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Biocontrol of *Fusarium* root rot in squash using mycorrhizal fungi and antagonistic microorganisms

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Abstract

Background: The study amid to investigate the influence of *Trichoderma album*, *T. harzianum, T. koningii*, *Bacillus subtilis* (EF1) and *Pseudomonas fluorescens* against *Fusarium solani*, the causal agent of root rot in squash under in vitro conditions. Field experiment was conducted to evaluate the effects of arbuscular mycorrhizal fungi *i.e.*, (*Glomus intraradices*, *G. monosporum*, *G. etunicatu,* AMF) and *T. harzianum* (TZ) as well as *B. subtilis* (BS) either as individual or combined treatment against *Fusarium* root rot of squash in two successive seasons of study 2020 and 2021 using New Eskandrani cultivar.

Results: *Trichoderma harzianum* caused the greatest reduction in mycelial growth of *F. solani* (75.17%), followed by *T. album* and *T. koningii*. Amongst the tested 4 cultivars (Sakata, Galaxy, New Eskandrani H1 hybird, Hollr Queen F1 hybrid) in glasshouse, Hollr Queen was the most resistant to the infection of *F. solani* with survival rate (84.92%), whereas New Eskandrani c.v was the most susceptible. Results of filed experiments proved that the combined inoculation of AMF + TZ + BS, AMF + BS, AMF + TZ, TZ + BS resulted in significant elevation of total chlorophyll, carotenoids, free phenolic compounds, free amino acids, total protein as well as the antioxidative enzyme activities (*i.e.* Superoxide dismutase, Peroxidase and Polyphenol-oxidase) and contents of macro and micro elements. Results further showed that the combined treatments caused a significant decrease in disease severity in both seasons with subsequent significant increase of plant growth parameters as well as total fruit yield/plant and total fruit yield/feddan.

Conclusion: It could be concluded that the combined inoculations of the tested bioagents proved to have potentials in control of *Fusarium* root rot but large scale field experiments should be conducted before any ultimate conclusion or recommendation was drawn.

Keywords: Squash, Biological control, *Fusarium solani*, Mycorrhizal fungi, *Trichoderma*, *Bacillus*, *Pseudomonas*

Background

Summer squash (*Cucurbita pepo* L.) belongs to Cucrbitaceae family. Root rot of squash is a soil borne disease caused by several fungal pathogens including *Fusarium* spp., *Rhioctonia solani, Pythium* spp. and *Phytophthora* spp. (Nawar 2007). *Fusarium* root rot caused by *F. solani* has a major concern in many squash growing areas (Hernández et al. 2017) leading to economic yield losses. The pathogen is capable of surviving in infested fields for a long period. Symptoms of *Fusarium* root rot appears as damping-off, long red to brown streaks on the hypocotyls and taproot. Taproot later turns dark brown and cracks. Longitudinal cracks might develop in lesions and the cortical tissues be discolored and decayed (Gómez et al. 2008).

The extensive use of fungicides may cause toxic effects on non- target microorganisms and inflicts the undesirable changes to the environment and might induce the selection of pathogen resistance (Levy et al. 1983).

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Biological control using antagonistic microorganisms is considered an alternative method for existing chemical treatments and a key component in the development of sustainable agriculture for suppressing phytopathogenic fungi. *Trichoderma* spp. are great potential bioagents used for management of different diseases, especially against soil-borne pathogens in squash and other crops (Hamed et al. 2015). Mechanisms of *Trichoderma* include mycoparasitism, induction of systemic resistance, competition and production of metabolites such as tricholin, heptelidic acid, gliovirin, peptaibols and glisoprenins which are lethal to plant pathogens (Qualhato et al. 2013). *Bacillus* and *Pseudomonas* are the most investigated genera of the biocontrol agents. *B. subtilis* suppress many soil phytopathogens include *F. solani* (Bhattacharjee and Dey 2014) by dipeptide compounds, lysis, production of more antibiotics (bacteriocin and subtilisin), producing antifungal substances like lipopeptides and competition for limited nutrients (Elkahoui et al. 2012). *Pseudomonas fluorescens* suppresses root rot fungi by producing microbial metabolites like siderophores and production of extracellular degrading enzymes, competition and colonization in the rhizosphere (Weller 2007).

