**Impact of seed treatment with biocontrol agents on soybean (*Glycine max* L.) enzyme activity and phenol content under greenhouse and field conditions**

**Running Title**: **Seed treatment with biocontrol agents**

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**Novelty:** Fungal biocontrol agents increase the plant resistance against*M. phaseolina* by improving enzymatic and phenolic content of soybean plants

**Abstract**

Fungi in the genus *Trichoderma* are widely used as biological control agents because they can suppress plant pathogens and activate plant defense systems. The present study was conducted to determine the impact of different combinations of *Trichoderma* spp. on activity of pathogen’s defense enzymes and phenol content in soybean (*Glycine max* L.) plants. Soybean seeds were inoculated with *Trichoderma harzianum*, *T*. *viride* alone or their combination i.e *T*. *viride* + *T*. *harzianum*, then sown in pots under greenhouse and field conditions. Host enzyme activity and phenol levels were measured at 14, 28 and 42 days after sowing (DAS) in both field and greenhouse experiments. Seed treatments with *T. harzianum*, *T*. *viride* or their combination increased the peroxidase, polyphenol oxidase, β-1,3-glucanase activity and total phenol content in soybean leaves compared to an untreated control treatment. Peroxidase and β-1, 3-glucanase peaked at 14 DAS and decreased thereafter in all treatments under greenhouse and field conditions. All treatments showed highest level of total phenols and polyphenol-oxidase at 28 DAS under both greenhouse and field conditions. At 14 DAS in both trials, the treatment combining *T*. *viride* + *T*. *harzianum* resulted in the highest level of peroxidase and β-1,3-glucanase activity. This combination also resulted in the highest levels of total phenols and polyphenol oxidase content at 28 DAS. Our findings demonstrate that application of *Trichoderma* species as seed treatments have potential to trigger key mechanisms of systemically acquired resistance in soybean, and thereby enhance efficacy of disease management tactics.

**Keywords:** *Trichoderma*, Soybean, Seed inoculation, Peroxidase, Phenolics

**1. Introduction**

Soybean (*Glycine max* L.) is an important leguminous crop and a major source of vegetable oil and proteins worldwide (PARC, 2018). Soybeans are prone to many economically important fungal diseases of which charcoal rot caused by *Macrophomina phaseolina* is the major constraint causing colossal losses every year. Different management methods such as physical, chemical (fungicides), regulatory, cultural and biological have been used to eliminate the phytopathogenic fungus. But these methods are helpful only when used well in advance as precautionary measures (Ganeshamoorthi et al. 2010). In addition, conventional use of chemical fungicides for *M. phaseolina* infections may be less helpful due to soil-borne nature of disease, and further may interrupt the balance of beneficial microbes in soils (Anis et al. 2010). Furthermore, the uninterrupted application of chemical pesticides and fungicides may develop resistance to strains and can cause harmful environmental risks and health hazards (Afouda et al. 2012). Moreover, fungicides are too expensive and economically not affordable by low-income farmers (Aboshosha et al. 2007). Efforts to manage the disease in soybean through crop rotation has also been suggested as means of control (Mengistu et al. 2007) but it may be inadequate for control of soil borne fungal diseases with long-surviving propagules (such as charcoal rot). Therefore, alternative methods of disease control are needed.

