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Investigating the activity of *Bacillus subtilis* and *Trichoderma harzianum* to mitigate Fusarium wilt disease of diverse cultivars of *Vicia faba*

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***Fusarium* wilt causes destructive effects on *Vicia faba* (VF). This study is innovative because it evaluates the comparative effects of various biological treatments on different VF cultivars to determine the therapeutic potential of each treatment against *Fusarium* wilt. In this study, the suppression of *F. verticillioides* (FV) by *Bacillus subtilis* and *Trichoderma harzianum* was compared at both the pot level and in vitro. The findings indicated that both *B. subtilis* and *T. harzianum* have antifungal effects on *F. verticillioides* in vitro, exhibiting mycelium inhibition percentages of 45.88% and 68.20%. Our results showed that treatments with *T. harzianum* significantly reduced the disease incidence (DI) in both the *V. faba* 'Giza-716' (G) and 'Sakha-101' (S) cultivars, providing comparable protection at 70.58% and 72.22%, respectively. After the afflicted plant received biological therapies, a notable increase in all yield components was observed. The G and S cultivars treated with *T. harzianum* exhibited the highest PPO activity, measuring 116.70% and 227.70%. The S cultivar treated with *T. harzianum* showed the highest POD activity at 289.60%. In conclusion, *T. harzianum* and *B. subtilis* as potential bio pesticides exhibited high efficacy against *F. verticillioides*, which causes wilt in several *Vicia faba* cultivars, due to their effectiveness and environmental friendliness.**

Keywords *Fusarium*, *Vicia faba*, *Trichoderma*, Biocontrol, Fungicidal

The *Vicia faba* plant has great economic importance in Egypt as a major legume crop for human and animal consumption, and it is also exported. It serves as a valuable source of protein, carbohydrates, essential minerals, fats, phosphorus, iron, calcium, and vitamins. Moreover, *Vicia faba* contributes to improving soil quality through nitrogen fixation and effective crop rotation. It helps break disease cycles caused by various plant pathogens[1,2]. Fava beans are susceptible to many diseases, especially fungal infections that affect the roots. *Fusarium* is a fungal pathogen that can cause significant damage to crops, including *Vicia faba*. The fungus can infect seeds, leading to reduced germination, seedling vigor, and yield. In mung beans, *Fusarium* wilt significantly reduces yield by impairing the plant's photosynthetic capacity and can result in yield losses ranging from 5 to 30%, with severe infections causing up to 100% yield loss[3]. *Fusarium verticillioides* (FV) is the most common phytopathogenic fungus affecting cereals[4]. The fungus infects the plant through its roots, colonizing the vascular tissues, blocking the passage of nutrient and water. Consequently, the plant exhibits yellow leaves, stunted growth, and wilting. In severe cases, the plant may complete die.

Traditional methods of managing *F verticillioides*, such as the use of disease-resistant crop varieties, physical methods have been employed to separate damaged or contaminated crops from healthy ones, chemical fungicides. However, these methods must be cost-effective, simple, and easily applicable for farmers. Physical

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Gravels	Fine gravels	Coarse sand	Medium sand	Fine sand	Silt	Clay	Texture class
2.2	4.7	5.2	45.3	21.2	7.5	15.7	

Table 1. Physical properties of the soil.

pH	E.C. mmhos/cm	Cations meq/L				Anion meq/L			
		Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	$SO4^{2-}$	HCO_3^-	CO_3^{2-}
7.8	1.44	2.7	0.7	2.2	1.2	4.2	1.4	1.3	0.02

Table 2. Chemical properties of the soil.

methods have shown limited effectiveness and specificity, whereas chemical methods tend to be expensive and can negatively impact the quality of food products Therefore, researchers explored alternative approaches, such as biocontrol agents, to manage fungal diseases[5,6]. Utilizing microorganisms that promote plant growth (PGPM) is considered a sustainable environmental approach to increasing agricultural productivity and reducing the dependence on chemical fertilizers. PGPM are helpful microorganisms that promote plant development and safeguard plants from diseases. PGPM, including bacteria and fungi, promote plant growth through various mechanisms such as phosphate solubilization, nitrogen fixation, production of plant hormones, and synthesis of antimicrobial components[7,8].

T. harzianum (TH) and *Bacillus subtilis* (BS) are well-known beneficial microorganisms extensively studied for their biological control properties. Biocontrol agents employ various mechanisms to suppress plant diseases, including competition for resources, production of antagonistic compounds, induction of systemic resistance in plants, and parasitism of pathogens, all of which contribute to a more resilient plant immune response and a healthier ecosystem[9]. *T. harzianum* could suppress *Fusarium* wilt by enhancing photosynthetic pigments, malondialdehyde, proline, and phenols[10]. Biocontrol agents not only suppress plant diseases but also play a crucial role in enhancing plant growth through the stimulation of nodule formation. These beneficial microorganisms can promote the activity of nitrogen-fixing bacteria, leading to increased nitrogen availability for the host plant. As a result, enhanced nodule development contributes to improved nutrient uptake and overall plant vigor, ultimately boosting growth and yield[11,12]. The present study aimed to evaluate the efficacy of *T. harzianum* and *Bacillus subtilis* in suppressing seed-borne FV in *Vicia faba*, using both in vitro and in vivo experiments, and to investigate the comparative effect of the tested biocontrol agents on the physiological and biochemical changes in faba cultivars.

Materials and methods

Sampling process

Two recommended cultivars of *Vicia faba* seeds, cv. Sakha-101 and cv. Giza-716, were obtained from the Department of Legume Crops Research, at the Field Crops Research Institute, Agricultural Research Center, Giza, Egypt (ARCGE).

The pathogenic fungus's origin

A pure culture of *Fusarium verticillioides*, designated as RCMB (008 005 "2" or FV, was obtained from the Regional Center for Mycology et al.-Azhar University. It was characterized morphologically according spores' shape; size and the produced hyphae. FV was sub-cultured on potato dextrose agar (PDA) plates (Difco, Georgia, GA, USA) and incubated at 27 °C for 7 days. The culture was further confirmed through a pathogenicity test, following the method outlined by[13–15].

Preparation of treatments

Bacillus subtilis MT110635 (30×10^6 cfu/g), and *Trichoderma harzianum* MT110634 (TH), (30×10^6 cfu/g), isolated from cultivated soil were used at the rate of 5 g/kg seeds (Both of the tested bioagents' was kindly received from Central Lab. of Organic Agriculture., Agricultural Research Center, Giza, Egypt (ARCGE)). The tested fungicide Rizolex-T 50 WP, was applied at 3 g/kg seeds. The seeds were sown in 40 cm pots filled with sterilized clay loam soil at the rate of 10 seeds per pot. Physical and chemical Soil characteristics shown in Tables 1 and 2. Seeds of the two tested cultivars, S and G, were sown separately. For comparison, an equal number of untreated seeds (10 seeds per pot) were sown in soil infested with FV.

In vitro study

To evaluate the antagonistic effects of *B. subtilis* and *T. harzianum* on *F. verticillioides*, a dual culture approach was carried out[16,17] with minor modifications. *F. verticillioides* was grown on agar plate was placed at a distance of 2 cm from the edge of the PDA agar plate and challenged on the other end of the plate at 2 cm from edge with *T. harzianum*. Streaking was carried out in case of *B. subtilis*. Petri plates were incubated for incubation at 28 ± 2 °C for 7 days. The following formula was used to calculate the mycelial growth reduction percentage:

$$\mathrm{I}\,(\%) = (\mathrm{Dc} \;- \mathrm{Dt}\;/\mathrm{Dc}) \times 100$$

I (%): inhibition percentage; Dc: average diameter of the pathogen (control); Dt: average diameter of the treated colonies.

In vivo assessment of FV on *Vicia faba*
Two cultivars of *Vicia faba*, G and S, were used. An equal number of seeds from the two cultivars were surface sterilized by submerging them for 3 min in 2% NaOCl, followed by washing with distilled, sterilized water. The pathogenic FV was applied to the soil at a rate of 5 mL per application, with each mL containing (3×10^7 cfu/mL). The treatments were designed with the three replicates for each treatment cultivated in separate pots or under the same environmental conditions:

T1: Ten healthy control seeds per pot were sown in sterilized soil.
T2: Ten seeds per pot were sown in soil infested with *F. verticillioides* for the control-infected group.
T3: *B. subtilis* (BS) + 10 seeds/pot were sown in soil infested with *F. verticillioides.*
T4: *T. harzianum* (TH) + 10 seeds/pot were sown in soil infested with *F. verticillioides.*
T5: Rizolex-T 50 WP was applied to the soil, and 10 seeds per pot were sown in soil infested with *F. verticillioides*.