Arbuscular mycorrhiza fungi (AMF) have also been reported in combating the soil-borne diseases via plant defense proteins and physical barriers. Synergistic interaction among AMF, plant growth-promoting rhizobacteria (PGPR) and *Trichoderma* spp. induce systemic resistance to soil-borne pathogens (Atalla et al. 2020) that triggers the growth of plants as compared to single inoculation with one of them (Cely et al. 2016) through increasing nutrition, hyphal permeability in plant roots and protection against biotic and abiotic stresses.

This study aimed to investigate the antagonistic of tested bioagents against *F. solani* (lab. experiment), cultivar reaction (glasshouse experiment). Also, the potentials of arbuscular mycorrhizal fungi (AMF), PGPR (*Trichoderma harzianum* and *Bacillus subtilis* EF1) in individual or combined treatment against *Fusarium* root rot of squash and the effectiveness on metabolism of plant, plant growth characters and fruit yield were studied under field conditions.

Methods

Isolation and identification

Fusarium solani was originally isolated from naturally diseased squash plants with typical symptoms of root rot disease. Infected parts of (stems and roots) were washed with running tap water, cut into small parts, disinfected by sodium hypochloride solution (0.5%) for 2 min., passed in sterilized distilled water, dried between two sterilized filter papers and then transferred onto antibiotic amended PDA plates and incubated at 28 °C for 3–7 days (Nawar 2007). The hyphal tip technique was used. The pathogen was identified as *F. solani* based on morphological characters (Booth 1977). The identification was confirmed by Mycological Research and Disease survey Department, Plant Pathology Research Institute, ARC, Giza, Egypt.

In vitro experiment

Effect of antagonists on linear growth of F. solani

Trichoderma album, *T. harzianum*, *T. koningii* were obtained from the Department of Vegetable Diseases Research, Plant Pathology Research Institute, Agricultural Research Centre. *Bacillus subtilis* (EF1) strain and *Pseudomonas fluorescens* were obtained from Department of Botany, Faculty of Agriculture, Suez Canal University. These bioagents were tested against *F. solani*. Five mm diameter disc of 7 day-old-growth of *F. solani* on PDA was placed from the edge of the Petri plate. On the opposite side of the Petri plate, a five mm disc of the tested PGPR or a streak of the tested bacterium was placed. Control treatment was inoculated only with *F. solani*. Three replicates for each treatment were used. All Petri dishes were incubated at 28 ± 2 °C (Nawar 2007). The linear growth was measured when *F. solani* almost covered the medium surface in control treatment, then the percentage of mycelial growth reduction was calculated according to Kucuk and Kivanc (2003).

Glasshouse experiment

Inoculum preparation and varietal reactions

Four squash cultivars were tested in this experiment namely (Sakata, Galaxy, New Eskandrani H1 hybird, Hollr Queen F1 hybird). Plastic pots were sterilized by 5% formalin solution then filled with soil. Soil infection was performed using *F. solani* grown at 15 days on sterilized sorghum medium at the rate of 2% of soil weight (w/w) (El-Sharkawy et al. 2016). Each treatment was performed in six replicates. Control treatment was conducted using non-sterilized sandy soil mixed with the sterilized sorghum medium at the rate of 2% (w/w) using six replicates. Pots were watered daily after inoculation. One week later, 5 seeds/pot of each squash cultivars were sown. Percentages of pre-and post-emergence damping-off were recorded after 15 and 30 days of seed sowing. Meanwhile, the survived plants and disease severity were recorded 60 days post sowing. Disease severity Index (DSI) and symptoms were recorded according to the scale proposed by Filion et al. (2003).

In vivo experiment

A biocontrol agent that produced the highest antagonistic effect against *F. solani* in vitro was selected to

investigate its ability to reduce the incidence of *Fusarium* root rot in squash plants under field conditions.

T. harzianum inoculum

T. harzianum was inoculated into the Petri dishes containing PDA media and incubated at 27 ± 1 °C for 7 days (Srivastava et al 2010). Spores were harvested from agar plates by flooding with sterile distilled water. The spore solution was standardized to10^7 spores/ml using a hemocytometer.

B. subtilis (EF1) inoculum

B. subtilis (EF1) strain was cultured in 100 ml flasks containing 40 ml of sterilized tryptic soy agar (TSA) media (Starr et al. 1981). The stock culture was incubated at 30 °C for 3 days. The viable cell count used was 10^8 CFU/ml.

Arbuscular mycorrhizal fungal inoculum (AMF)

The inoculum of AMF was obtained from Microbiological Resource Center (MIRCN), Ain Shams University. The inoculum was prepared as a spore suspension containing three different species (i.e., *Glomus intraradices*, *G. monosporum*, *G. etunicatum*) with a concentration of 50 spores/ml.