During the past few decades, different probable biocontrol agents have been identified, characterized and commercialized. Biocontrol organisms have gained more attention as component of integrated disease management programme (Shali et al. 2010; Ghasemi et al. 2010) It is an effective way to enhance resistance in plants against pathogens, and this technique may play a significant role in sustainability of agricultural system. Biocontrol organisms are helpful against seed and soil borne fungal diseases of several crops (Kubicek et al. 2001). The fungus *Trichoderma harzianum* has been documented to suppress many soil borne fungal pathogens (Ziedan et al. 2005; Moataza et al. 2005). Alyet al. (2007) enlisted different antagonists of *Trichoderma* sp. against *M. phaseolina*. Sreedevi et al. (2011) depicted that *T. viride* and *T. harzianum* isolates had antifungal activity against *M. phaseolina*. *Trichoderma* spp. act as biocontrol organisms and also stimulate the plant resistance and growth resulting in overall improvement in yield. The biocontrol activity related to antibiotics and mycoparasitism also improves defense response or systemic resistance in plants (Naher et al. 2014). The germination percentage of melon was 96.7% when seeds were treated with commercial *T. harzianum* + *M. phaseolina* compared to *M. Phaseolina* (46.7%) and showed excellent results against charcoal stem rot of water melon (Etebarian 2006). The antagonistic characteristics of the biocontrol species depend on multiple mechanisms that are involved in activation of specific properties.

The most important mechanism of *Trichoderma* spp. is the induction of plant defense response to specific disease (Harman, 2006). Other than chemical and physical obstructions, plant have unpredictable immune systems. The system is able to identify motifs that contain common structural features of all microbes but not present in their host plants. The defense response of plant is rapid and transitory. Early response entails ion influx across the plasma membranes, nitric oxide, defense-related genes, generation of ROS (reactive oxygen species), different phytohormones, proteins synthesis and also, but later, the callose deposition and production of antimicrobial chemicals such as phenolics. Different biocontrol species may cause molecular and cellular transformations in plants that enhance resistance to biotic and abiotic stress (Brotman et al. 2013; Kumar 2013). The activity of defense-related enzymes such as phenylalanine ammonia lyase, polyphenol oxidase and peroxidase were documented to be progressively enhanced in plants of green gram when inoculated with sole *T. viride* or combined with *Pseudomonas fluorescens* against *M. phaseolina* (Thilagavathi et al. 2007). Tomato plants treated with *T. arundinaceum* showed early expression of defense-related genes against *Rhizoctonia* *solani* and *Botrytis cinerea* (Malmierca et al. 2012).

Although there are reports on role of *Trichoderma* spp. which act as biological control agent and induce defense related enzymes in plants. However, there is little information available on combined effect of *T. harzianum* and *T. viride* to induce defense related enzymes in soybean plants. Therefore, the main objective of the present investigation was to determine the suitable combination of *Trichoderma* spp. in improving the enzymatic and phenolic contents of soybean under greenhouse and field conditions.

**2. Materials and methods**

**2.1 Collection of antagonistic and phytopathogenic fungal isolates**

Soybean plants infected with *M. phaseolina* were collected from soybean growing areas of Punjab province of Pakistan. These infected samples showing typical charcoal rot disease symptoms were kept in polythene bags and brought to plant pathology laboratory for isolation and further processing. Potato dextrose agar (PDA) medium was used to culture *M. phaseolina*. For this purpose, 200 g peeled and sliced potatoes, 20 g agar and 20 g dextrose (C6H12O6) were used. The potatoes were sliced, boiled in 400 ml distilled water and their extract was used after filtration with muslin cloth. Likewise, agar was boiled in distilled water (400 ml); after boiling, 20 g of melted agar and 20 g of dextrose were mixed with potato extract. After preparation, the medium was autoclaved at 121oC and 15 psi pressure for 30 minutes. Symptomatic portions of stems were chopped into 5- to 7-mm-long segments. The segments were disinfested with mercuric chloride (0.1%) and then washed with sterilized distilled water and then placed on PDA plates with the help of sterilized forceps. These PDA plates were incubated at 27±1°C for 4 days to get suitable growth of *M. phaseolina*. Characteristics of *M. phaseolina* were identified on the basis of formation of sclerotia and morphology of colony by following guidelines of Ashby (1927), Goidanich, (1947), Mayek-Pérez *et al*. (2002), Beas-Fernandez *et al*. (2006) and Mahdizadeh *et al*. (2011). To maintain fungal culture in a viable condition, the PDA plates containing fungus were placed in a refrigerator at 4°C until used.