During the winter, pots were stored in the greenhouse at garden of Botany and microbiology department, faculty of Science, Al-Azhar University (Latitude 30°03′15.48″N, longitude 31°19′12.75″E), which had day and night temperatures of 22 and 18 °C and relative humidity levels of 70 to 85%.

Disease symptoms and disease index
The disease symptoms were observed 45 days after seeding with minor modifications. A five-class score analogous to that published by Farrag et al.[18] was used to assess the disease index and plant protection. The possible outcomes included lower leaf yellowing (1), moderate yellowing of the plant (2), wilted plant with browning of vascular bands (3), and severely stunted and damaged plants (4). To compute the percent disease index (PDI) and the percentage of protection (P%).

Photosynthetic pigment determination
The photosynthetic pigments were evaluated to ascertain the presence of carotenoids, chlorophyll a, and chlorophyll b in fresh broad beans (BB) leaves. Using 50 mL of 80% acetone, the process involved extracting photosynthetic pigments from 0.5 g of fresh leaves. The greenness at 665, 649, and 470 nm was measured in the filtrate after filtration[1].

Proline content determination
The dried BB shoot's proline content was ascertained according to method used by[19]. In this method, 10 mL (3%) of sulfosalicylic acid was used to digest 0.5 g of dried shoots BB. In a boiling water bath, 2 mL of the filtrate was mixed with 2 mL of ninhydrin and 2 mL of glacial acetic acid. The mixture was left to sit in an ice bath for an hour to halt the reaction. After adding 4 mL of toluene, the absorbance at 520 nm was measured.

Total phenol determination
The technique of[20] was used to determine the BB phenols. Five milliliters of 80% ethyl alcohol were used to extract one gram of dried BB shoots for a whole day. After mixing 0.5 mL of the extract with 0.5 mL of Folin's reagent, the mixture was stirred for three minutes. Then, 1 mL of saturated Na_2CO_3 solution and 3 mL of purified water were added and well combined. After an hour, a blue color was observed at 725 nm.

mg chlorophyll (a)/g tissue = 11.63 (A665) − 2.39(A649).
mg chlorophyll (b)/g tissue = 20.11(A649) − 5.18(A665). Carotenoids = $1000 \times O.D_{470} - 1.82\ C_a - 85.02\ C_b/198$ = mg/g fresh weight. "A" denotes the reading of optical density.

Antioxidant enzymes
PPO and POD are well-documented markers of plant stress responses and pathogen resistance, making them suitable choices for assessing plant defense activation in our study. PPO is involved in the oxidation of phenolic compounds, leading to the formation of quinones, which contribute to pathogen resistance. Similarly, POD is essential in the oxidative burst response, catalyzing the formation of ROS that enhance defense signaling and reinforce plant cell walls. Peroxidase (POD) activity and polyphenol oxidase (PPO) of fresh BB were assayed by the method described by Elbasuney, El-Sayyad[21]. PPO and POD are well-documented markers of plant stress responses and pathogen resistance, making them suitable choices for assessing plant defense activation in our study. PPO is involved in the oxidation of phenolic compounds, leading to the formation of quinones, which contribute to pathogen resistance. Similarly, POD is essential in the oxidative burst response, catalyzing the formation of ROS that enhance defense signaling and reinforce plant cell walls.In this method; In a cooled centrifuge, 2 g of the BB shoot were homogenized with 10 mL of phosphate buffer pH 6.8 (0.1 M), and the then centrifuged at 2 °C for 20 min at 20,000 rpm. POD measured as the following; 2 mL of 20 mM pyrogallol was added, 5.8 mL of 50 mM phosphate buffer pH 7, 200 µL of the enzyme extract, and 2 mL of 20 mM hydrogen peroxide were added. The rate of increase in absorbance as pyrogallol was measured by UV spectrophotometer (Jenway) within 60 s at 470 nm and 25 °C. The amount of enzyme needed to catalyze the conversion of one micromole of hydrogen peroxide per minute at 25 °C was established as one unit of enzyme activity. Rather than employing enzyme extract, buffer was used to create the blank sample. PPO determined as the following; 2 mL of enzyme extract, 125 µmol of phosphate buffer (pH 6.8), and 100 µmol of pyrogallol. Add 1 mL of 5% Sulfuric

acid to terminate the reaction after the incubation period of 5 min at 25 °C. The developed color was measured at 430 nm for the blank sample, which was prepared using an extremely well-boiled enzyme extract. The variations in optical density/gram fresh weight/hour were used to express the enzyme activity (U/g F.wt/h).

Estimation of leghemoglobin (LHb) content in root nodules

LHb in the BB nodules was measured by an addendum to the determination of LHb[22]. In each treatment, four samples of frozen tissue were collected. The nodules were homogeneously mixed with 5 mL of 0.1 N potassium hydroxide, centrifuged for 10 min at 7500 rpm, and 1.5 mL of the supernatant water was mixed with 0.5 mL of 5 N KOH, and 0.1 mL of sodium dithionite ($Na_2S_2O_4$) was added to reduce it. The optical density of LHb was measured for 10 min at wavelengths of 537, 557, and 577 nm. The OD of leg LHb was calculated using the formula LHb = O.D.557 – 1/2(O.D.537–O.D.577).

Statistical analyses

One-way analysis of variance (ANOVA) was used to analyze the obtained findings. CoStat used the LSD test at $p < 0.05$ to show the significant differences between treatments. The findings ($n = 3$) were presented as means with ± standard errors.

Results

In this study, biological agents, namely *Trichoderma* and *Bacillus*, were used on two different cultivars of faba bean plants and compared to the fungicide Rezolex in the in-vitro. The study was then completed in vivo, and the effect of these agents on the extent of the pathogen's virulence was examined in their presence and absence. The results were confirmed by monitoring physiological processes inside the plant as indicators of disease, starting with measuring photosynthetic pigment content, proline, phenol, and antioxidant enzymes, and ending with the ability of these agents to promote the plant to form root nodules. The ability of these agents to directly affect the productivity of the faba bean plant has been confirmed.

Confirmation of pathogen

Fusarium verticillioides confirmed by morphological characters, The mycelium pink, with a cottony or fluffy appearance on PDA medium The colony diameter around 5–7 cm in diameter after 7 days of incubation at 27 °C. Microconida that are typically 2–5 µm in length and 1–2 µm in width. They are usually found in short chains and have a curved apical cell (Fig. 1).

Antifungal activity

In the current study, the efficacy of *T. harzianum* (TH) and *B. subtilis* (BS) in controlling seed-borne *F. verticillioides* (FV) was evaluated using the dual culture technique as shown in Fig. 2. Results showed that, the promising efficacy of both BS (Fig. 2B) and TH (Fig. 2C) against *F. verticillioides* compared to the growth of *F. verticillioides* alone as a control (Fig. 2A). Additionally, growth inhibition percentages were calculated, as shown in Fig. 2D. Results illustrated that, TH has more antifungal activity than BS compared to control, with inhibition percentages were 68.20% and 45.88%, respectively.

Disease severity and index

Table 3 and Fig. 3 displays the outcomes of three distinct treatments (BS, TH, and Rizolex), together with their impact on disease symptoms and cultivar protection against *F. verticillioides* wilt of *Vicia faba* G and S. The degree of disease symptoms and severity varies across the control groups, with S being 90% sensitive compared to G at 85%.

Differences in the degree of protection against *F. verticillioides* wilt among treatments, including BS, TH, and Rizolex have been observed. The BS treatment demonstrates moderate protection with a disease incidence (DI) of 47.50% for Giza-716 and 45.00% for Sakha-101, with protection percentages of 44.11% and 50%, respectively. The TH treatment exhibits superior protection with a DI of 25% for both G and S, and protection percentages of 70.58% and 72.22%, respectively. The Table 3 indicates that the applications of TH and rizolex on BB seeds yield promising results in mitigating disease symptoms and providing defense against *F. verticillioides*.

Photosynthetic pigments as observed in Fig. 4, the statistics indicate that the healthy control group had the highest concentrations of both chlorophyll a and b. Compared to the infected control plants, treatments with BS, TH, and rizolex have varying impacts on chlorophyll levels. Addition of BS led to a significant increase in both chlorophyll a (53.90%, 42%) and chlorophyll b (78.48%, 136.92%) for G and S respectively. TH treatment also increases both chlorophyll a (40.60, 78.80%) and chlorophyll b (120.08% for G and 87.15% for S). Figure 4 suggests that the BS and TH treatments had a beneficial impact on the recovery of chlorophyll a and b in *Vicia faba* G and S with *Fusarium* wilt. The results indicate that treatments with TH and BS significantly increased carotenoid levels compared to the infected control group, with TH demonstrating the largest increase 84.16% for cultivars G and 121.63% for cultivars S respectively.

