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Biostimulant impact of *Trichoderma* species on physiological characteristics of beans

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ABSTRACT

This research project is dedicated to investigating innovative strategies for reducing agrochemical usage and promoting sustainable agriculture. The primary focus of this study revolves around the development and evaluation of biostimulant products derived from *Trichoderma* fungus. To enhance the biostimulant properties of *Trichoderma,* gamma irradiation was employed to induce mutations in various *Trichoderma* species. Subsequently, three distinct biological fertilizers were formulated using five different *Trichoderma* species and their respective mutants. These bio-fertilizers underwent rigorous testing to evaluate their effects on the physiological characteristics of pinto bean plants. In total, seven experimental treatments were compared to a control group. Key parameters such as soluble protein content, chlorophyll levels, carotenoid concentrations, peroxidase, and polyphenol oxidase activities were measured and analyzed. Moreover, protein profiles and enzyme subunit activities were investigated to gain deeper insights into the mechanisms underlying the observed effects. The results of this study indicate that bio-priming seeds with a combination of *Trichoderma* spores resulted in the most significant improvements in chlorophyll content, carotenoid levels, and peroxidase activity. Additionally, mutants of *Trichoderma* species exhibited greater biostimulant effects compared to their wild-type counterparts. Notably, treatments involving kaolin-based granules demonstrated higher polyphenol oxidase activity. This research emphasizes the significant impact of *Trichoderma*-based treatments on the physiology of pinto bean plants. The induced mutations in *Trichoderma* species play a crucial role in enhancing efficacy.

***Key words*:** Bio priming, Biostimulants, *Trichoderma*, *Phaseolus vulgaris*.

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INTRODUCTION

Beans are an essential source of nutrition for both humans and animals. They are an excellent source of protein, dietary fiber, essential minerals, fats, and carbohydrates. Legumes play a crucial role in global food security, as they contribute approximately 22% of vegetable protein, 32% of fat, and 7% of carbohydrates in human diets (FAO, 2020). In addition to their nutritional benefits, legumes have significant agricultural advantages, particularly in crop rotation systems (Sepehri *et al.,* 2021). Grain legumes, such as pinto beans, have a protein content ranging from 18% to 32%, making them a valuable alternative to animal protein. Additionally, the deep roots of beans contribute to biological soil aeration, increasing soil fertility, and enhancing the soil structure, particularly in low-productivity areas (Majnoun Hosseini, 2017). Crop rotation involving beans can enhance soil nitrogen levels, thereby benefiting other crops in the following seasons (Giri *et al.,* 2019). Despite their agricultural and nutritional benefits, legumes can face several challenges during their growth, including pest infestations, disease outbreaks, and adverse environmental conditions. These challenges can impact crop yield and quality, leading to economic losses for farmers and food shortages for consumers. As a result, researchers have focused on developing strategies to enhance legume growth and productivity (Sepehri *et al.,* 2021).

The use of biostimulants is emerging as a promising strategy to enhance plant growth and development. Biostimulants refer to substances or microorganisms that can stimulate plant growth and productivity. Compared to traditional fertilizers and pesticides, biostimulants offer several advantages. They can enhance crop growth and yield while being environmentally friendly and non-toxic to human health (Li *et al.,* 2022). Among biostimulants, *Trichoderma* species have been identified as potential agents to enhance legume growth and productivity. *Trichoderma* is a ubiquitous soil fungus known for its beneficial effects on plant growth and development, including promoting root growth, increasing nutrient uptake, and enhancing plant tolerance to abiotic and biotic stresses (Vinale *et al.,* 2012).

Several studies have investigated the impact of *Trichoderma* species on legume growth and productivity, specifically pinto beans. For instance, *Trichoderma harzianum* has been shown to increase shoot length, root length, and biomass (Rezaloo *et al.,* 2020). Similarly, *Trichoderma viride* has been shown to improve the morphological and physiological characteristics of pinto beans, such as increasing root length, shoot length, and chlorophyll content (Vargas-Mendoza *et al.,* 2019). Despite the potential benefits of *Trichoderma* in enhancing legume growth and productivity, there is still limited understanding of the underlying mechanisms that govern their interactions with plants. Further research is needed to elucidate the specific modes of action of *Trichoderma* on legumes, including the molecular and biochemical pathways involved in their beneficial effects (Eslahi *et al.,* 2021; Das *et al.,* 2022). Considering the potential of *Trichoderma* as biostimulants holds the potential to enhance legume growth and productivity, thereby promoting sustainable agriculture and food systems, a more thorough understanding is required to fully elucidate the mechanisms through which *Trichoderma* acts on legumes and to develop effective strategies for integrating them into agricultural systems.

