plant defense response to diverse pathogen attack (Harman *et al.*, 2004). Among various plant defenses, antioxidant system plays crucial role in mitigating...
the pathogen mediated oxidative stress. Therefore, in our study various antioxidant enzymes such as superoxide dismutase, catalase, ascorbic acid oxidase were assessed in pathogen infested plants treated with *Trichoderma*. The study revealed that *Trichoderma viride* significantly stimulated the activities of antioxidant enzymes in *Trichoderma* treated plants when compared to their corresponding controls. These findings are in agreement with earlier studies on the activity of antioxidant enzymes during biological control by fungi in legumes (Wang et al., 2004). The induction of antioxidant enzymes by *Trichoderma* against *Fusarium* and *Alternaria* might be due to the increase in the levels of reactive oxygen species (ROS) such as hydroxyl and peroxide ions during pathogen attack. Suppression of CAT activity and induction of SOD activity might have led to the induction of the accumulation of a higher level of $H_2O_2$. Accumulation of ROS might have resulted in alleviating the levels of antioxidant enzymes. These findings were further substantiated by measuring lipid peroxidation levels during infestation of legume by pathogens. The results were expressed in terms of MDA which were comparatively lower than controls implying that *Trichoderma* had potent efficacy in alleviating pathogen-induced oxidative damages in legumes. It was found that lipid peroxidation significantly decreased in plants pretreated with *Trichoderma* as compared to controls.

**CONCLUSIONS**

Outcomes of our study indicate that spore suspensions of *T. viride* have the potential to suppress both wilt and blight diseases in black gram. Spore suspensions of *Trichoderma* can act as potential biological control agent against *Alternaria* than *Fusarium*. It can decrease not only disease incidence but can also enhance seed germination, growth and vigour index of legume plants infested with *Alternaria* and *Fusarium*. The biological control activity of spore suspension of *Trichoderma* might have induced systemic disease resistance, probably by the activation of the induced systemic resistance pathway in plants. Black gram plants pretreated with spore suspensions of *Trichoderma viride* showed the alleviated levels of antioxidant defense enzymes. The decrease in lipid peroxidation during the infestation by *F. oxysporum* and *A. alternata* revealed the orchestration of these mechanisms playing an important role in mitigating pathogen-induced oxidative stress in legumes. Therefore, it can be concluded that the amelioration of oxidative damage during plant–pathogen interactions can be induced by spore suspensions of *Trichoderma* in legumes. In conclusion, application of spore suspension of *T. viride* to seeds or fresh-faced plants as described herein can be recommended as BCAs for controlling wilt and blight in legumes. Results of our study hold important inferences for developing effective strategies in formulating *Trichoderna spp.* based biocontrol agents in mitigating the infestations against fungal borne pathogens.

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