Effect of treatments on nodule formation and legheamoglobine in Giza-716 and Sakha-101 varieties

Table 4 illustrates the effects of BS and TH on nodule production in G and S varieties. Compared to the healthy control group, nodule numbers in the *F. verticillioides*-infected control group are significantly reduced by 73.57% and 56.87% respectively. However, both varieties exhibit a marked increase in nodule development following treatment with BS (53.90% and 106.37%) and TH (40.60% and 85.48%) compared to infected control. Additionally, these values indicate a significant increase compared to the infected control group, suggesting that TH treatment benefits the nodule production of *F. verticillioides*-infected varieties. The treatment with rizolex

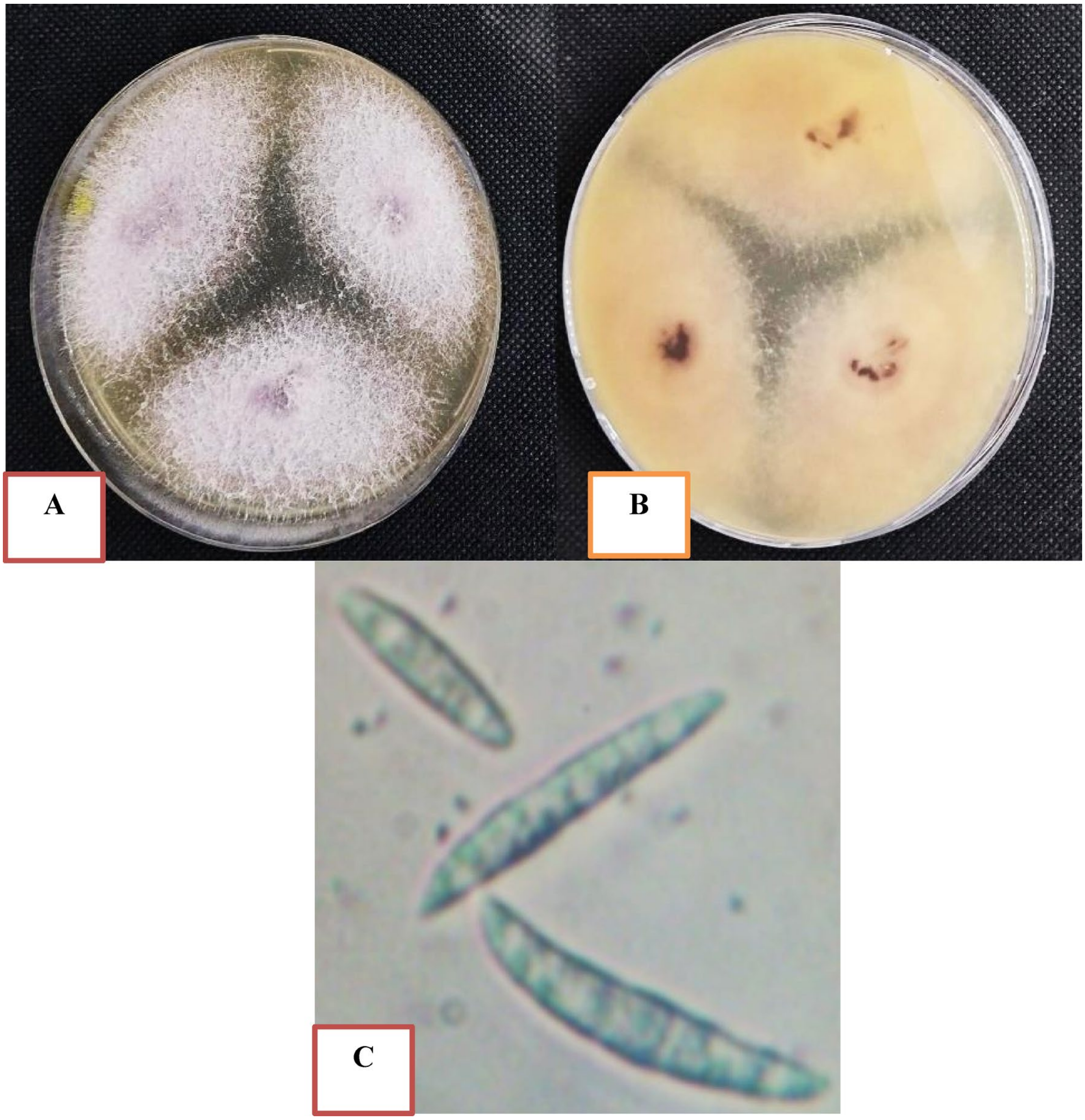

Fig. 1. Morphological characteristics of a *Fusarium verticillioides*. (**A**) Surface colony grown on a PDA medium showing characteristic radial growth with cottony, white mycelium and darker central regions. (**B**) Reverse colony on a PDA medium, displaying pigmentation and sporulation with dark yellow to orange. (**C**) Microscopic view of the *Fusarium verticillioides* conidia, which are elongated, multi-septate, and slightly curved under light microscope. 20 × 40 Ex.

has variable impacts; while it decreases nodules in group G, it increases them in group S. The results indicate that the leg hemoglobin levels in the infected control group are significantly lower than those in the healthy control group. However, the leg hemoglobin levels in infected plants treated with BS, and TH are significantly higher compared to the infected G and S control. The healthy control group exhibited the highest levels of leg hemoglobin for both plants.

Effect of BS and TH on yield

The findings (Table 5) indicate that the control group infected with *F. verticillioides* wilt had significantly fewer pods, seeds per plant, and seed weight per plant than the healthy S and G plant groups. The number of pods, number of seeds per plant, and seed weight were all significantly higher in the infected S plants treated with BS than in the untreated S plants by (130.20%, 115.60% and 114.40%) and G plants by 107.20%, 85.16% and 27.22%. These results of yield correlated to protection of treatments against Fusarium wilt. Similarly, TH, Sakha-101 showed increases of 14.80%, 90.98% and 127.35%, while G plants exhibited significant increases of 124%, 97.37% and 34.91%.