In this study, we assessed the effects of different *Trichoderma* treatments as biostimulants on the physiological responses of bean plants to the biomaterial treatment. The analysis included total soluble protein, chlorophyll, carotenoids, peroxidase, and polyphenol oxidase activity in pinto beans. For this aim, bean plants were treated with 2 types of spore mixtures of *Trichoderma* species: *T. harzianum, T. lixii, T. ghanens, T. virens,* and *T. atroviride* in 3 different methods: seed biopriming, talc-based powder, and kaolin-based granule. The Wild-type biological material and spore mixture of the selected mutant isolates (*T. harzianum* M1, *T. lixii* M17, *T. ghanensis* M1, *T. virens* M17, and *T. atroviride* M2) were used for the experiment. The study evaluated the enhanced effectiveness of biological treatment by utilizing mutant isolates of Trichoderma to improve the physiological responses of bean plants, compared to their wild-type isolates.

MATERIALS AND METHODS

Plant materials, statistical design, and treatments

In this experiment, bean plants were exposed to *Trichoderma* fungi using 3 different application methods. The plant materials used included pinto beans (*Phaseolus vulgaris*), specifically the Talash variety, sourced from the Vegetable Crops Research Department (VCRD) of the Seed and Plant Improvement Institute (SPII) in Karaj, Iran. The experiment followed a completely randomized statistical design with 3 replicates. The treatments involved 2 factors. Factor A included the types of *Trichoderma* fungi

with two variations: A1- a blend of spores from five wild type species: *T. harzianum* (MW718882), *T. lixii* (MW719563), *T. ghanensis* (MW719590), *T. virens* (MW719876), and *T. atroviride* (MW719255), and A2- a blend of spores from mutant isolates of the same species: *T. harzianum* mutant (NAS108 M1), *T. lixii* mutant (NAS114-M17), *T. ghanensis* mutant (ON545796), *T. virens* mutant (NAS115 M17), and *T. atroviride* mutant (NAS112M2). Factor B involved the application methods with three levels: B1- seed bio-priming, B2- kaolin-based granule, and B3- talc-based powder. Additionally, a control treatment was included alongside the 6 specified treatment combinations. All *Trichoderma* strains were provided by the Nuclear Agriculture Research School–Nuclear Science and Technology Institute (NSTRI), Karaj, Iran. The details of Factor B levels are outlined below:

Seed bio-priming with *Trichoderma* spores

Seeds were superficially disinfected in 70% ethanol for 1 minute and 2% sodium hypochlorite for 40 seconds, and finally, washed 3-4 times with sterile distilled water. The spore suspension of each *Trichoderma* species was separated, and an equal amount of spore suspension from each species was mixed. The mixed spore suspension population in *Trichoderma* Complete Medium (TCM) was adjusted at 1×10^8 spore.mL^{-1}. Then, 40% v/v gum Arabic was added, and the bean seeds were coated with this thick liquid containing fungal spores (wild type as SW and mutant isolates as SM) and dried at room temperature (Soufi *et al.,* 2021).

Kaolin-based granule preparation

The production of kaolin-based powder containing the wild-type strain (KW) and mutant strains (KM) was conducted following the method outlined by Oancea *et al.* (2016) with some modifications. The granule's base composition (TCM) includes a solution of spores containing *Trichoderma* fungi (1×10^8 spore.mL^{-1}) with 50% kaolin and 2% sodium alginate (Soufi *et al.,* 2021). The mixture was added dropwise into a $CaCl_2$ solution (0.1M) at pH 4.8. All materials were sterilized before the test, and the spherical seeds were dried with distilled water at room temperature and stored at 4 °C before use (Oancea *et al.,* 2016). The substance was added to the soil in the form of granules 14 days before planting the seeds because the granules require time to open and release the spores. Each of the cases mentioned above was prepared for two types of fungal spores: wild type and mutant.

The soil needed for the pots was placed in a bag and exposed to 50 pounds of pressure at a temperature of 121 °C in an autoclave for 1 hour. The mixture was then combined with perlite, soil, and peat moss in a 1:1:1 ratio. The seeds were planted in soil in pots weighing two kg each, which contained biofertilizer added to the soil on the same day of seeding. The pots were irrigated immediately after seeding in a flower bed. In the pre-flowering stage, a leaf sample was taken to conduct enzymatic and protein analyses. The sample was crushed in liquid nitrogen and stored in a freezer at -70 °C.

Talc-based powder preparation

For this treatment, the method was implemented with a series of modifications. *Trichoderma* spores were cultured on wheat, dried at room temperature, and then milled. The spores were collected using a sieve with a screen size of 150 µ (U.S. Standard Sieve No. 100). The isolates were added to talc powder until their final population in talc powder reached 1×10^8 spores per gram. The moisture content of talc powder was reduced to 8% (Jeyarajan and Nakkeeran, 2000). The talc-based powder was prepared by mixing spore suspensions of wild-type *Trichoderma* isolates (TW) and mutant strain (TM). The biomaterial was applied in the soil in powder form along with seeds (Orojnia *et al.,* 2021).

Soluble protein extraction from leaves

During the pre-flowering stage, a leaf sample was collected for enzymatic and protein analyses. The samples were crushed in liquid nitrogen and stored in a freezer at -70 ºC. To prepare, the plant tissues (1 g) were homogenized in a mortar using a 100 mM potassium phosphate buffer (10 mL) at pH 7. Following centrifugation, the resulting supernatant was collected for physiological measurements. The protein concentration of the samples was assessed using the Bradford method, as outlined by Bradford (1976).