Field experiment

Field experiment was carried out during two successive growing summer seasons 2020 and 2021 in natural soil infested with *F. solani* in the Experimental Farm, Faculty of Agriculture, Suez Canal University, Ismailia, Egypt. Randomized complete block design with three replicates was used. Each plot (3×5 m^2) included 3 rows (each 5 m in length and 1 m in width). Eight treatments were tested: control (without treatment), *Arbuscular mycorrhiza* fungi (AMF), *T. harzianum (*TZ*)*, *B. subtilis* (BS), TZ + BS, AMF + TZ, AMF + BS, AMF + TZ + BS.

Squash seeds (New Eskandrani H1 cv.) were planted on the second week of April during both seasons. A flooding irrigation system was used for this study. Seeds were surface sterilized by dipping in 95% ethanol solution for 5 min and then washed with sterilized water and dried. Inoculums of AMF, TZ and BS either as individual or combined inoculation were applied as a band to each seed row prior to sowing (Duc et al. 2017). Thirty ml of (AMF) fungal at 50 spores/ml or either 10 ml of BS (10^8 CFU/ml) or 10 ml of TZ suspension (10^7 spores/ml) were applied into the planting hole then seeds were planted. The application of tested bioagents was repeated 3 weeks post sowing at the same rate. Control plots had no inoculum. The weeds were removed by hand-howling once a week after sowing. Fertilizer was applied to plants when needed.

Data collections

After 45 days from sowing, leaves form each treatment in each replication were randomly selected and tagged for determining non- enzymatic compounds and antioxidant enzyme activity. After the end of the growing seasons, five plants were randomly removed from each plot for determining disease severity index (DSI), plant height (cm), number of leaves/plant, as well as plant fresh weight and plant dry weights (g). Also, the average of fruit weight/plant (kg) was calculated from the fruit harvested during both seasons. The total fruit weight/feddan (ton) for each plot in a given treatment was calculated.

Determination of non-enzymatic compounds

Photosynthetic pigments

Total chlorophyll (a, b) and carotenoids were estimated spectrophotometrically in leaves according to Lichenthaler and Wellburn (1983). Half gram of fresh leaves was mixed in 85% acetone with calcium carbonate, homogenized then centrifuged at 3000 rpm for 5 min. The sample was measured at optical density of 662, 644 and 440.5 nm.

Free phenolic compounds

Fresh leaf was extracted with ethanol (Abdel-Rahman et al. 1975). One ml of ethanolic extract was added to 1 ml of 2 N Folin–Ciocalteu reagent, 1 ml of Na_2CO_3 solution (14%) and 7 ml distilled water in test tube. These test tubes were heated (70 °C) in water bath. The contents of free phenolic compounds were measured by Folin–Ciocalteu method (Horwitz et al. 1970) at 650 nm using the correction factor 0.0042 from catechol standard curve.

Total free amino acids

Total free amino acids were estimated using the method of Rosen (1957). Here, 0.1 ml of ethanolic extract was added to 5 ml methanol + 1 ml ninhydrin reagent then placed in water bath (60 °C) for 20 min. Samples were measured against blank sample at 570 nm. Concentrations were calculated as mg/g fw of the extracted leaves using correction factor 0.0042 from glycine standard curve.

Total protein

Leaf extract was prepared using 0.2 g fresh leaves homogenized with 1 ml of 0.1 M phosphate buffer (pH 7). Suspension was filtered and centrifuged at 10000 rpm for 15 min (Urbanek et al 1991). The supernatant leaf extract was used to determine soluble protein concentration according to Bradford (1976) as

mg/g fw of the extracted leaf using Bovine serum albumin standard curve with correction factor 0.00233.

Determination of antioxidant enzyme activity

Enzyme extracts were prepared according to (Urbanek et al 1991).

Superoxide dismutase (SOD) activity

The reaction mixture consisted of 0.25 ml of methionine (13 m M), NBT (80 µM), EDTA (0.1 m M) the mixture was completed to 3 ml with buffer. Finally, 0.25 ml of riboflavin (50 mM) was added in the test tube, shaked well and placed 30 cm away of light source. Reaction was measured for 20 min then it was stopped by switching the light off. Enzyme activity of SOD was assayed by measuring its ability to inhibit reduction of nitro blue tetrazolium (NBT) at 560 nm (Beauchamp and Fridovich 1971).

