Full Length Article

Impact of Seed Treatments with Fungal Biocontrol Agents on Enzymatic Activities and Phenolic Content of Soybean under Greenhouse and Field Conditions

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Abstract

Fungi in the genus *Trichoderma* are widely used as biological control agents because they can suppress plant pathogens and activate plant defense systems. In the present study, efficacy of microbial antagonists *viz.*, *T. harzianum* and *T. viride* or their combination was evaluated against the pathogenic fungus *Macrophomina phaseolina* and their effect on enzymatic activities and phenol content of soybean [*Glycine max* (L.) Merr.] plants. Soybean seeds were inoculated with *T. harzianum* and *T. viride* separately or in combination, and sown in pots under green house and under field conditions. Host enzymatic activities 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 peroxidase, polyphenol oxidase and β-1, 3-glucanase activities, and also the total phenol content in soybean leaves as compared to a non-treated control treatment. Concentration of peroxidase and β-1, 3-glucanasepeaked at 14 DAS and decreased thereafter in all the treatments under greenhouse and field conditions. All the treatments showed the highest levels of total phenols and polyphenol-oxidase at 28 DAS under both greenhouse and field conditions. At 14 DAS in both trials, the combination of *T. viride* + *T. harzianum* resulted in the highest level of peroxidase and β-1, 3-glucanase activities. This combination also resulted in the highest levels of total phenols and polyphenol oxidase content at 28 DAS. Our findings demonstrated that application of *Trichoderma* species as seed treatment has potential to trigger key mechanisms of systemically acquired resistance in soybean, and thereby enhanced efficacy of disease management tactics. © 2021 Friends Science Publishers

Introduction

Soybean is an important leguminous crop and a major source of vegetable oil and proteins worldwide. The crop is prone to many economically important fungal diseases of which charcoal rot caused by *Macrophomina phaseolina* is the major constraint causing significant losses every year (Marquez *et al*. 2021). This pathogen also causes diseases in other legumes such as chickpea, mungbean and mashbean (Banaras *et al.* 2021; Javed *et al.* 2021). Different management methods such as physical, chemical (fungicides), regulatory, cultural and biological have been used to control *Macrophomina phaseolina* (Khan and Javaid 2020a; Khan *et al.* 2021; Um-e-Aiman *et al.* 2021). However, these methods are helpful only when used well in advance as precautionary measures (Ganeshamoorthi *et al.* 2010; Marquez *et al*. 2021). In addition, conventional chemical fungicides for *M. phaseolina* infections may be less helpful due to soil-borne nature of the pathogen, and

may interrupt the balance of beneficial microbes in the soils (Anis *et al.* 2010). Furthermore, the indiscriminate use of chemical pesticides and fungicides may develop resistance to pathogenic strains and can cause harmful environmental risks and health hazards (Afouda *et al*. 2012). Efforts to manage the disease in soybean through crop rotation has also been suggested (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 need of the time.

During the past few decades, different biocontrol agents have been identified, characterized and commercialized (Javaid *et al.* 2021; Sharf *et al.* 2021). Biocontrol organisms have gained more attention as component of integrated disease management programs (Shahid and Khan 2019; Ali *et al.* 2020). Biological control is an effective way to enhance resistance in plants against pathogens, and this technique may play a significant role in sustainability of agricultural systems. Biocontrol organisms

are helpful against seed- and soil-borne fungal diseases of several crops (Akhtar and Javaid 2018; Javaid *et al.* 2018; Yasmin *et al*. 2020). The fungus *Trichoderma harzianum* has been documented to suppress many soil-borne fungal pathogens including *M. phaseolina* (Mukhopadhyay and Kumar 2020; Khan and Javaid 2020b). Aly *et al.* (2007) enlisted different antagonists of *Trichoderma* spp. 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 (Javaid *et al.* 2017; Shoaib *et al.* 2018). 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* as compared to *M. phaseolina* alone (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 (Khan and Javaid 2020b).