Peroxidase enzyme (POD) activity assay

Peroxidase activity was evaluated by extracting soluble proteins from leaves and utilizing guaiacol as the substrate. The reaction mixture comprised 800 µL of a 10 mM guaiacol solution in a 50 mM phosphate buffer at pH 6. It contained 100 µL of enzyme extract, diluted at a ratio of 1:20 in protein extraction buffer, and 100 µM H_2O_2 at a concentration of 35 mM. The absorbance changes were monitored at a wavelength of 470 nm, with readings taken every 10 seconds, over 2 minutes at 25 °C temperature (Dazy *et al.,* 2008).

Polyphenol oxidase (PPO) activity assay

Polyphenol oxidase activity was assessed by measuring soluble protein extraction from leaves using pyrogallol as a substrate. The reaction mixture comprised 900 µL of 10 mM phosphate buffer (pH 6.5) and 100 µL

of enzyme extract diluted at 1:2 in protein extraction buffer. The reaction commenced with the addition of pyrogallol, and absorbance changes were monitored at 420 nm for 2 minutes and 10 seconds (Rostaminia *et al.,* 2021).

Chlorophyll and carotenoid content determination

Chlorophyll and carotenoid content were assessed from 0.5 g of fresh leaf homogenized with 20 mL of 80% acetone. The homogenate was centrifuged at 6000 rpm for 10 minutes. Absorbance readings were taken at 663 nm for chlorophyll a, 645 nm for chlorophyll b, and 470 nm for carotenoids using a spectrophotometer. Pigment concentrations were determined using the formulas outlined by Arnon (1973):

$$(1) \qquad chl\ a = \frac{(19.3 * A663 - 0.8 * A645)v}{100w}$$

$$(2) \qquad chl\ b = \frac{(19.3 * A645 - 3.6 * A663)v}{100w}$$

$$(3) \qquad caretenoides = \frac{100(A470) - 3.27(mg\ chl\ a) - 104\ (mg\ chl\ b)}{227}$$

v=volume of filtered solution (upper solution obtained from centrifugation)
a=absorption of light at wavelengths of 663, 654, and 470 nm
w=sample fresh weight in grams

SDS-PAGE

For SDS-PAGE, Soluble proteins were extracted from leaf samples using gel plates that were 1 mm thick, following Laemmle's protocol (1970). Electrophoresis was carried out in a UVP Vertical Electrophoresis Unit using a discontinuous buffer system, running gels at a constant current of 20 mA until the bromophenol blue marker reached the bottom. Protein molecular weights were estimated by comparing them to Protein Ladder SDS-PAGE standards. Post-electrophoresis, gels were rinsed with an isopropanol-acetic acid-water solution followed by a methanol-acetic acid-water solution. Gels were then stained with Coomassie Brilliant Blue R-250 (0.01% w/v) and destained using a mixture of methanol, acetic acid, and water until the protein bands became visible. Finally, Gels were scanned using a GelDoc System to determine the protein molecular weights based on the results.

Extraction and assay of peroxidase

Peroxidase activity was assessed with pyrogallol as a substrate, following the procedure outlined by Rostaminia *et al.* (2021). The activity of the enzyme was quantified in Enzyme Units (E.U.). Peroxidase isoenzymes were examined in bean leaves.

Polyphenol oxidase zymogram

Polyphenol oxidase enzyme bands were visualized on polyacrylamide gels using the method described by Mardani-Mehrabad *et al.* (2020).

Data analysis

The results were analyzed based on the one-way analysis of the variance algorithm. The means were compared using Tukey's honestly significant difference (HSD) test at a significance level of 5% using SPSS 26 statistical software.

RESULTS

Biochemical analysis of fresh leaf

Soluble protein

Soluble protein extracted from *P. vulgaris* 40 days post-treatment leaves was measured by establishing the regression relationship between BSA and absorption rate. Analysis of protein concentrations indicated a significant treatment effect at the 5% level. Mean squares results for protein measurements showed SW with the highest protein concentration at 4.13 ($mg.g^{-1}$ leaf fresh weight). The method involved attaching *Trichoderma* spores to seeds using Arabic gum, likely enhancing colonization from the seed germination onset due to the proximity of spores to the seed. Protein concentrations in all treatments exceeded the control (Table 1), except for KM at 2.12 ($mg.g^{-1}$ leaf fresh weight), displaying the lowest protein levels significantly different from the others (Table 1).

Peroxidase enzyme activity assay

Statistical analysis showed a significant difference ($P \leq 0.05$) in soluble peroxidase activity between treatments as indicated by the F-test. A comparison of the mean peroxidase activity value showed that the SW treatment exhibited the highest activity (25.28 $U.g^{-1}$ leaf wb), whereas the TW treatment displayed the lowest activity (17.03 $U.g^{-1}$ leaf wb). Other treatments also differed significantly from the control (Table 1). Considering the high soluble protein content in SW and its correlation with the peroxidase activity, it can be concluded that the accumulation of soluble protein in this treatment was related to this enzyme.