For mass culturing of *M. phaseolina*, rice seeds were washed with distilled water, placed in narrow glass flasks of 250 ml, and soaked with enough water to cover the seeds. The flasks were plugged with cotton and wrapped with aluminum foil. After 12 hrs., seeds were autoclaved at 121°C for 30 min. After cooling, 5 mm mycelial discs were taken from 7 days old culture of *M. phaseolina,* which had been prepared in PDA medium. These discs of *M. phaseolina* were placed in flasks containing rice seeds and incubated at 27±1°C for 15 days in dark. From 3rd day on, flasks were stirred daily to avoid aggregate formation. After 15 days, the seeds were completely colonized showing black color and became ready for use. After incubation, the inoculum was kept at 4°C till further utilization in the experiments.

**2.2. Application of fungal antagonists under greenhouse and field conditions**

Greenhouse experiment was conducted using plastic pots (17 × 20 × 20 cm) filled with clay, sand and peat (1: 1: 1 v/v). Soil was autoclaved at 121°C for 30 min at 100 kPa (15 psi) for 2 successive days prior to use. For fungal bio-control agents, the treatments of experiment were: *T. harzianum* (alone), *T*. *viride* (alone) and *T. harzianum + T*. *viride* (combination) with different concentrations viz. 2×104, 2×106 and 2×107 spores/mL (Karthikeyan *et al*., 2015).

In field trial, fungal bio-control agents or their combination i.e. *T. harzianum*, *T*. *viride* and *T. harzianum* +*T*. *viride* at 2×104, 2×106 and 2×107 spores/mL were used. Seeds of soybean variety NARC-3 keeping seed rate 80 kg ha-1 were treated with bacterial and fungal bio-control agents using Arabic gum as sticky material. Both experiments were conducted in research area of University of Agriculture Faisalabad, Pakistan using RCBD with factorial arrangement and three replications. The net plot size for each treatment unit was 3 × 3 meter. The inoculum of the pathogen *M. phaseolina* developed on rice grains (procedure given in section 3.4 (Mass culturing of *M*. *phaseolina*) was added along the length of the lines @ 6 g/ meter along with sowing seeds. Crop was sown with the help of hands in rows in first week of February, 2017 and 2018. The distance between rows was 25 cm while between plants was 5 cm. Fertilizers like nitrogen, phosphorus and potassium were used @ 25, 60, 50 kg ha-1, respectively. Irrigation and plant protection measures were given accordingly.

**2.3. Observations**

Peroxidase activity**,** total phenol content (TPC), polyphenol-oxidase (PPO) and β-1, 3-glucanase activity were determined in leaves of NARC-3 14, 28 and 42 days after sowing (DAS) in the field studies.

**2.3.1. Peroxidase (PO) activity**

The procedure for determining the activity of peroxidase was adopted as discussed by Fehrmann and Dimond (1967). Peroxidase activity was determined using treated and non-treated (control) soybean leaves. Approximately 0.5 g fresh leaves were ground in a pre-chilled mortar with 0.1 M ice cold phosphate buffer (20 ml) having pH 7.1. Later on, it was kept for centrifugation (3000 rpm) for 15 min. The supernatant (25 ml) was used for assay. Freshly prepared pyrogallol, reagent, enzyme extract and phosphate buffer were mixed in a cuvette tube and the blend was instantly tuned to zero absorbance on a spectrophotometer. The activity of enzyme was measured as the alteration in absorbance per minute (ΔA / min) at 430 nm.

**2.3.2. Total phenol content (TPC)**

TPC was estimated by the Folin-Ciocalteu reagent method (Bray and Thorpe, 1954). Folin-Ciocalteu reagent (1 ml) and 20% sodium carbonate (2 ml) were added together with ethanol extract (1 ml) in tube and then heated for 1 min on boiling water bath. After cooling, distilled water was added and final volume was kept up to 25 ml. The absorbance of the blue color that developed was determined with Spectronic–20 colorimeters at 725 çm. Total phenol content was noted from standard curve used for catechol.