The most important mechanism of *Trichoderma* spp. is the induction of plant defense response to specific pathogens (Harman 2006; Inayati *et al.* 2020). Other than chemical and physical obstructions, plants have 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 plants is rapid, transitory and generalized. During biotic stress, host plant shows various cellular and physiological changes such as ion influx across the plasma membranes; activation of nitric oxide, defense-related genes; high production of ROS (reactive oxygen species), different phytohormones; biosynthesis of specific stress related proteins and production of antimicrobial chemicals such as phenolics (Wu *et al.* 2014; Nishad *et al.* 2020). Different biocontrol organisms may cause distinct molecular and cellular transformations in plants that enhance the 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 was documented to be progressively enhanced in plants of green gram (*Vigna radiata*) when inoculated with *T. viride* alone or in combination 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. as a biological control agent and induced defense-related enzymatic changes 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 a suitable combination of

Trichoderma spp. in improving the enzymatic and phenolic contents of soybean under greenhouse and field conditions.

Materials and Methods

Collection of fungal isolates

Soybean plants infected with *M. phaseolina* were collected from soybean growing areas of Punjab, Pakistan. These infected samples 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 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 121°C for 30 min. Symptomatic portions of stems were chopped into 5- to 7-mm long pieces. The chopped pieces were disinfested with mercuric chloride (0.1%), washed with sterilized distilled water and then placed on PDA plates with the help of sterilized forceps. These PDA plates were incubated at $27 \pm 1°C$ 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 Mahdizadeh *et al*. (2011). To maintain fungal culture in a viable condition, the PDA plates 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 h 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 $27 \pm 1°C$ 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.

Application of fungal antagonists

Greenhouse experiment: Plastic pots ($17 \times 20 \times 20$ cm^3) were filled with a mixture of clay, sand and peat (1:1:1). Soil was autoclaved at 121°C for 30 min for 2 successive days prior to use. For fungal bio-control agents, treatments were: *T. harzianum* alone, *T. viride*alone and *T. harzianum* + *T. viride* in combination at three levels of concentration of conidia (2×10^4, 2×10^6 and 2×10^7 spores mL^{-1}) (Karthikeyan *et al.* 2015). Equal concentration of each species was used

in the combined treatment. The spore concentration was determined using hemocytometer.

Field experiment: Fungal bio-control agents or their combination *i.e.*, *T. harzianum*, *T. viride* and *T. harzianum* + *T. viride* were used at the same concentrations as for greenhouse. Seeds of soybean variety NARC-3 (80 kg ha⁻¹) were treated with fungal bio-control agents using gum Arabicas sticky material. Seeds were coated with 1% gum Arabic (10 g for 1 kg of soybean seed) as an adhesive and suspended in the conidial suspension and kept at $25 \pm 2°C$ in a rotary shaker for 6 h to ensure uniform coating. After coating, seeds were dried in shade, and then used for sowing. Both the experiments (greenhouse and field) 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 was added along the length of the lines @ 6 g m⁻¹ 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 such as nitrogen, phosphorus and potassium were used @ 25, 60, 50 kg ha⁻¹, respectively. When the crop needed water, it was irrigated and weeds were controlled manually during growing season.

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.

Peroxidase (PO) activity

The procedure for determining the activity of peroxidase was adopted from Fehrmann and Dimond (1967). Approximately 0.5 g fresh leaves of treated or non-treated (control) soybean leaves ground in a pre-chilled mortar with 0.1 *M* ice cold phosphate buffer (20 mL) at 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 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.

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 a test tube and then heated for 1 min in a boiling water bath. After cooling, distilled water was added and final volume was made up to 25 mL. The

absorbance of the blue color was determined with Spectronic-20 colorimeters at 725 nm. Total phenol content was noted from the standard curve used for catechol.

Polyphenol oxidase (PPO)

Enzyme extract (0.5 mL) and 0.1 *M* phosphate buffer (2.3 mL) were added to a cuvette, which was adjusted to zero absorbance on a spectrophotometer (Mahadevan and Sridhar 1982). A 0.2 mL aliquot of 0.1 *M* catechol was added and then reactants were rapidly mixed. The activity of enzyme was noted as variation in absorbance instantaneously after adding 0.1 *M* catechol (0.2 mL).

β-1, 3-glucanase activity

Approximately 1 g soybean leaves 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⁻¹ fresh weight leaves for 1 min. This preparation was then passed through cheese cloth and filterate was centrifuged at 3000 rpm for 15 min at 6°C. The clear supernatant was collected and considered to be a crude extract for enzymes assay. The supernatant was stored in the refrigerator at -20°C until determination of *β*-1, 3-glucanase activity by following the procedure of El-Gamal *et al.* (2016).

Statistical analysis

Data were statistically analyzed using Statistix 8.1 software and means were compared by least significant difference test (LSD) at 5% probability level.