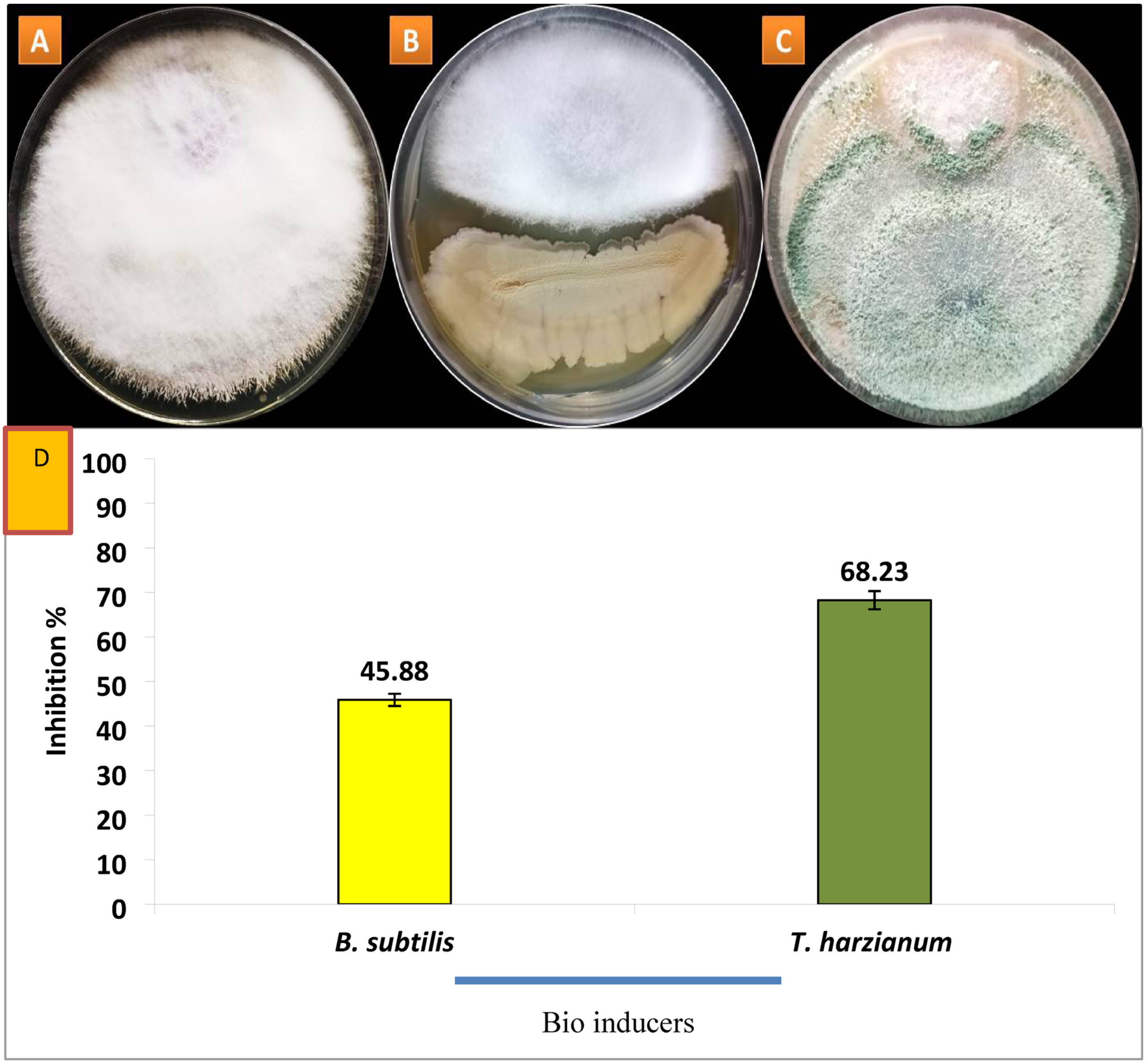

Fig. 2. In vitro antifungal activity of *B. subtilis* and *T. harzianum* against *F. verticillioides* using the dual culture method. (**A**–**C**) represent control, BS vs FV, and TH vs FV, respectively. (**D**) Percentages of FV inhibition under BS and TH conditions.

Effect of *B. subtilis* and *T. harzianum* on antioxidant enzymes

Enzymes PPO and POD are involved in stress reactions and defense processes in plants. Plants infected with *F. verticillioides* generally showed significant increased PPO and POD activity compared to the G uninfected ones. More precisely, the highest POD activity was observed in infected plants treated with Rizolex, while the significant highest PPO activity was noted in infected plants treated with TH. These results suggest that different treatments have varying effects on the activities of these enzymes, indicative of the plant's defense mechanisms against infection and stress. The ability of various treatments to modulate the physiological and biochemical processes in plants is highlighted by the differences in enzyme activity among the treatments (Fig. 5).

The findings show differences in the activities of these enzymes among the various treatments, suggesting unique physiological and biochemical responses to the interventions. Since enzyme levels often increase in response to infection or stress, it is not surprising that the healthy control group exhibits lower PPO and POD activities compared to the infected groups.

When compared to the infected control, significantly altered enzyme activities were observed in the infected Sakha-101 plants treated with BS, TH, and Rizolex, indicating that these treatments may affect the plants defense mechanisms. The Rizolex therapy demonstrates the highest PPO activity (289.70%), while the *T. harzianum* treatment exhibits the highest POD activity (289.60%). These results suggest that the various treatments uniquely affect these enzymes' activities, potentially influencing the plants' ability to combat *F. verticillioides* wilt. The recovery of the wilt disease associated with improvement of antioxidant enzymes PPO, POD.

Treatment	cultivars of *Vicia faba* seeds	Disease symptoms Classes of *Fusarium verticillioides*					DI (disease index) (%)	Protection (%)
		0	1	2	3	4		
Control healthy	Giza-716	10	0	0	0	0	0	–
Control Infected		0	0	1	4	5	85.00	0
Infected + *B. Subtilis*		0	3	5	2	0	47.50	44.11
Infected + *T. harzianum*		2	6	2	0	0	25	70.58
Infected + *Rizolex*		6	3	1	0	0	12.50	85.29
Control healthy	Sakha-101	10	0	0	0	0	0	–
Control Infected		0	0	0	4	6	90.00	0
Infected + *B. Subtilis*		1	6	0	0	3	45.00	50.00
Infected + *T. harzianum*		4	2	4	0	0	25	72.22
Infected + *Rizolex*		6	2	0	2	0	20.00	77.77

Table 3. Effect of treatments on *F. verticillioides* disease index on cultivars of *Vicia faba*. Protection % calculated as the following ((PDI of control – PDI of treated)/(PDI of treated)) × 100.

Effect of *B. subtilis* and *T. harzianum* on phenols

Figure 6 data indicates that the levels of phenol increase in the *F. verticillioides*- infected G and S plants compared to the healthy control, with a more significant increase in the presence of the biocontrol agents *T. harzianum* and *B. subtilis*. The highest level was recorded at TH at both varieties S and G (227.70% and 68.80%). Thus, these treatments may induce a higher level of stress or defense response in the plants since phenols are involved in plant defense mechanisms against *F. verticillioides*.

Effect of *B. subtilis* and *T. harzianum* on proline content

Figure 7 indicates that the levels of proline significant increase in the *F. verticillioides*-infected compared to the healthy controls G and S, with a more pronounced increase observed when biocontrol agents TH and BS are present. The highest levels were recorded with TH in both varieties at 136.88% and 121.79%, respectively.

Discussion

The detrimental effects of *Fusarium* on plants highlight the need for efficient management strategies and research efforts to mitigate the damage caused by this pathogen. To minimize the disastrous impacts of *Fusarium*, it is crucial to maintain crop yields and preserve the financial stability of agricultural systems through the implementation of biological disease management strategies[9].

Several species of *Trichoderma* have been identified as potential sources of bioactive metabolites and are recognized for their effectiveness as biocontrol agents against fungal plant diseases. Additionally, numerous *Bacillus* species, including *B. amyloliquefaciens*, *B. licheniformis*, and *B. subtilis*, have been identified as plant-growth-promoting bacteria and biocontrol agents (BCAs)[23].

In the current study, results confirmed that TH and BS have antifungal activity toward *F. verticillioides* where inhibition percentages were 68.20% and 45.88% respectively. Previous study evaluated the antifungal activity of *T. viride* and TH strains against *F. verticillioides* strains with inhibition of 70.46%, while TH exhibited inhibition of 60.64%. A recent study assessing *T. asperellum*'s antagonistic effect on *Fusarium* spp[24]. The dual culture technique found that *Trichoderma* significantly impeded *F. verticillioides*' growth[25]. Also, another study assessed the antifungal activity of TH toward *Fusarium sudanense* where results showed the promising activity of TH against *Fusarium sudanense*[26]. These findings suggest that the effectiveness of TH, BS and Rizolex in reducing the severity of *F. verticillioides*-induced disease in BB seeds varies. TH and Rizolex show the most promising results regarding disease control, with Rizolex providing the highest level of protection, 12.5%, 20%, with protection 85.29%, 77.77% at Giza-716, and Sakha-101 respectively. TH is one of the most potent biological resistance components, defending against fungal plant diseases through a complex network of interrelated mechanisms. Studies have shown that biocontrol agents such as *Trichoderma* spp. produce hydrolytic enzymes (e.g., chitinases, glucanases) that degrade *Fusarium* cell walls, as well as antimicrobial secondary metabolites that inhibit its growth[27]. TH can produce plant physiological and structural immunity against various harmful plant microbes and diseases. These results can be explained by Abdelaziz et al.[28] reported that, due to the formation of hydrocyanic acid, siderophore, and indole-3-acetic acid (IAA), as well as phosphate solubilization, TH enhanced protection against *Fusarium* wilt by 50% in pepper plants. Additionally, extracts from TH contain chemicals with antifungal activity, antioxidants, and plant growth stimulators. BS improved plant development and results in 40% inhibition of *Fusarium* in vitro through distortions and uncharacteristic swellings in fusarial mycelium and by secretion of chitinase, xylanase, pectinase, volatiles, and other fungicidal components[29]. The results suggest that treatments with BS and TH have beneficial effects on the recovery of chlorophyll a, chlorophyll b, and carotenoid levels in *Vicia faba* plants infected with FV wilt.