Peroxidase (POD) activity

POD activity was measured by adding 0.1 ml of enzyme extract with 4 ml of guaiacol solution (3 ml of 0.1 M potassium phosphate (pH 6.5), 0.5 ml of 2% guaiacol and 0.5 ml of 0.3% H_2O_2). POD activity was expressed as the change in absorbance at 425 nm/gram fresh weigh/5 min using a spectrophotometer (Allam and Hollis 1972).

Polyphenol-oxidase (PPO) activity

The reaction mixture contained 0.1 ml enzyme extract, 1 ml of 0.2 M of potassium phosphate buffer (pH 7) and 1 ml of 10^{-3} M catechol and completed to 6 ml with daistilled water. This mixture was incubated for 30 min at 30 °C. PPO activity was expressed as the change in the absorbance each 0.5 min at 430 nm/g fresh weigh/5 min using a spectrophotometer (Matta and Dimond 1963).

Determination of macro and micro-elements

Leaf sample of squash plants were dried at 70 C for 48 h and grounded. Half gram of sample was digested by sulphoric acid hydrogen peroxide according to Jackson (1973). After proper dilution of digested materials, N was determined using modified kheldahl method (Jackson 1973). Phosphorus was determined calorimetrically using spectrophotometer (Black et al.1965). Potassium was determined by using flame photometer (Jackson 1973). The Fe, Zn and Mn contents were determined using a nitric, sulfuric and perchloric acid mixture (4:1:8v/v) (Jackson 1973). The concentration of Fe, Zn and Mn was measured by the atomic absorption spectrophotometer (Thermo-electron, S Series GE 711,838).

Statistical analysis

Analysis of variance (ANOVA) of mean values of the samples from each treatment was subjected to statistical analyses using CoStat software (version 6.311). Means were separated using LSD at $P < 0.05$.

Results

Effect of PGPRs on the linear growth of *F. solani*

All tested PGPRs differed significantly in reducing the mycelial growth of *F. solani* than the control (Fig. 1). *T. harzianum* was the most effective PGPR in reducing *F. solani* mycelial growth with reduction (75.17%), whereas *P. fluorescens* was the least effective causing 50.58% reduction in *F. solani* growth (Fig. 1).

Reactions of squash cultivars to *F. solani*

The tested squash cultivars differed significantly in their susceptibility to *F. solani*. Hollr Queen F1 cv. was the most resistant squash cultivar with the highest percentage of survival plants (84.92%) and the lowest pre- emergence (7.06%) while post-emergence (8.02%) damping-off,

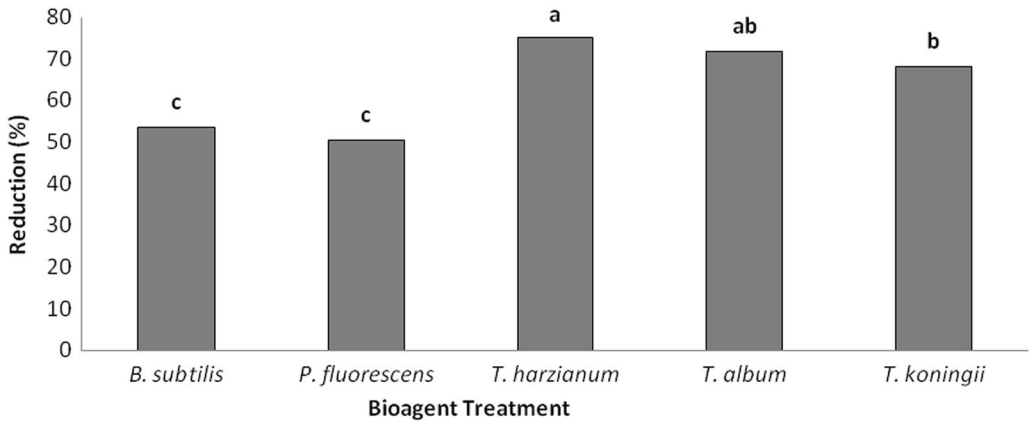

Fig. 1 Effect of PGPRs on mycelial growth reduction of *Fusarium solani*. Bars with different letters are significantly different (LSD, $P > 0.05$)

Table 1 Reaction of different squash cultivars to infection with the *Fusarium solani* under glasshouse conditions