Results

β-1, 3-glucanase activity of soybean plants

Fungal bio-control agents significantly increased *β*-1, 3-glucanase activity in soybean compared to the control under both greenhouse and field conditions. Among fungal bio-control agents, *T. harzianum* + *T. viride* greatly increased *β*-1, 3-glucanase activity (6.07 and 2.98 *µ*g g⁻¹ under greenhouse and field conditions, respectively) followed by *T. harzianum*, whereas plants treated with *T. viride* expressed the least *β*-1, 3-glucanase activity (3.75 and 1.37 *µ*g g⁻¹ under greenhouse and field conditions, respectively). At 14 DAG, plants exhibited maximum *β*-1, 3-glucanase activity where *T. harzianum* + *T. viride* was applied (9.88 and 3.16 *µ*g g⁻¹ under greenhouse and field conditions, respectively) which had not changed at 28 DAG. At 42 DAG, plants expressed the lowest *β*-1, 3-glucanase activity where *T. viride* was applied (Fig. 1–4).
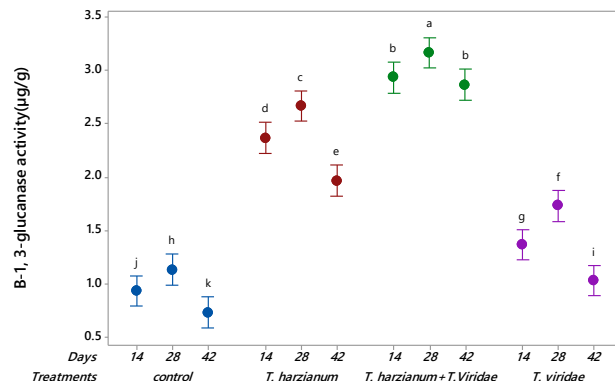
Peroxidase activity of soybean plants

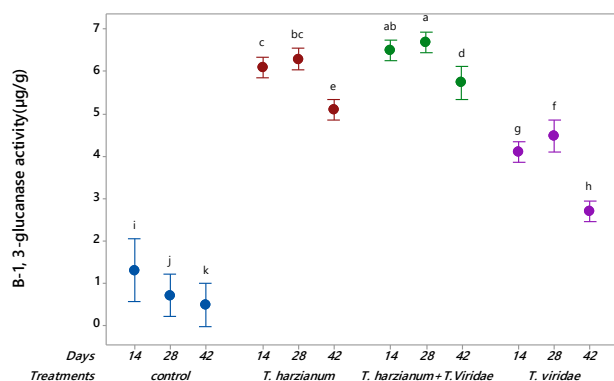Control plants had less peroxidase activity than bio-control
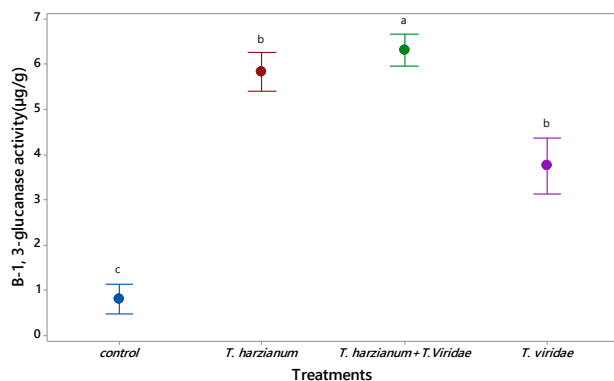
Individual standard deviations were used to calculate the intervals.

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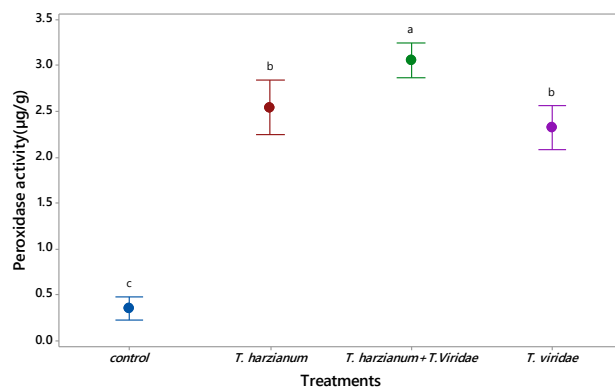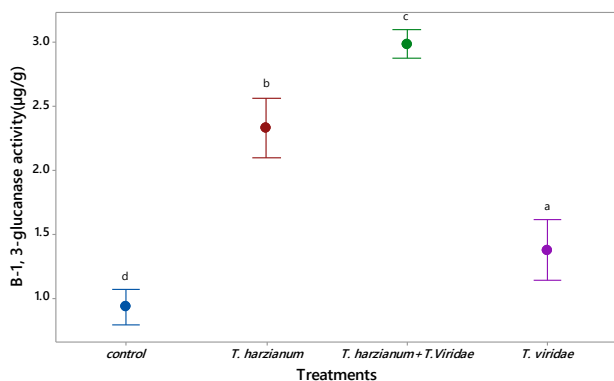