In BB cultivars, rhizobacteria and the plant exchange signaling chemicals during the intricate process of root nodule formation. The bacteria supply the BB with fixed nitrogen, while the BB provides the bacteria with

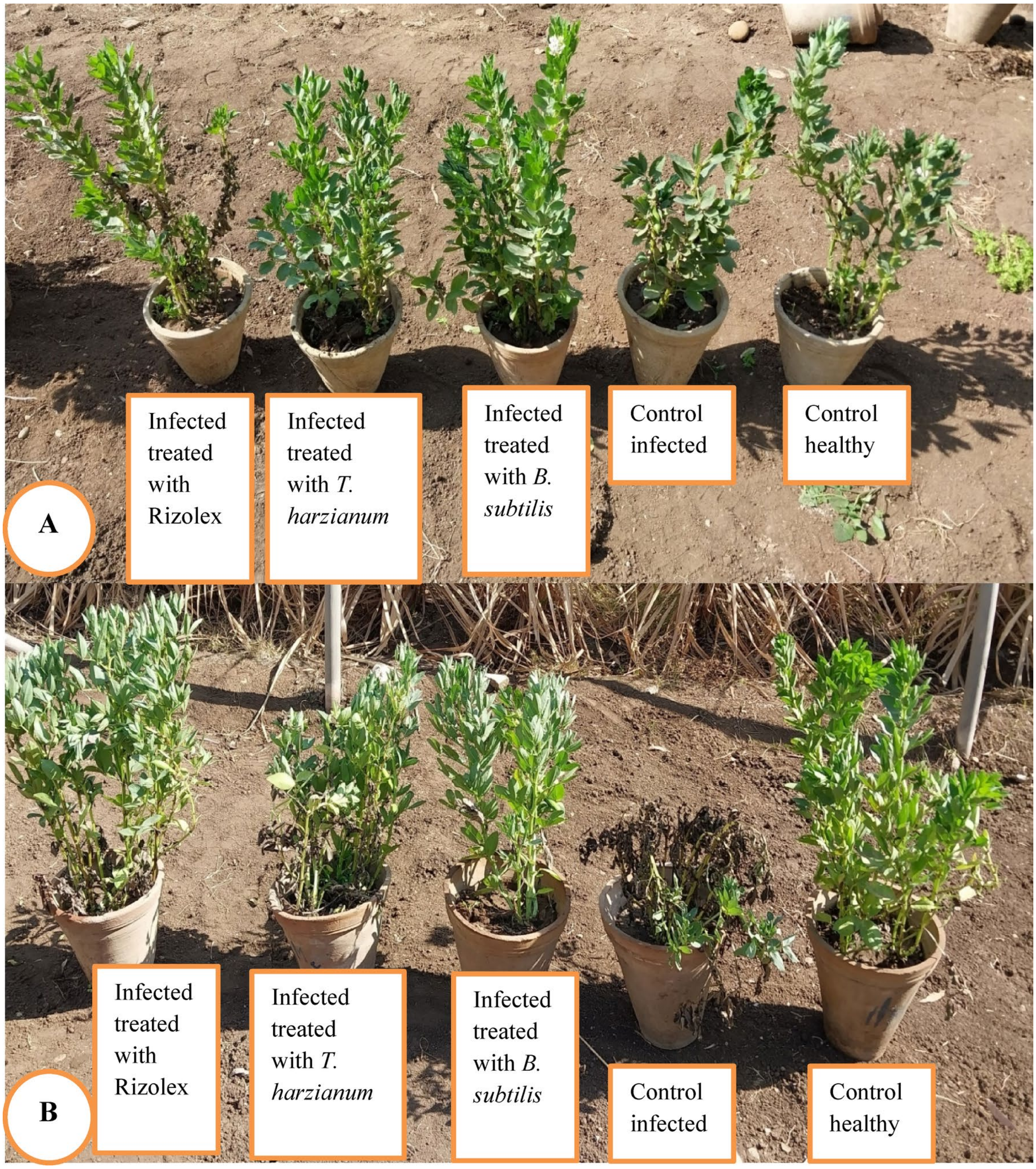

Fig. 3. Effect of *F. verticillioides* and treatments on different cultivars; (**A**) Giza-716, and (**B**) Sakha-101.

carbohydrates and other nutrients[30]. Hemoglobin levels were significantly decreased in the infected control group, suggesting that the infection adversely affected the hemoglobin synthesis in both BB cultivars. The BS and TH treatments showed improvements compared to the infected control group. For both BB cultivars, the number of nodules per plant increased, indicating that these treatments could partially mitigate the negative effects of the infection on nodule formation. Our study is consistent with another that emphasized the importance of TH in inducing root nodule formation and leghemoglobin production in BB[31].

Comparing the infected control plants with the healthy control BB cultivar plants, the findings reveal a significant decrease in the pod number, seeds per plant, and seed weight. When BS and TH are applied to infected plants, there is an increase in the number of pods, seeds per plant, and seed weight, as well as improvement in the plants' growth and seed yield. Thus, the detrimental effects of *F. verticillioides* on both BB cultivars G and S can be mitigated with the aid of these bioagents. Although not to the same extent as BS and TH, the application of Rizolex to infected plants also enhances the growth and seed production of both BB cultivars. The preceding investigation demonstrated that the application of bioagents increased the survival rates of *Vicia faba*, which can explain the observed results: an increase in the number of pods, as well as growth parameters[32].

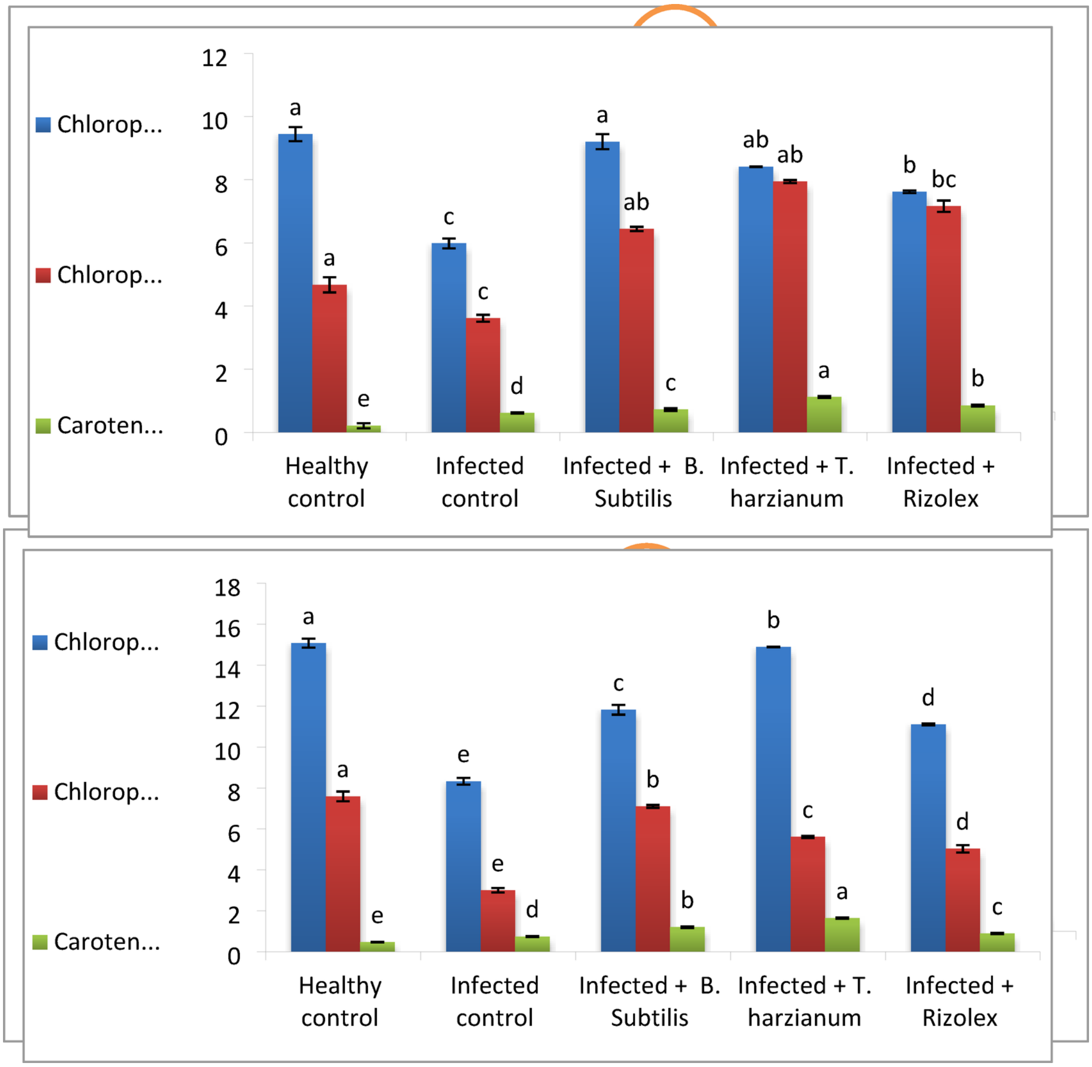

Fig. 4. Photosynthetic pigments of infected cultivars and treatments (*B. Subtilis*, *T. harzianum* and Rizolex); (**A**). Giza-716 and (**B**) Sakha-101, a, b, c, d, e, represent the significant letters.