**2.3.3. Polyphenol oxidase (PPO)**

Enzyme extract (0.5 ml) and 0.1M phosphate buffer (2.3 ml) were added in cuvette and attuned to zero absorbance of a spectrophotometer (Mahadevan and Sridhar 1982). An aliquot of 0.2 ml of 0.1 M catechol was used and then reactants were rapidly mixed. The activity of enzyme was noted as variation in absorbance instantaneously after adding of 0.1M catechol (0.2 ml).

**2.3.4. β-1,3-glucanase activity**

Approximately 1 g soybean leaves taken from each treatment were homogenized separately in a mortar containing 0.1 M. sodium phosphate buffer at pH 7.1 at the rate of 2 ml/g fresh weight leaves for 1 min. This preparation was then passed through cheese cloth and resultants were centrifuged at 3000 rpm for 15 minutes at 6oC. The clear supernatant was collected and considered as a crude extract for enzymes assay. The supernatant was stored in the refrigerator at -20oC until determination of β-1,3-glucanase activity by following the procedure of El-Gammal (2013).

**2.3.5 Statistical Analysis**

Data were analyzed using co-state and means were compared by standard errors.

**3. Results**

**3.1 β-1,3-glucanase**

Fungal bio-control agents significantly increased the β-1, 3-glucanase activity in soybean as compared to control under greenhouse and field conditions. Among fungal bio-control agents, *T. harzianum* + *T. viride* greatly increased the β-1, 3-glucanase activity (6.07 and 2.98 under greenhouse and field conditions, respectively) followed by *T. harzianum* while plants grown where *T. viride* was mixed in soil expressed minimum β-1, 3-glucanase activity (3.75 and1.37 under greenhouse and field conditions, respectively). The interactive effect of fungal biocontrol agents with days revealed that at 14 DAG, plants exhibited maximum β-1, 3-glucanase activity where *T. harzianum* + *T. viride* was applied (9.88, 3.16 greenhouse and field conditions, respectively) which was at par with *T. harzianum* + *T. viride* at 28 DAG. At 42 DAG, plants expressed the lowest β-1, 3-glucanase activity where *T. viride* was applied (Figures 1-4).

# 3.2 Peroxidase activity of soybean plants

Plants of control treatment revealed minimum peroxidase activity than bio-control agents (Figures 5-8). Among fungal bio-control agents, *T. harzianum* + *T. viride* progressively improved peroxidase activity in soybean (3.05 and 1.98 under greenhouse and field conditions, respectively) while soil application of *T. viride* expressed minimum peroxidase activity (2.32). Interactive effect of fungal bio-control agents and days indicated that at 28 DAG, plants where *T. harzianum* + *T. viride* was mixed with soil showed maximum peroxidase activity (3.10, 2.16 under greenhouse and field conditions, respectively). Peroxidase activity decreased with passage of time and minimum was recorded at 42 DAG where *T. viride* was soil mixed.

**3.3. Polyphenol oxidase (PPO)**

Concentrations of PPO in leaves of soybean were considerably higher when seeds were inoculated with different fungal antagonists before sowing (Figures 9-12) than in the non-inoculated controls. Fungal bio-control agents also improved the polyphenol-oxidase in soybean leaves compared to control. Among fungal bio-control agents, *T. harzianum* + *T. viride* greatly enhanced polyphenol-oxidase activity (1.26 and 2.90) while *T. harzianum* expressed least polyphenol-oxidase activity (0.63 and 1.27) under greenhouse and field conditions, respectively. Interactive effect of fungal bio-control agents with days expressed that at 42 DAG, polyphenol-oxidase activity was maximum where *T. harzianum* + *T. viride* was applied (1.50). Polyphenol-oxidase activity was minimum (0.50 and 3.10) at 42 DAG where alone *T. harzianum* was soil mixed under greenhouse and field conditions, respectively.