Individual standard deviations were used to calculate the intervals.

Fig. 2: Impact of interaction between fungal biocontrol agents and days on β-1, 3-glucanase activity of soybean under greenhouse conditions

Individual standard deviations were used to calculate the intervals.

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

Individual standard deviations were used to calculate the intervals.

Fig. 4: Impact of interaction between fungal biocontrol agents and days on β-1, 3-glucanase activity of soybean under field conditions
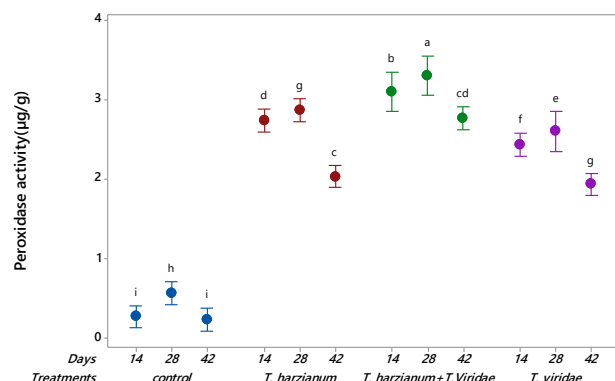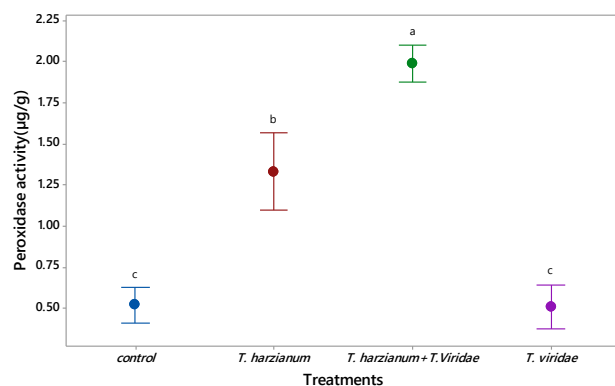

Individual standard deviations were used to calculate the intervals.

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

Individual standard deviations were used to calculate the intervals.

Fig. 6: Impact of interaction between fungal biocontrol agents and days on peroxidase activity of soybean under greenhouse conditions
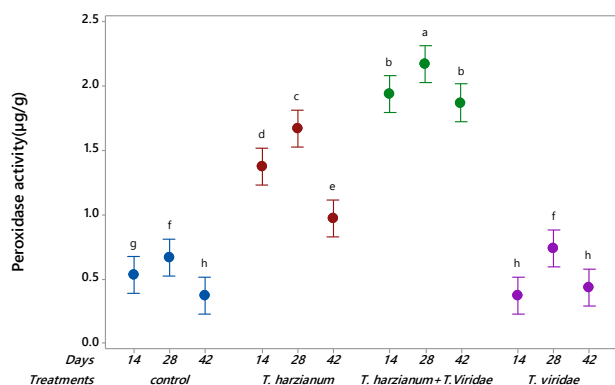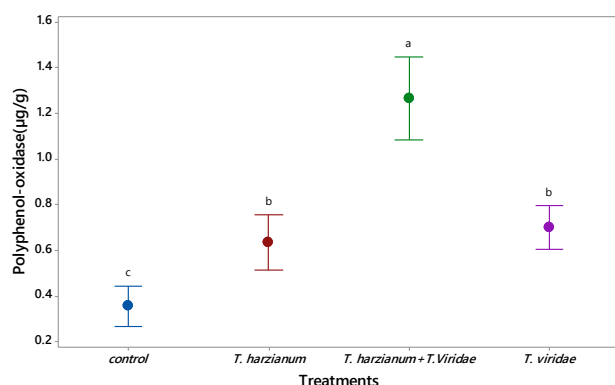
agents (Fig. 5–8). Among fungal bio-control agents, *T. harzianum + T. viride* progressively improved peroxidase activity in soybean (3.05 and 1.98 μg g^{-1} under greenhouse and field conditions, respectively), while soil application of