Treatments	Number of nodules /plant		Leg hemoglobin	
	Giza-716	Sakha-101	Giza-716	Sakha-101
Healthy control	28.00 ± 1.01^{a}	36.40 ± 1.01^{a}	0.35 ± 0.001^{a}	0.59 ± 0.004^{a}
Infected control	7.40 ± 1.04^{c}	15.70 ± 1.02^{d}	0.13 ± 0.004^{e}	0.22 ± 0.002^{e}
Infected + BS	22.40 ± 1.05^{ab}	32.40 ± 1.03^{b}	0.32 ± 0.009^{b}	0.47 ± 0.001^{c}
Infected + TH	22.40 ± 1.27^{ab}	29.12 ± 1.019^{bc}	0.28 ± 0.004^{c}	0.54 ± 0.002^{b}
Infected + Rizolex	12.13 ± 0.73^{b}	27.12 ± 0.53^{c}	0.25 ± 0.003^{d}	0.43 ± 0.004^{d}

Table 4. Effect of *B. subtilis* and *T. harzianum* on Nodule Formation and leg hemoglobin. BS: *B. Subtilis* and TH: *T. harzianum*, a, b, c, and d are significant letters. (The findings (n = 3) were presented as means with ± standard errors).

Infected plants generally exhibit higher PPO and POD values than the healthy control. The *F. verticillioides* can cause the BB plant to produce these enzymes to eliminate free radicals resulting from the pathogen infection, which explains these results. Proline and phenol are essential for improving BB plant defense and infection resistance. Their accumulation and participation in various physiological functions help BB resist pathogen invasion, control the spread of disease, and maintain overall health and survival[33]. The growth and productivity of BB depend on the symbiotic relationship between rhizobia bacteria and BB cultivars G and S. By producing root nodules, BB can access a nitrogen source not readily available in the soil, reducing their need for nitrogen fertilizers and supporting sustainable agriculture[34,35]. The findings indicate that the BB cultivars, G and S, as

Treatments	Number of pods (per plant)		Number of seeds/plant		Weight of seeds/plant	
	Giza-716	Sakha-101	Giza-716	Sakha-101	Giza-716	Sakha-101
Healthy control	30.10 ± 1^{a}	48.16 ± 0.1^{a}	66.13 ± 0.1^{a}	92.58 ± 0.08^{a}	159 ± 0.09^{a}	270.30 ± 0.1^{a}
Infected control	12.50 ± 0.01^{c}	18.00 ± 0.02^{c}	30.53 ± 0.01^{d}	36.70 ± 0.011^{d}	116.0 ± 0.01^{d}	117.20 ± 0.009^{c}
Infected + BS	25.90 ± 0.82^{a}	41.44 ± 0.28^{a}	56.53 ± 0.2^{b}	79.14 ± 0.19^{ab}	147.58 ± 0.02^{b}	250.89 ± 0.21^{b}
Infected + TH	28.00 ± 0.58^{a}	44.80 ± 0.05^{a}	60.26 ± 0.04^{b}	70.09 ± 0.05^{b}	156.50 ± 0.08^{a}	266.05 ± 0.03^{a}
Infected + Rizolex	18.20 ± 1.02^{b}	29.12 ± 1^{b}	44.26 ± 0.01^{c}	61.97 ± 0.21^{c}	137.00 ± 0.2^{c}	249.90 ± 0.02^{b}

Table 5. Effect of *B. subtilis* and *T. harzianum* on yield.

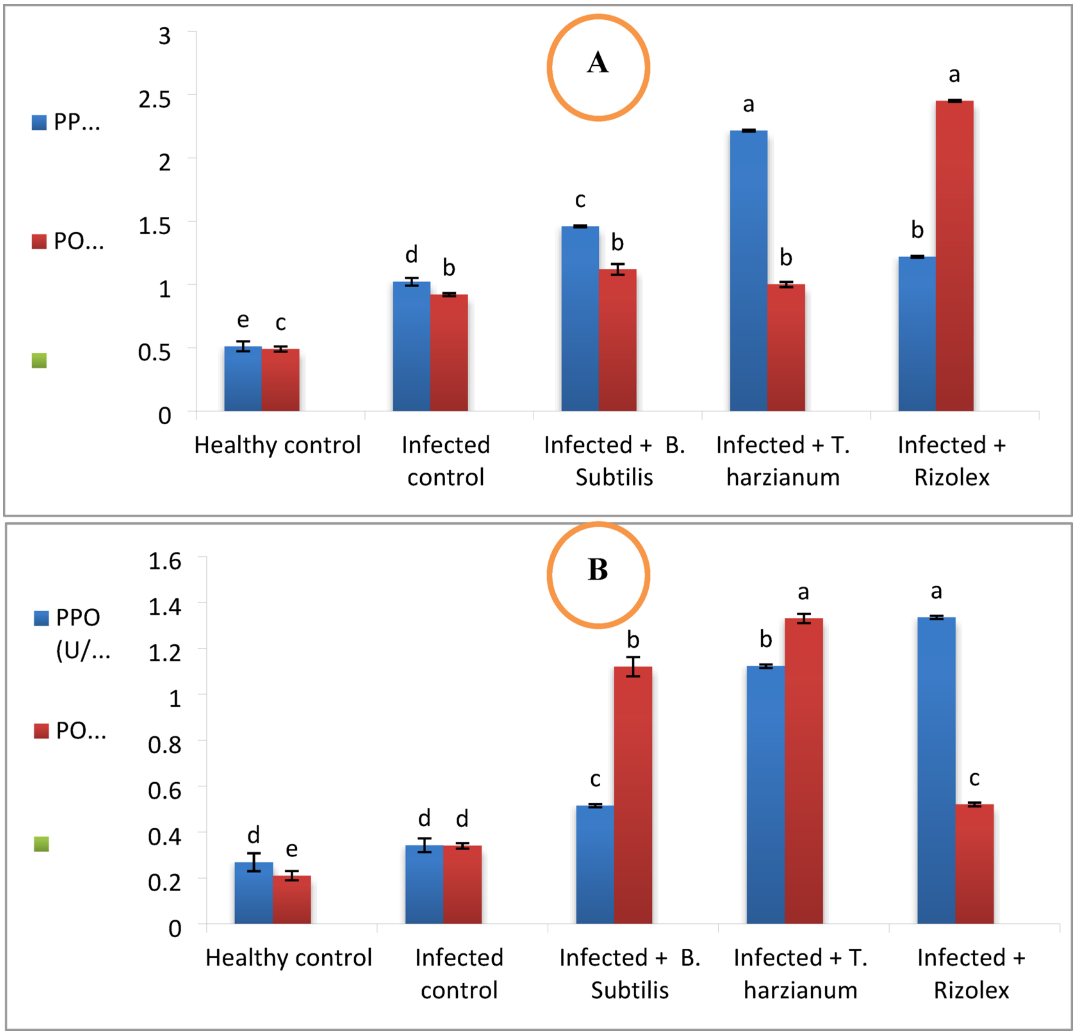

Fig. 5. Effect of BS and TH on antioxidant enzymes in Giza-716 (**A**) and Sakha-101 (**B**). The activity of each enzyme is shown in the figure as U/g F. wt./h. a, b, c, d, e, represent the significant letters.

well as the treatment, impact the levels of phenol and proline. G and S exhibit similar phenol levels in the healthy control group, with values of 0.42. However, proline levels differ, with S at 0.23 and G at 0.38. Both cultivars exhibit higher phenol and proline levels in the infected control group than in the healthy control. The combination of BS infection among treatment groups results in increased amounts of proline and phenol, with values of 0.96 for both G and S. Further increases in phenol and proline levels were observed following infection and treatment with TH, with values reaching 1.16 for both G and S. The possibility of the effect of biological factors based on what was studied in previous studies, which is due to the production of these biological factors some substances that stimulate plant growth, so they induce defensive substances that inhibit the action of the pathogen inside the plant and the plant's resistance improves[36]. TH is one of the most potent biological resistance components, defending against fungal plant diseases through a complex network of interrelated mechanisms.