Polyphenol oxidase enzyme activity assay

Table 1 illustrates the impact of different *Trichoderma* treatments and the control on leaf polyphenol oxidase enzyme activity. The findings reveal that *Trichoderma* strains TW, KM, and KW notably increased the levels

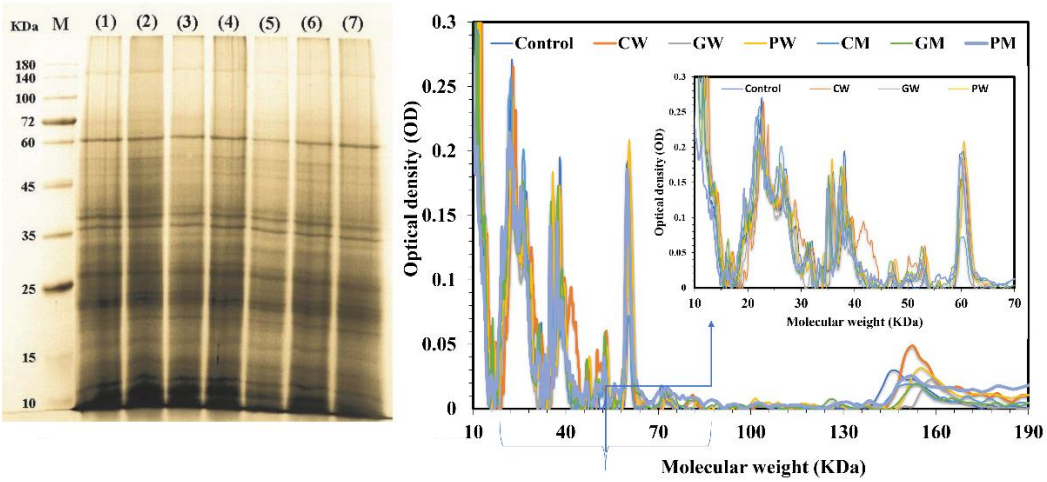

Figure 1. A: SDS-PAGE Gel Extractive proteins and **B:** SDS-PAGE gel densitometry from pinto bean leaf samples. (1): Control plant, (2): Blend of wild-type *Trichoderma* spores via seed bio-priming, (3): Blend of mutant-type *Trichoderma* spores via seed bio-priming, (4): Blend of wild-type *Trichoderma* spores via the kaolin-based granule, (5): Blend of mutant-type *Trichoderma* spores via the kaolin-based granule, (6): Blend of wild-type *Trichoderma* spores via the talc-based powder, (7): Blend of mutant-type *Trichoderma* spores via the talc-based powder.

of polyphenol oxidase enzyme activity compared to other treatments. The enzyme activity levels were 167.36, 165.12, and 162.55 U.g^{-1} fresh weight for TW, KM, and KW, respectively. In contrast, the control with 162.93 U.g^{-1} fresh weight exhibited lower levels of this enzyme (Table 1). Both KW and KM contribute to increased polyphenol oxidase enzyme levels in bean plants by gradually releasing *Trichoderma* spores in the rhizosphere throughout the plant's lifespan.

Chlorophylls a, b, and carotenoids contents

The mean comparison results (Table 1) indicate that the highest values of chlorophyll a, b, and carotenoids were observed in the Seed treatments with spore mixture SM (Chl a: 11.23, Chl b: 3.53, and carotenoids: 2.69 (mg.g^{-1} leaf fresh weight), and the values were significantly different from other treatments. The carotenoid content in KM was 2.69, which was not significantly different from SW at the 5% probability level. The control had lower levels of pigments (chlorophyll a: 6.26, chlorophyll b: 2.03, and carotenoids: 1.69 (mg.g^{-1} fresh weight) compared to all treatments (Table 1). The results of this study are consistent with previous findings which confirmed that *Trichoderma* promotes chlorophyll production.

In this study, we analyzed the protein bands of pinto bean plants treated with different *Trichoderma* species, as well as control plants without *Trichoderma*. All treatments, including the control plants, showed a variety of protein bands ranging from 10 to 180 kDa in molecular weight (Figure 1). Unlike other treatments, seeds treated with a combination of mutant isolates in the soil exhibited a distinct absence of a well-defined band within the range of 62 to 66 kDa (Figure 1). Furthermore, a protein band at 62.7 kDa was identified in seeds treated with the mutant isolate granule (KM), but its intensity was weaker compared to other treatments (Figure 1). there were no significant differences in the presence of other sharp bands at molecular weights ranging from 36 to 40 kDa, 25 to 29 kDa, and 20 to 23 kDa in samples of seeds treated with different *Trichoderma* fungal biofertilizers and control plants (Figure 1). However, the highest levels of bands were observed in the SW, TM, and KW treatments (Figure 1).