T. viride expressed minimum peroxidase activity (2.32 μg g^{-1}). At 28 DAG, plants with *T. harzianum + T. viride* showed maximum peroxidase activity (3.10 and 2.16 μg g^{-1} under

Fig. 7: Effect of fungal biocontrol agents on peroxidase activity of soybean under field conditions

Fig. 8: Impact of interaction between fungal biocontrol agents and days on peroxidase activity of soybean under field conditions

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

greenhouse and field conditions, respectively). Peroxidase activity decreased with passage of time to a minimum at 42 DAG in the *T. viride* treatment.

## Polyphenol oxidase (PPO) activity of soybean plants

Concentration of PPO in leaves of soybean was considerably higher when seeds were inoculated with fungal antagonists before sowing (Fig. 9–12) than in the non-inoculated controls. Fungal bio-control agents also increased the PPO concentration in soybean leaves compared to control. Among fungal bio-control agents, *T. harzianum + T. viride* greatly enhanced PPO activity (1.26 and 2.90 μg g^{-1}), whereas *T. harzianum* showed the least activity (0.63 and 1.27 μg g^{-1}) under greenhouse and field conditions, respectively. At 42 DAG, PPO activity was the highest when *T. harzianum + T. viride* was applied (1.50 μg g^{-1}). Polyphenol-oxidase activity was the lowest (0.50 and 3.10 μg g^{-1}) at 42 DAG for the *T. harzianum* treatment under greenhouse and field conditions, respectively.

Total phenol content (TPC)

Fungal bio-control agents significantly enhanced total phenol content in soybean leaves compared to control (Fig. 13–16). The combination of *T. harzianum + T. viride* significantly improved total phenol content (3.41 and 4.20 μg g^{-1} under greenhouse and field conditions, respectively), whereas *T. viride* exhibited the least total phenol. At 28 DAG, total phenol content was increased substantially when *T. harzianum + T. viride* was applied (3.83 and 4.66 μg g^{-1} under greenhouse and field conditions, respectively), which was equivalent to the values at 14 DAG. Total phenol content was the least at 42 DAG in the *T. viride* treatment (2.23 μg g^{-1}).

Discussion

In the present study, higher β-1, 3-glucanase, peroxidase, polyphenol oxidase activities and maximum total phenol content were observed in the soybean plants when seeds were sown after treatment with *T. harzianum + T. viride*, while the minimum values of these parameters were observed in the plants sown with untreated seed under both greenhouse and field conditions. These results indicate that combinations of *T. harzianum + T. viride* triggers stronger soybean defense signals than alone *T. harzianum* and *T. viride*. Increased activity of these host enzymes during plant-fungus interactions have been reported previously by several researchers (Khaledi and Taheri 2016; Yusnawan *et al.* 2019; Inayati *et al.* 2020). Peroxidase and β-1, 3-glucanase play a significant role to initiate the plant defense response against various pathogens through production of highly toxic phenolic compounds and higher production of reactive oxygen species or establishment of structural barriers such as lignin accumulation (Yusnawan *et al.* 2019; Inayati *et al.* 2020). β-1,3-glucanase degrades the cell wall polysaccharides of fungal pathogens and kills the pathogens (Ueki *et al.* 2020). Khaledi and Taheri (2016) reported significant increase in peroxidase activity and
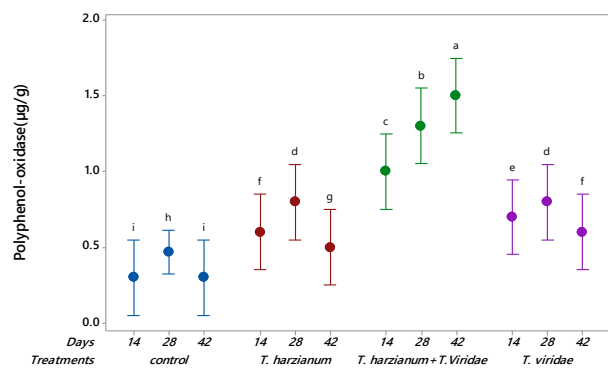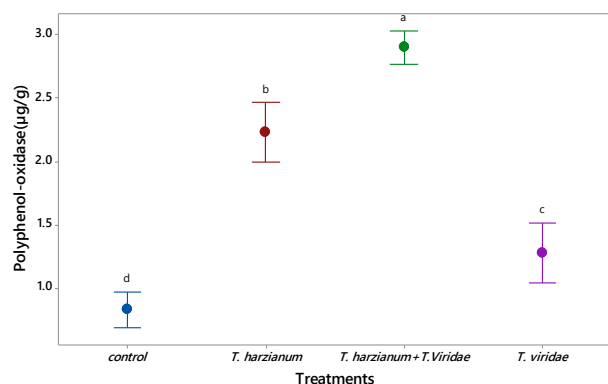
Individual standard deviations were used to calculate the intervals.