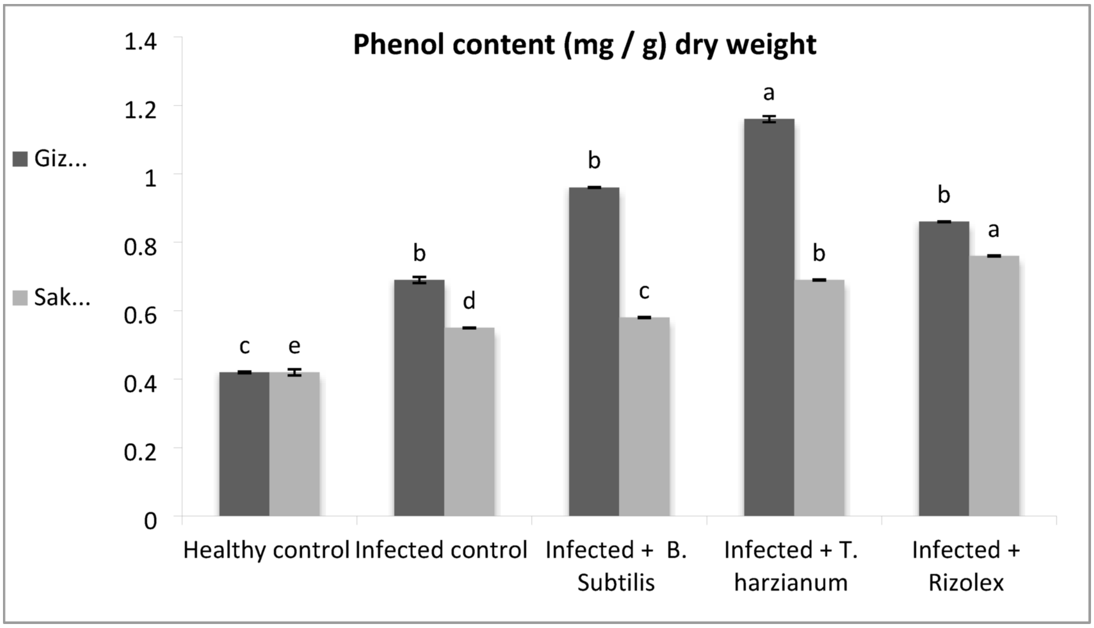

Fig. 6. Effect of BS and TH on phenols Giza-716 and Sakha-101. a, b, c, d, e, represent the significant letters.

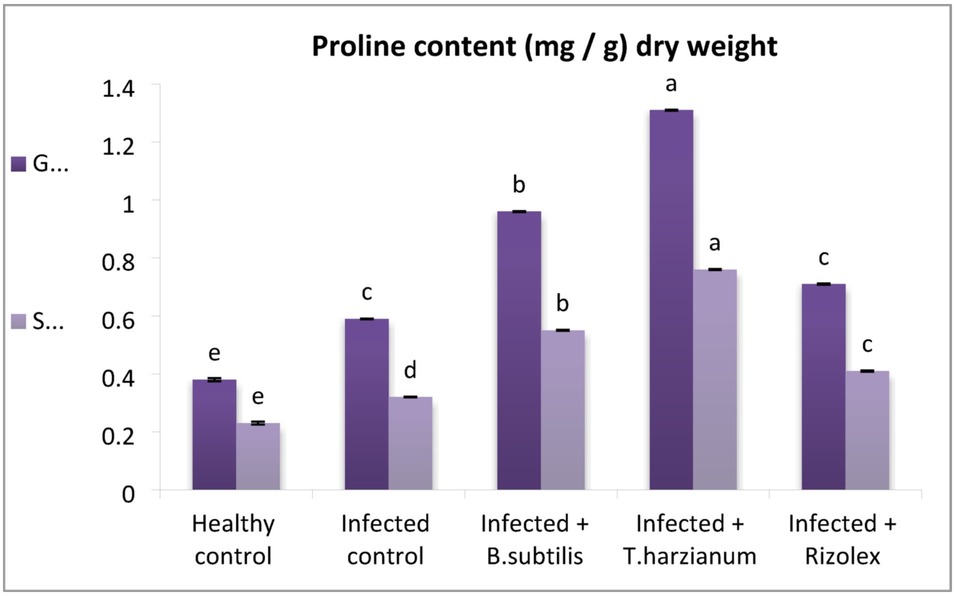

Fig. 7. Effect of *B. subtilis* and *T. harzianum* on proline in Giza-716 and Sakha-101.

TH can produce plant physiological and structural immunity against various harmful plant microbes and diseases. Finally, our results demonstrated the high severity of *F Verticillioides* on both BB cultivars. Bioagents highly dramatically migrated the harmful effects on both cultivars, but the Bioagents' effects mainly depend on BB cultivars. Our findings indicated superior antifungal activity of *Trichoderma* may arise from its diverse modes of action, including mycoparasitism, production of hydrolytic enzymes, and secondary metabolites, in contrast to *Bacillus* which primarily rely on antimicrobial lipopeptides and induction of systemic resistance in plants. Additionally, environmental factors such as soil composition, humidity, and temperature may have enhanced *Trichoderma* efficacy[37].

Conclusion

This study highlights the significant potential of biological agents in managing *Fusarium verticillioides* wilt in *Vicia faba* cultivars. The comparative evaluation revealed that both *B. subtilis* and *T. harzianum* exhibit significant antifungal activity against *F. verticillioides*, with *T. harzianum* demonstrating superior inhibition rates. The in vitro and in vivo experiments highlighted that *T. harzianum* significantly reduced disease incidence and improved plant growth and yield components in both 'Giza-716' and 'Sakha-101' cultivars. The application of *T. harzianum* resulted in the highest reductions in disease incidence, alongside notable increases in chlorophyll content, carotenoid levels, and nodule formation, which are critical indicators of plant health and productivity. Additionally, *T. harzianum* treatments enhanced the activity of antioxidant enzymes and increased phenol and proline content, suggesting an improved defense response in treated plants. The findings indicate that biological agents like *T. harzianum* and *B. subtilis* can effectively manage *Fusarium* wilt, offering an environmentally friendly alternative to chemical fungicides. These bioagents suppress pathogen growth and promote overall plant health. The future studies will concentrate on optimizing formulation techniques and delivery methods that could improve the stability, persistence, and field performance of *T. harzianum* and *B. subtilis*.

Data availability
All data generated or analyzed during this study are included in this manuscript.