The molecular weight range of bands, spanning from 290 to 35 kDa, was observed across all treatments compared to the control plants (Figure 2). Diverse molecular weight bands in the peroxidase zymogram imply the existence of distinct subunits of this enzyme within the leaf sample of the plant. The highest band intensity was observed in the KM treatment at a molecular weight of 265 to 210 kDa, followed by the KW and TM treatments (Figure 2). Samples treated with TM, KM, as well as TW showed the highest enzyme band intensity at a molecular weight of 65-68 kDa. Furthermore, the most robust band was observed at a molecular weight of 43 to 45 kDa in TM-treated samples, followed by SM (Figure 2). Elhelaly (2022) also observed bands in the same weight range in broad bean plants.

Various bands were observed in both the treatments and control plants within a range of molecular weights

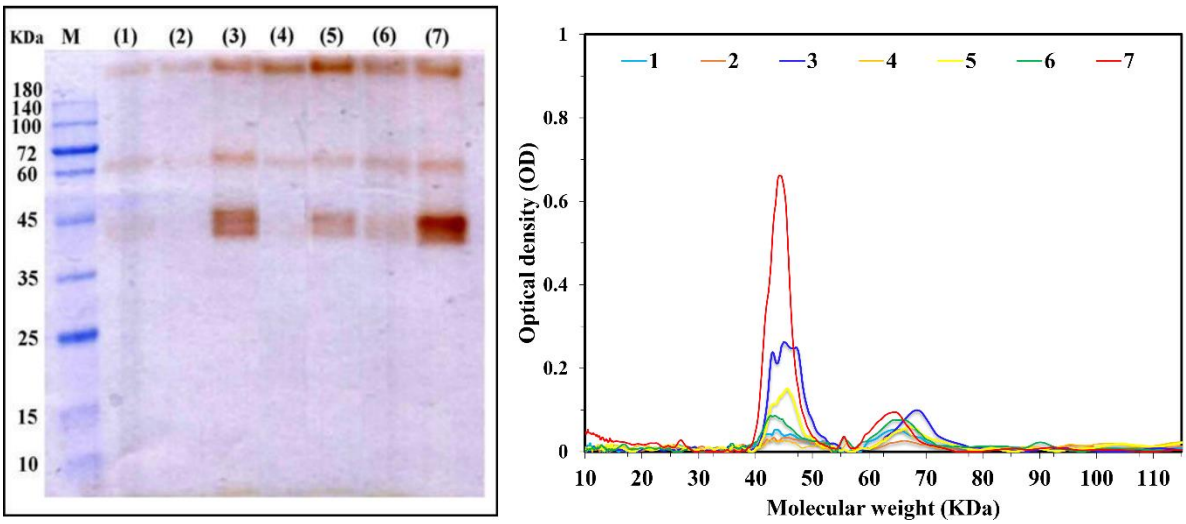

Figure 2. A: Zymogram gel Extracts of peroxide enzymes and **B:** Zymogram gel densitometry from pinto bean leaf samples.
(1): Control plant, (2): Blend of wild-type Trichoderma spores via seed bio-priming (SW), (3): Blend of mutant-type Trichoderma spores via seed bio-priming (SM), (4): Blend of wild-type Trichoderma spores via the kaolin-based granule (KW), (5): Blend of mutant-type Trichoderma spores via the kaolin-based granule (KM), (6): Blend of wild-type Trichoderma spores via the talc-based powder (TW), (7): Blend of mutant-type Trichoderma spores via the talc-based powder (TM).