Fig. 10: Impact of interaction between fungal biocontrol agents and days on polyphenol-oxidase of soybean under greenhouse conditions

Individual standard deviations were used to calculate the intervals.

Fig. 11: Effect of fungal biocontrol agents on polyphenol-oxidase of soybean under field conditions

Individual standard deviations were used to calculate the intervals.

Fig. 12: Impact of interaction between fungal biocontrol agents and days on polyphenol-oxidase of soybean under field conditions
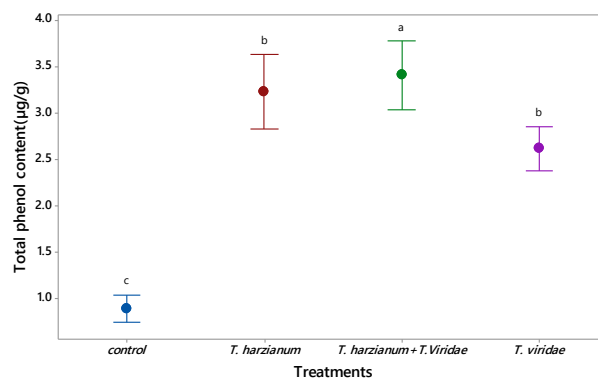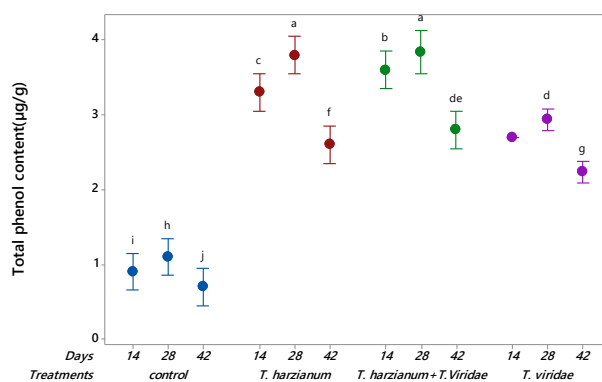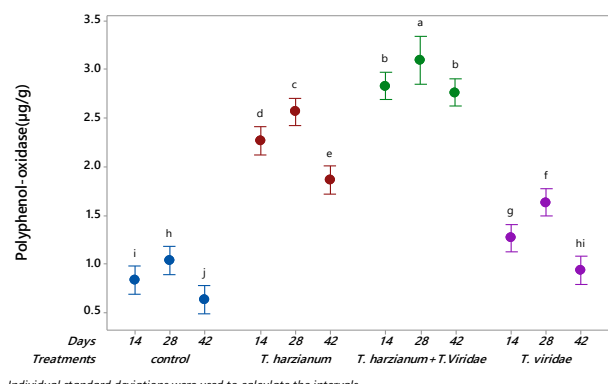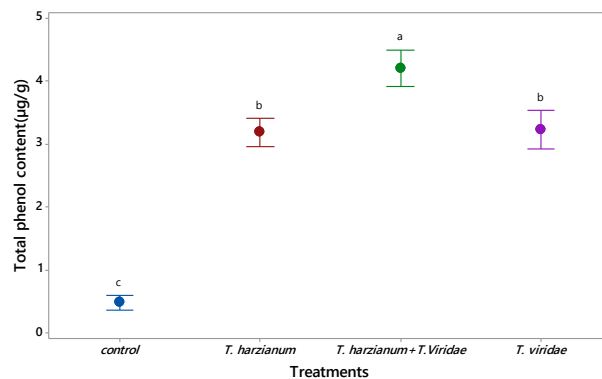

Individual standard deviations were used to calculate the intervals.