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References

1. Attia, M. S. et al. Protective role of mycosynthesized bimetallic ZnO-CuO nanoparticles as therapeutic nutrients to enhance the resistance of *Vicia faba* against fusarium wilt disease. *Agronomy* **13**(11), 2725. https://doi.org/10.3390/agronomy13112725 (2023).
2. Hashem, A. H. et al. *Bacillus megaterium*-mediated synthesis of selenium nanoparticles and their antifungal activity against *Rhizoctonia solani* in faba bean plants. *J. Fungi* **7**(3), 195. https://doi.org/10.3390/jof7030195 (2021).
3. Van Haeften, S. et al. Fusarium wilt constrains mungbean yield due to reduction in source availability. *AoB Plants* **16**(2), plae21. https://doi.org/10.1093/aobpla/plae021 (2024).
4. Vasić, T. et al. Phytopathogenic fungi causers fungal diseases of the faba bean (*Vicia faba* L.) in Serbia. Biol. Nyssana https://doi.org/10.5281/zenodo.3463990 (2019).
5. Khattab, A. M. et al. Daphnia magna and Gammarus pulex, novel promising agents for biomedical and agricultural applications. *Sci. Rep.* **12**(1), 13690. https://doi.org/10.1038/s41598-022-17790-z (2022).
6. Tanani, M. A. Haemogram changes in the desert locust *Schistocerca gregaria* (Orthoptera: Acrididae) by different extracts from the wild plant *Fagonia bruguieri* (*Zygoplyllaceae*). *Al-azhar Bull. Sci.* **21**(1), 67–95 (2010).
7. Soliman, E. A., Abdelaziz, A. M., El Dorry, M. A. & Attia, M. S. Protective role of biomass endophytic fungi to enhance potato plant responses against wilt disease caused by *Ralstonia solanacearum*. *Physiol. Mol. Plant Pathol.* **131**, 102287. https://doi.org/10.1016/j.pmpp.2024.102287 (2024).
8. Attia, M. S. et al. Application of *Rhizopus microsporus* and *Aspergillus oryzae* to enhance the defense capacity of eggplant seedlings against Meloidogyne incognita. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* **51**(3), 1–23. https://doi.org/10.15835/NBHA51313300 (2023).
9. Hashem, A., Abdelaziz, A. M. & Attia, M. S. Impact of plant growth promoting fungi on biochemical defense performance of tomato under fusarial infection. *Egypt. J. Chem.* **65**(132), 291–301. https://doi.org/10.21608/ejchem.2022.124008.5532 (2022).
10. Abdelaziz, A. M., Mohamed, A. S. & Attia, M. S. Protective role of *Claroideoglomus etunicatum* and *Trichoderma harzianum* to improve growth and physiological immune responses of *Olea europaea* tolerance against *Fusarium solani*. *Physiol. Mol. Plant Pathol.* **136**, 102593. https://doi.org/10.1016/j.pmpp.2025.102593 (2025).
11. Ismail, M. A. & Amin, M. A. Response of wheat (*Triticum aestivum* L.) plants to foliar spraying of some bio-stimulants (yeast extract, arginine, b12 and their interactions). *Al-Azhar Bull. Sci.* **24**(2–C), 1–10 (2013).
12. Goel, A., Sindhu, S. & Dadarwal, K. Stimulation of nodulation and plant growth of chickpea (*Cicer arietinum* L.) by *Pseudomonas* spp. antagonistic to fungal pathogens. *Biol. Fertil. Soils* **36**, 391–396. https://doi.org/10.1007/s00374-002-0554-5 (2002).
13. Elwakil, M. A., El-Refai, I. M., Awadallah, O. A., El-Metwally, M. A. & Mohammed, M. S. Seed-borne pathogens of faba bean in Egypt: Detection and pathogenicity. *Plant Pathol. J. Faisalabad* **8**(3), 90–97. https://doi.org/10.3923/ppj.2009.90.97 (2009).
14. Rahjoo, V. et al. Morphological and molecular identification of Fusarium isolated from maize ears in Iran. *J. Plant Pathol.* **90**, 463–468 (2008).
15. Attia, M. S., Salem, M. S. & Abdelaziz, A. M. Endophytic fungi *Aspergillus* spp. reduce fusarial wilt disease severity, enhance growth, metabolism and stimulate the plant defense system in pepper plants. *Biomass Convers. Biorefin.* **14**(14), 16603–16613. https://doi.org/10.1007/s13399-022-03607-6 (2024).
16. Singh, L. & Sati, S. C. Antagonistic activity of isolated root endophytic freshwater fungus *Anguillospora longissima* against pathogenic fungi. *Natl. Acad. Sci. Lett.* https://doi.org/10.1007/s40009-019-00818-w (2020).
17. Prachi Singh, P. S. et al. Exploration of multitrait antagonistic microbes against *Fusarium oxysporum* f. sp. *lycopersici*. *J. Appl. Nat. Sci.* https://doi.org/10.31018/jans.v11i2.2111 (2019).
18. Farrag, A., Attia, M. S., Younis, A. & Abd Elaziz, A. Potential impacts of elicitors to improve tomato plant disease resistance. *Al Azhar Bull. Sci.* **9**, 311–321 (2017).
19. Desouky, S. Effect of salicylic acid on photosynthesis and some antioxidant enzymes of stressed scenedesmus obliquus cultures. *Al-azhar Bull. Sci.* **19**(2–C), 91–108 (2008).
20. Abdelaziz, A. M. et al. Inhibition of *Aspergillus flavus* growth and aflatoxin production in *Zea mays* L. using endophytic *Aspergillus fumigatus*. *J. Fungi* **8**(5), 482. https://doi.org/10.3390/jof8050482 (2022).
21. Elbasuney, S. et al. Stable colloidal iron oxide nanoparticles: A new green nanofertilizer and therapeutic nutrient for eggplant immune response against fusarium wilt disease. *J. Cluster Sci.* **35**(4), 983–997. https://doi.org/10.1007/s10876-023-02527-3 (2024).
22. Sofy, A. R., Attia, M. S., El-Monem, M. A., Sharaf, A. E. M. A. & El-Dougdoug, K. A. Bean yellow mosaic potyvirus potential on nodulation and N2-fixation of faba bean plants. *J. N. Y. Sci.* **7**(9), 101–109 (2014).
23. Khan, N., Maymon, M. & Hirsch, A. M. Combating *Fusarium* infection using *Bacillus*-based antimicrobials. *Microorganisms* **5**(4), 75. https://doi.org/10.3390/microorganisms5040075 (2017).
24. Yassin, M. T., Mostafa, A. A. F., Al-Askar, A. A., Sayed, S. R. & Rady, A. M. Antagonistic activity of *Trichoderma harzianum* and *Trichoderma viride* strains against some fusarial pathogens causing stalk rot disease of maize, in vitro. *J. King Saud Univ.-Sci.* **33**(3), 101363. https://doi.org/10.1016/j.jksus.2021.101363 (2021).
25. Jambhulkar, P. P. et al. Potential native *Trichoderma* strains against *Fusarium verticillioides* causing post flowering stalk rot in winter maize. *Crop Prot.* **152**, 105838. https://doi.org/10.1016/j.cropro.2021.105838 (2022).
26. Larran, S., Santamarina Siurana, M. P., Roselló Caselles, J., Simón, M. R. & Perelló, A. In vitro antagonistic activity of *Trichoderma harzianum* against *Fusarium sudanense* causing seedling blight and seed rot on wheat. *ACS Omega* **5**(36), 23276–23283. https://doi.org/10.1021/acsomega.0c03090 (2020).
27. Harman, G. E., Howell, C. R., Viterbo, A., Chet, I. & Lorito, M. Trichoderma species—Opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* **2**(1), 43–56. https://doi.org/10.1038/nrmicro797 (2004).

28. Abdelaziz, A. M. et al. Biocontrol of Fusarium wilt disease in pepper plant by plant growth promoting *Penicillium expansum* and *Trichoderma harzianum*. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* **51**(3), 1–23. https://doi.org/10.15835/NBHA51313302 (2023).
29. Khan, N. et al. Antifungal activity of Bacillus species against Fusarium and analysis of the potential mechanisms used in biocontrol. *Front. Microbiol.* **9**, 2363. https://doi.org/10.3389/fmicb.2018.02363 (2018).
30. Omer, A. M., Osman, M. S. & Badawy, A. A. Inoculation with *Azospirillum brasilense* and/or *Pseudomonas geniculata* reinforces flax (*Linum usitatissimum*) growth by improving physiological activities under saline soil conditions. *Bot. Stud.* **63**(1), 15 (2022).
31. Abd-Alla, M. H., Al-Amri, S. M. & El-Enany, A. W. E. Enhancing rhizobium-legume symbiosis and reducing nitrogen fertilizer use are potential options for mitigating climate change. *Agriculture* **13**(11), 2092. https://doi.org/10.3390/agriculture13112092 (2023).
32. Osman, M. S., Badawy, A. A., Osman, A. I. & Abdel Latef, A. A. H. Ameliorative impact of an extract of the halophyte *Arthrocnemum macrostachyum* on growth and biochemical parameters of soybean under salinity stress. *J. Plant Growth Regul.* **40**, 1245–1256. https://doi.org/10.1007/s00344-020-10185-2 (2021).
33. Kumar, K., Debnath, P., Singh, S. & Kumar, N. An overview of plant phenolics and their involvement in abiotic stress tolerance. *Stresses* **3**(3), 570–585. https://doi.org/10.3390/stresses3030040 (2023).
34. Wheatley, R. M. et al. Lifestyle adaptations of Rhizobium from rhizosphere to symbiosis. *Proc. Natl. Acad. Sci.* **117**(38), 23823–23834. https://doi.org/10.1073/pnas.2009094117 (2020).
35. Kawaka, F. Characterization of symbiotic and nitrogen fixing bacteria. *AMB Express* **12**(1), 99. https://doi.org/10.1186/s13568-022-01441-7 (2022).
36. Abdel Latef, A. A. H., Omer, A. M., Badawy, A. A., Osman, M. S. & Ragaey, M. M. Strategy of salt tolerance and interactive impact of *Azotobacter chroococcum* and/or *Alcaligenes faecalis* inoculation on canola (*Brassica napus* L.) plants grown in saline soil. *Plants* **10**(1), 110. https://doi.org/10.3390/plants10010110 (2021).
37. Ongena, M. & Jacques, P. Bacillus lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol.* **16**(3), 115–125 (2008).

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Author contributions
Mohamed S. Attia, Deiaa A. El-Wakil, Amr H. Hashem, and Amer M. Abdelaziz Conceptualized the study, Designed the study, Conducted experiments, collected and analyzed data, provided resources and materials, wrote the manuscript, reviewed and edited the manuscript; Abdulaziz A. Al-Askar, Rawan Saif Alotaibi, Saad. A. Abdel–Kader: Assisted with experimental procedures, reviewed the manuscript; Hamada AbdElgawad: Performed data analysis, interpreted the results, contributed to writing and editing the manuscript, All authors reviewed the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Ethics approval and consent to participate
There are no experiments on people or animals in this study.

Plant collection
The plant collection and use were in accordance with all the relevant guidelines.

Additional information
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