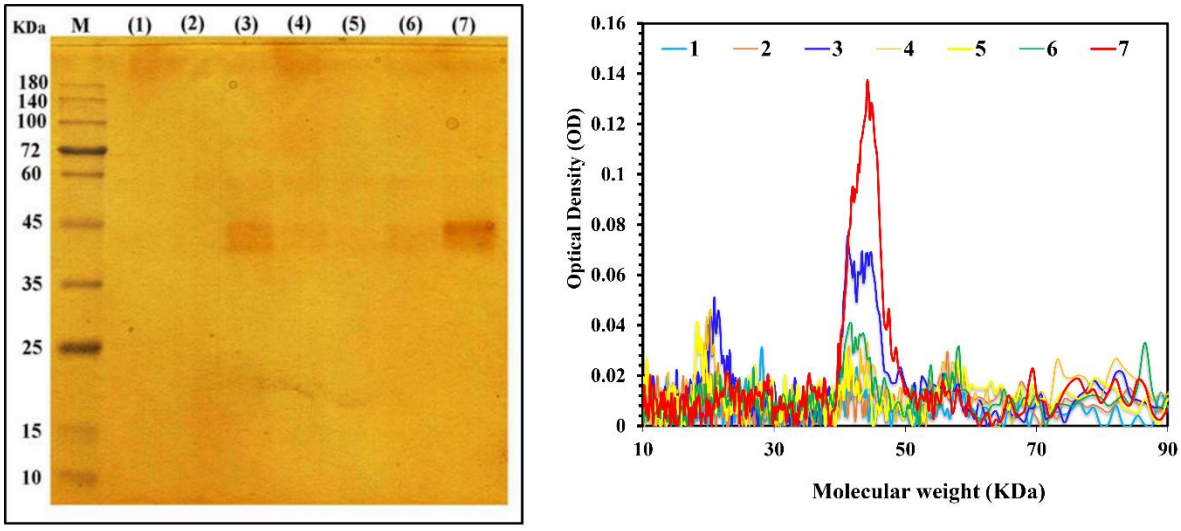

Figure 3. A: Zymogram gel Extracts of polyphenol oxidase enzymes and **B:** Zymogram gel densitometry from pinto bean leaf samples.
(1): Control plant, (2): Blend of wild-type Trichoderma spores via seed bio-priming (SW), (3): Blend of mutant-type Trichoderma spores via seed bio-priming (SM), (4): Blend of wild-type Trichoderma spores via the kaolin-based granule (KW), (5): Blend of mutant-type Trichoderma spores via the kaolin-based granule (KM), (6): Blend of wild-type Trichoderma spores via the talc-based powder (TW), (7): Blend of mutant-type Trichoderma spores via the talc-based powder (TM).

from 35 to 245 kDa (Figure 3). The presence of distinct molecular weight bands in the polyphenol oxidase zymogram suggests the presence of diverse subunits of this enzyme within the plant leaf sample (Figure 3). Notably, the Control, KW, and TM treatments displayed a color alteration, indicating enzyme activity, within the molecular weight range of 228 to 245 kDa (Figure 3). In the control plants, staining caused by enzyme activity was observed at molecular weights of 44.7 and 42.85 (Figure 3). No color change indicative of enzyme activity was observed in the KW treatment (Figure 3). Samples treated with TM and SM showed the highest activity of polyphenol oxidase enzyme with molecular weights of 41 and 44 kDa, respectively (Figure 3). In contrast, additional treatments such as KW, KM, and TW exhibited a minimal banding as a result of

polyphenol oxidase enzyme activity (Figure 3).

DISCUSSION

The production of secondary metabolites is genetically controlled; however, environmental factors significantly affect the quantity and quality of these substances (Hermosa *et al.,* 2012). *Trichoderma* species, widely present as beneficial symbionts in plants, exhibit rapid growth rates that make them useful in modifying plant metabolism in the agricultural sector (Amini *et al.,* 2014). These fungi primarily play a role in the mineralization of organic materials and the production of compost, which leads to increased nutrient absorption and plant growth (Rudresh *et al.,* 2005). As a result of these biochemical changes, plants acquire increased resilience to stress, including pathogenic infection (Van Loon *et al.,* 2006). Different *Trichoderma* species serve as triggers for these biochemical modifications within the cell, highlighting their potential as a tool to improve plant health (Gaderer *et al.,* 2015).

Recent studies indicate that these fungi play a crucial role in controlling soil pathogens, producing growth hormones, enhancing the bioavailability of elements in soil, and facilitating nutrient absorption by plants. They also contribute to improved sugar and amino acid transport in plant roots, boost resistance to environmental stress, and promote overall plant growth (Anshu *et al.,* 2022). Furthermore, research suggests that these fungi directly or indirectly stimulate plant growth through the production of various compounds and metabolites (Drikund *et al.,* 2015). In pea stems, the application of harzianolide and 6-pentyl-pyrone, both secondary metabolites produced by different *Trichoderma* strains, has shown an auxin-like effect (Khan *et al.,* 2010).

Our data revealed changes in protein expression in bean plant leaves, likely linked to mechanisms contributing to the induction of resistance against salt stress. Key mechanisms may involve metabolic pathways that enhance the cells' ability to cope with stressful conditions. Further experiments are required to identify these mechanisms more precisely. Among pathogenicity-related proteins, peroxidases are known to trigger the plant defense system and enhance resistance to plant pathogens (Djonovic *et al.,* 2006). Howell *et al.* (2000) showed that treating cotton seeds with *T. virens* fungus induces the peroxidase enzyme. Also, Mortezania *et al.* (2010) found that inducting resistance in cucumber seedlings with *T. harzianum* by pre and post-inoculation with *Pythium aphanidermatum* is related to maximum peroxidase enzyme activity. Overall, the results of this study show that seedlings treated with antagonistic fungi displayed higher peroxidase levels than the control and appeared healthier.