**3.2. Total phenol contents (TPC)**

Fungal bio-control agents significantly enhanced the total phenol contents in soybean leaves compared to control (Figures 13-16). Combination of *T. harzianum* + *T. viride* significantly improved total phenol contents (3.41, 4.20) under greenhouse and field conditions, respectively while *T. viride* exhibited least total phenol. Interaction of fungal bio-control agents with days showed that at 28 DAG, total phenol contents were increased substantially where *T. harzianum* + *T. viride* was applied (3.83, 4.66) under greenhouse and field conditions, respectively which was equal to same combination at 14 DAG. Total phenol contents were least at 42 DAG where *T. viride* was soil mixed (2.23).

**4. Discussion**

The current study revealed that seed treatment with various combinations of three *Trichoderma* species raised levels of several types of plant defense compounds - peroxidase, total phenolics, polyphenol oxidase and β-1,3-glucanase in soybean leaves for several weeks following sowing. Field and greenhouse trials showed consistently the combination of *T. harzianum* + *T. viride* elevated levels of these compounds to a greater extent than individual seed inoculation with *T. harzianum* and *T. viride*.Peroxidase, total phenolics, and β-1,3-glucanase function to facilitate plant growth as well as defense against fungal pathogens (Zhang, Kirkham et al. 1994). An upsurge in concentration of these enzymes in plants is often associated with induction of systemic resistance against phytopathogenic fungi (Sticher, Mauch-Mani et al. 1997). This study does not deal explicitly with mechanisms.

Increased activity of these host enzymes during plant-fungus interactions have been reported previously by several researchers (Heath 1996; Khaledi and Taheri 2016). Singh, Sindhan et al. (1998) reported higher phenol concentrations in chickpea plants when seeds were inoculated with *T. viride,* and that these phenols induced resistance against *M. phaseolina*. Khaledi and Taheri (2016) reported significant increase in peroxidase activity and phenolics in soybean root when seeds were sown after inoculation with *T. harzianum* isolates. Talaviya and Jadeja (2015) revealed that combined application of *T. viride* + *T. harzianum*+ *Pseudomonas fluorescens* was highly effective in reduction of cumin wilt disease (*Fusarium oxysporum* f. sp. *cumini*) and improved crop yield. Rajeswari and Science (2019) reported that when *T. viride* was used along with *Pseudomonas fluorescens* on ground nut (*Arachis hypogaea* L.). It effectively reduced Fusarium wilt caused by *Fusarium oxysporum*. As in our work, combinations of biocontrol agents have beneficial effect and resulted in more effective disease control than using single biocontrol species (Guetsky, Shtienberg et al. 2002).

Activation of polyphenol oxidase and peroxidase plays a major role in resistance of plants to pathogen attack (Mohammadi and Karr, 2002; Chérif, Arfaoui et al. 2007; Zhang, Zhang et al. 2008). The greater activity of PPO and PO along with higher amount of total phenols improved the host resistance (Nawar and Kuti 2003; Sreedevi, Charitha Devi et al. 2011). Nawar and Kuti (2003) documented that there was positive relationship between peroxidase levels and improvement in resistance. In a finding similar to our results, Rajeswari (2019) observed that leaves of *Arachis hypogaea* sprayed with combinations of *T. viride* and *T. harzianum* (Tv+Th) showed significantly high level of phenols. Inoculation with *Trichoderma* species can also enhance host physiological functions. For example, chickpea seed treated with a mixture of *T. harzianum*, *T. viride* and *Trichoderma virens* was helpful in increasing plant height as well as nitrogen and phosphorus uptake (Rudresh, Shivaprakash et al. 2005). The combination of *T. atroviride* and *T. viridescens* significantly increased the health and vigor of cuttings, their establishment and increased plant survival by 12%. Possible mechanisms of plant enhancement include increased nutrient uptake (Yobo, Laing et al. 2011), siderophore production, and synthesis of plant growth promoters (Yobo 2005, Yobo, Laing et al. 2011). Chickpea treated with a mixture of *Trichoderma, Rhizobium* and *Bacillus* exhibited higher germination, nutrient uptake, and yield and yield components than treatment with any of these fungi individually (Rudresh, Shivaprakash et al. 2005). The β-1,3-glucanases degrade the cell wall polysaccharides of fungal pathogens and release elicitors of additional plant defenses (Bishop, Ripoll et al. 2005). Our results also showed that maximum concentration of β -1,3-glucanase was recorded in soybean leaves when seeds were sown after treating with *T. viride* + *T. harzianum* (C3).Similarly, roots of coconut (*Cocos nucifera* L.) treated with *P. fluorescens* + *T. viride* + chitin showed significantly high β -1,3-glucanase activity (Karthikeyan, Radhika et al. 2006).