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

Individual standard deviations were used to calculate the intervals.

Fig. 14: Impact of interaction between fungal biocontrol agents and days on total phenol contents of soybean under greenhouse conditions

Individual standard deviations were used to calculate the intervals.

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

phenolics in soybean roots when seeds were sown after inoculation with *T. harzianum* isolates. Similarly, Rajeswari (2019) observed that leaves of *Arachis hypogaea* sprayed with combinations of *T. viride* and *T. harzianum* significantly increased phenols concentration. The study of Yusnawan *et al.* (2019) showed that the activity of

peroxidase increased in soybean plants treated with *T. virens*. Phenolics are one of the largest and most diverse groups of plant active substances involved in the plant growth regulation, and also play important role in defense

Individual standard deviations were used to calculate the intervals.

Fig. 16: Impact of interaction between fungal biocontrol agents and days on total phenol contents of soybean under field conditions
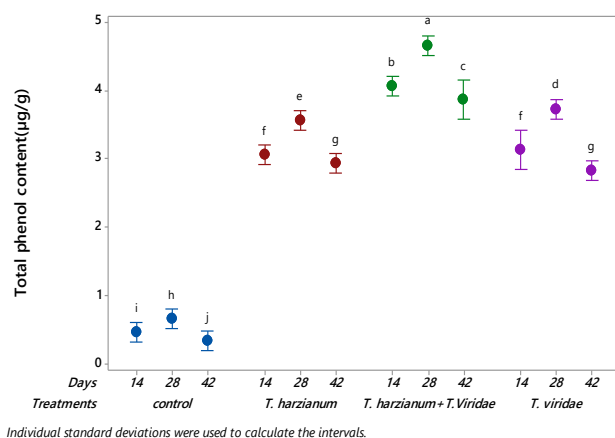
responses during pathogen infection and abiotic stress (Kubalt 2016). Phenolic compounds are produced by plant when the plant recognizes harmful pathogens or beneficial microbes. Polyphenol oxidase is involved in synthesis of phytoalexin and phenolic compounds, and the studies show that activity of polyphenol oxidase increases in legumes when treated with *T. viride* (Surekha *et al.* 2014). Seed treatment with *T. virens* increased the accumulation of total phenols in legumes (Inayati *et al.* 2020). PPO has been suggested to play important role in disease resistance due to its ability to catalyze oxidation of phenolic compounds into quinones and lignin biosynthesis (Kavitha and Umesha 2008; Inayati *et al.* 2020). According to the reported study, it is suggested that the induction of plant resistance in different hosts may require different signaling, and the induction is represented in different manner (Martínez-Medina *et al.* 2014). An increase of phenolic contents was also observed in soybean when seeds were treated with *T. virens* (Yusnawan *et al.* 2019). *Trichoderma* species have been studied for decades as effective bio-control agents against many pathogens through various modes of action (Inayati *et al.* 2020) and these *Trichoderma* spp., can induce systemic resistance in various plant species and pathogens (Angel *et al.* 2016; Małolepsza *et al.* 2017). The study of Dubey *et al.* (2018) showed that there were up-regulated expression of some defense-related genes and catalase in response to the presence of *T. virens* and *R. solani*. Numerous studies indicate the ability of *Trichoderma* spp. to reprogram plant genes expression that changes plant proteome and metabolome which alleviates physiological and biochemical change, and improve plant resistance to biotic and abiotic stresses (Mazzei *et al.* 2016). In plants, *Trichoderma* is able to activate plant defense mechanisms mostly for induced systemic resistance. Studies show that *Trichoderma* colonization triggers the plant defense systems (Pieterse *et al.* 2014; Inayati *et al.* 2020).

Conclusion

The present study showed that combining of *T. harzianum* with *T. viride* significantly increased the peroxidase, polyphenol oxidase, phenolics, polyphenol and β-1, 3-glucanase concentration in soybean compared to *T. harzianum* or *T. viride* alone. The increase in peroxidase, polyphenol oxidase, phenolics, polyphenol and β-1,3-glucanaseactivity and phenols concentration demonstrates that *T. harzianum* + *T. viride* are synergistic and have beneficial impact on growth of soybean plants.

Author Contributions

ZI and STS planned the experiments and AI interpreted the results and wrote the manuscript.

Conflicts of Interest

All authors declare no conflicts of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable in this paper

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