Different *Trichoderma* species employ various biological control mechanisms, such as modulating the level of host plant hormones, synthesizing and secreting phenolic compounds both intracellularly and extracellularly, and synthesizing proteins related to pathogenesis, thereby inducing systemic host resistance (Amini *et al.,* 2014). Phenolic compounds' accumulation and oxidation have been suggested as crucial mechanisms of plant resistance, especially against fungal contamination. These compounds play an essential role in absorbing and neutralizing free radicals and neutralizing active oxygens, or peroxidases that decompose (Taheri and Tarighi, 2011). Our experiment's results also show that *Trichoderma* treatments cause the accumulation of phenolic compounds in the plant, which leads to plant resistance (Table 1). The highly expressed polyphenol oxidase enzymes in tomato plants are associated with increased pathogen resistance (Li and Steffens, 2002). In the *A. thaliana* plant, salinity affects the growth and development of plants, including primary root length, the formation of secondary roots, and the formation of hairy roots (Deng *et al.,* 2022). These effects significantly increased the plant's tolerance to salinity. The mechanisms related to the accumulation of abscisic acid, ascorbic acid, antioxidants, and L-proline provide conditions for stress control (Deng *et al.,* 2022). Also, according to reports, L-Pro accumulation is regulated by *T. asperelloides* T203 in *A. thaliana* (Musin *et al.,* 2022). Among the secondary metabolites of Trichoderma fungi, indole acetic acid plays an important role in adapting to salt stress. It is the mechanism through which microorganisms alter the hormonal pathway to enhance tolerance to salt stress (Eslahi *et al.,* 2020).

Under drought stress conditions, the plant produces carotenoids because they act as antioxidants and protect the photosynthetic system by scavenging the free radicals generated (Bae *et al.,* 2002). Edreva (2016) attributed the increase in carotenoids in plants inoculated with *Trichoderma* to improved nutrient absorption. In plants inoculated with *Trichoderma*, iron absorption and chlorophyll levels are higher than in those not inoculated (Salahi Ostad and Salahvarzi, 2021). Altomare *et al.* (1999) reported that *Trichoderma* in symbiosis with plant roots enhances the solubility of iron, zinc, and manganese elements in the root environment, resulting in increased absorption.

Table 1. Comparison of the mean of effects of *Trichoderma* mixture on some enzymes, protein, and photosynthetic pigments in pinto beans.

Treatment	Protein (mg/g fw±SD)	PPO (U/g fw±SD)	POD (U/g wb±SD)	Chl a	Chl b	Carotenoids
				(mg/g fw leaf±SD)		
SW	4.13±0.02^{a}	127.08±4.25^{d}	25.28±1.01^{a}	11.23±0.26^{a}	3.53±0.03^{a}	2.69±0.01^{a}
SM	2.35f±0.07^{bc}	139.68±3.53^{c}	17.03±0.70^{def}	6.73±0.07^{d}	2.33±0.05^{e}	1.77±0.05^{e}
TW	2.25±0.06^{de}	167.36±2.08^{a}	23.40±0.79^{b}	8.36±0.07^{c}	2.86±0.03^{bc}	2.30±0.01^{b}
TM	2.47±0.06^{cd}	147.68±2.15^{b}	19.93±0.23^{de}	8.66±0.07^{c}	2.60±0.00^{d}	2.18±0.01^{c}
KW	2.41±0.04^{bc}	162.93±2.32^{a}	22.50±0.18^{bc}	8.46±0.03^{c}	2.73±0.03^{cd}	2.09±0.0^{d}
KM	2.12±0.05^{e}	165.12±6.39^{a}	21.78±0.18^{cd}	9.43±0.13^{b}	2.96±0.03^{b}	2.69±0.03^{a}
Control	2.22±0.06^{de}	106.62±3.95^{e}	20.02±0.30^{e}	6.26±0.09^{e}	2.03±0.14^{f}	1.69±0.01^{f}

In each column, means followed by the same letter are not statistically different according to Tukey's ($P \leq 0.05$). Each number is the average of three repetitions.
SW: Blend of wild-type *Trichoderma* spores via seed bio-priming, SM: Blend of mutant-type *Trichoderma* spores via seed bio-priming, TW: Blend of wild-type *Trichoderma* spores via the talc-based powder, TM: Blend of mutant-type *Trichoderma* spores via the talc-based powder, KW: Blend of wild-type *Trichoderma* spores via the kaolin-based granule, KM: Blend of mutant-type *Trichoderma* spores via the kaolin-based granule, POD: Peroxidase activity, PPO: Polyphenol oxidase activity, Chl a: Chlorophyll a, Chl b: Chlorophyll b.

They also found that *Trichoderma* produces chelating substances that enhance the absorption of this element by binding with iron ions, thereby increasing chlorophyll levels. Irannezhad *et al.* (2010) reported that *Trichoderma* increased chlorophyll levels in olive seedlings. Guler *et al.* (2016) also found that plants inoculated with *Trichoderma* had a more balanced chlorophyll and carotenoid production, indicating a less disturbed photosynthetic system. Yedidia *et al.* (2001) presented a similar report. Entesari *et al.* (2012) reported that all three types of chlorophyll (Chl a, b, and carotenoids) had the highest amount in seeds primed with zinc sulfate and *T. harzianum* fungus, showing significant differences compared to other treatments. The results are in line with the reports of other researchers regarding the increase in plant growth by *T. harzianum* by increasing the dissolution of phosphates and available micronutrients (Ali *et al.,* 2022). Most Trichoderma strains acidify their surroundings by releasing organic acids such as gluconic acid, citric acid, and fumaric acid. This process helps dissolve phosphate, micronutrients, iron, manganese, and magnesium, ultimately promoting plant growth. (Ali *et al.,* 2022).