**5. Conclusions**

The present study showed that compatible combination of *T. harzianum* + *T. viride* significantly increased the peroxidase, polyphenol oxidase, phenolics, polyphenol and β-1,3-glucanase concentration in leaves of soybean compared to alone *T. harzianum* and *T. viride*. This improvement in the peroxidase, polyphenol oxidase, β -1,3-glucanase activity and phenols concentration demonstrate that *T. harzianum* + *T. viride* are synergistic in their beneficial impact on soybean plants. This combination may provide a more consistent level of growth promotion and a broader spectrum of activity than using single *Trichoderma* species, thus increasing the management options for soybean growers seeking to control charcoal rot and potentially other fungal diseases.

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Figure 1: Effect of fungal biocontrol agents on β-1, 3-glucanase activity of soybean under greenhouse conditions



Figure 2: Impact of interaction between fungal biocontrol agents and days on β-1, 3-glucanase activity of soybean under greenhouse conditions. 1, 2, 3 show 14, 28 and 42 days, respectively



Figure 3: Effect of different fungal biocontrol agents on β-1, 3-glucanase activity of soybean under field conditions



Figure 4: Impact of interaction between fungal biocontrol agents and days on β-1, 3- glucanase activity of soybean under field conditions. 1, 2 and 3 shows 14, 28 and 42 days, respectively



Figure 5: Effect of fungal biocontrol agents on peroxidase activity of soybean under greenhouse conditions



Figure 6: Impact of interaction between fungal biocontrol agents and days on peroxidase activity of soybean under greenhouse conditions. 1, 2, 3 show 14, 28 and 42 days, respectively



Figure 7: Effect of different fungal biocontrol agents on peroxidase activity of soybean under field conditions



Figure 8: Impact of interaction between fungal biocontrol agents and days on peroxidase activity of soybean under field conditions. 1, 2 and 3 show 14, 28 and 42 days, respectively



Figure 9: Effect of fungal biocontrol agents on polyphenol-oxidase of soybean under greenhouse conditions



Figure 10: Impact of interaction between fungal biocontrol agents and days on polyphenol-oxidase of soybean under greenhouse conditions. 1, 2, 3 show 14, 28 and 42 days, respectively

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Figure 11: Effect of different fungal biocontrol agents on polyphenol-oxidase activity of soybean under field conditions

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Figure 12: Impact of interaction between fungal biocontrol agents and days on polyphenol-oxidase activity of soybean under field conditions. 1, 2 and 3 show 14, 28 and 42 days, respectively



Figure 13: Effect of fungal biocontrol agents on total phenol contents of soybean under greenhouse conditions



Figure 14: Impact of interaction between fungal biocontrol agents and days on total phenol contents of soybean under greenhouse conditions. 1, 2, 3 show 14, 28 and 42 days, respectively



Figure 15: Effect of fungal biocontrol agents on total phenol contents of soybean under field conditions



Figure 16: Impact of interaction between fungal biocontrol agents and days on total phenol activity of soybean under field conditions. 1, 2 and 3 show 14, 28 and 42 days, respectively