Pink beans, pinto beans, and small red beans exhibit distinct bands at a molecular weight of 27 kDa (Rui *et al.,* 2011), which aligns with the protein profile we observed in our bean samples (25-29 kDa). These results are consistent with previous studies by Barker *et al.* (1976), which show that some proteins with a molecular weight of 23 kDa from *Phaseolus vulgaris* seeds have similar properties to Phaseolin. In our study, we also observed a distinct band at 20 to 23 kDa. In line with the protein profiles obtained in our experiment, similar bands with a molecular weight of 18 kDa have been identified across six bean species, namely white, pink, black, white, large northern, and small red beans. Furthermore, a band at 17 kDa, attributed to the β-subunit of α-amylase inhibitor, was observed in cranberry, light red kidney, and dark red kidney bean PIs, as reported by Rui *et al.* (2011). Previous studies on diverse bean genotypes have also revealed bands ranging from 36 to 40 kDa (Karaman *et al.,* 2022). Pinto beans have been found to contain smaller proteins with a molecular weight of 14 kDa. Interestingly, our study demonstrated a lower number of bands within the 24 to 38 kDa range, which aligns with the observations made by Rui *et al.* (2011). Soil microorganisms have been found to improve plant growth through various activities, including the production of metabolites, breaking down organic compounds, production of growth enhancers, and increasing nutrient availability through cooperative relationships. The present study confirms the beneficial role of *Trichoderma* fungi in promoting plant growth, as the treated plants exhibited better overall condition than the control plants. The peroxidase enzyme is typically associated with a molecular weight of 44 kDa, as reported by Arredondo-Peter and Escamilla (1993). In our study, the *Trichoderma* treatments exhibited a distinct band within the same molecular weight range. Marzol *et al.* (2022) identified a band with a molecular weight of 44 kDa in *Arabidopsis thaliana*, which was attributed to peroxidase. Notably, clear banding of peroxidase was observed between 35 and 50 kDa, consistent with

the specified molecular weight of 45 kDa reported by Nar *et al.* (2013), Akıncıoğlu *et al.* (1985), and Elhelaly (2022). Li *et al.* (2022) previously reported the presence of bands with molecular weights of 35.0 and 35.5 kDa, indicating the existence of PPO isomers.

Yıldız *et al.* (2022) observed a molecular weight of approximately 50 kDa for polyphenol oxidase isomers through SDS-PAGE and Native-PAGE electrophoresis. Li *et al.* (2022a) similarly obtained comparable results, demonstrating the molecular weight range of polyphenol oxidase (PPO) and peroxidase (POD) within the range observed in our study. According to Zenin and Park (1978), four isoforms of PPO exhibited molecular weights ranging from 39.0 to 57.5 kDa based on SDS-PAGE. In our investigation, SDS-PAGE revealed two bands at 35 and 50 kDa for PPO isozymes, while Native-PAGE showed visible and slightly visible bands at 50-70 kDa and 100 kDa, respectively, in agreement with the findings of Moeini Alishah *et al.* (2023). In black bean plants, Michel-Lopez *et al.* (2022) and Rui *et al.* (2011) reported similar findings. This discovery aligns with the outcomes of Rezvankhah *et al.* (2022), who likewise identified bands within the aforementioned molecular weight range during their investigation of bean plants.

CONCLUSION

The results of this experiment demonstrate the efficacy of *Trichoderma* fungi in promoting the growth of bean plants. Using a mixture of *Trichoderma* spores for seed treatment is suggested for improving physiology parameters in bean plants. In addition, seed coating with biological agents such as *Trichoderma* can be considered a promising biotechnological approach to improve growth and resistance to biotic and abiotic stresses. Our study also highlights that inducing gamma-ray mutations and selecting suitable mutants can mitigate the initial response of some plants to *Trichoderma* in the ecological root niche, leading to increased positive effects of this fungus. This approach holds potential as a promising technique to improve crop yield and quality in agriculture. Although the physiological changes of the plant depend on the method of inoculation and the use of different *Trichoderma* isolates, it is important to note that before recommending this biological agent, the interactions of different species of this fungus should be investigated independently, and then with each plant, and even with any varietal variety.

In general, the results of the studies showed that physical stimuli, such as varying doses of gamma rays, can effectively induce positive responses by eliciting physiological and biochemical changes. By genetically modifying these microorganisms, we were able to improve the quality of their action as growth-stimulating factors.

The results showed that the method used to increase the antagonist ability to increase the biocontrol ability of *Trichoderma* and improve the antagonistic power of *Trichoderma* by inducing mutation in the genome by irradiating specific doses of gamma rays has been effective By affecting the fungal spores of Trichoderma species, they increase the functional potential of the species.

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Competing interests

The authors declare that there are no competing interests.

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