Varity	*Fusarium solani*				Control			
	Damping-off (%)		Survival (%)	Severity (%)	Damping-off (%)		Survival (%)	Severity (%)
	Pre-emergence	Post-emergence			Pre-emergence	Post-emergence		
Sakata	11.73c	12.08b	76.19b	33.7b	4.01bc	2.7a	93.29b	0.0b
Galaxy	14.12b	12.62b	73.26b	35.02b	4.36b	0.0b	95.64a	0.0b
New Eskandrani	22.40a	18.31a	59.29c	52.32a	7.11a	3.02a	89.87c	3.27a
Hollr Queen	7.06d	8.02c	84.92a	24.15c	3.14c	0.0b	96.86a	0.0b
LSD 0.05	2.34	2.08	3.41	2.64	1.14	0.51	1.44	0.623

Means in a column followed by the same letters are not significantly different (LSD, $P < 0.05$)

Table 2 Effect of AMF and PGPRs individually or in combinations on total chlorophyll and carotenoids in squash leaves

Treatments	Photosynthetic pigments content	
	Total chlorophyll (mg/100 g fw)	Carotenoids (mg/100 g fw)
Control	26.15f	3.51c
AMF	50.82b	7.21ab
TZ	46.17cd	8.24a
BS	30.27e	5.41bc
TZ + BS	54.47a	6.29ab
AMF + TZ	45.16d	7.81ab
AMF + BS	47.67c	7.89ab
AMF + TZ + BS	50.88b	8.30a
LSD 0.05	2.01	2.55

Means in a column followed by the same letters are not significantly different (LSD, $P < 0.05$). Arbuscular Mycorrhizal fungi (AMF), *Trichoderma harzianum* (TZ), *Bacillus subtills* (BS), control (without treatment), fw (fresh weight)

followed by Sakata, and Galaxy. In contrast, New Eskandrani cv. was the most susceptible cultivars (Table 1).

Effects on total chlorophyll and carotenoids in squash leaves

The treatment of squash seeds (New Eskandrani H1 cv.) with mycorrizal fungi and bioagents (*T. harzianum* and *B. subtilis*) individually or in combinations significantly elevated the total chlorophyll and carotenoids as compared to control. The highest total chlorophyll was observed in BS + TZ treatment (54.47 mg/100 g fw), followed by AMF + TZ + BS (50.88 mg/100 g fw) whereas the lowest was recorded for *B. subtilis* at (30.27 mg/100 g fw). The level of carotenoids in all tested treatments was higher than control (3.51 mg/100 g fw). On contrast, combined treatments of AMF + TZ + BS recorded the highest level of carotenoids (8.3 mg/100 g fw) compared to control (3.51 mg/100 g fw), (Table 2).

Effects on free phenolic compounds, total free amino acids and protein content

Data in (Fig. 2) indicate that most of the studied non-enzymatic compounds, *i. e.,* free phenols, total free amino acid and total protein were significantly affected by the applied treatments than the control. Free phenols content was the highest in AMF + TZ + BS treatment at 2.66 mg/g fw followed by AMF + TZ at 2.33 mg/g fw compared to the lowest Free phenols content in control at 1.27 mg/g fw. Pertaining to total free amino acid, BS treatment showed the highest level at 5.99 mg/g fw, followed by AMF + TZ + BS at 3.67 mg/g fw compared to control at 1.82 mg/g fw. As for total protein, significant differences were also found among all tested treatments and control. TZ treatment recorded the lowest level of total protein at 39.48 mg/g fw, whereas AMF + TZ + BS showed the highest level at 48.69 mg/g fw.

Effects on peroxidase, polyphenol-oxidase and superoxide dismutase activity

The tested PGPRs and AMF either individually or in combinations increased POD, PPO and SOD compared to control treatment (Fig. 3). The highest POD activity was observed in AMF + TZ + BS followed by AMF + BS and TZ + BS at 0.498, 0.437 and 0.414 unit/g fresh weight/5 min, respectively compared to 0.073 unit/g fresh weight/5 min in control treatment. Similarly, the highest PPO activity was recorded with AMF + TZ and AMF + TZ + BS at 1.137 and 1.026 unit/g fresh weight/5 min, respectively as compared to control (0.049 unit/g fresh weight/5 min). Also, AMF + TZ + BS showed the highest SOD activity at 1.621 unit/g fresh weight/5 min followed by AMF + BS and AMF + TZ at 1.42 and 0.925 unit/g fresh weight/5 min, respectively. Statistical analyses showed that POD, PPO and SOD activity differed significantly among tested PGPRs and

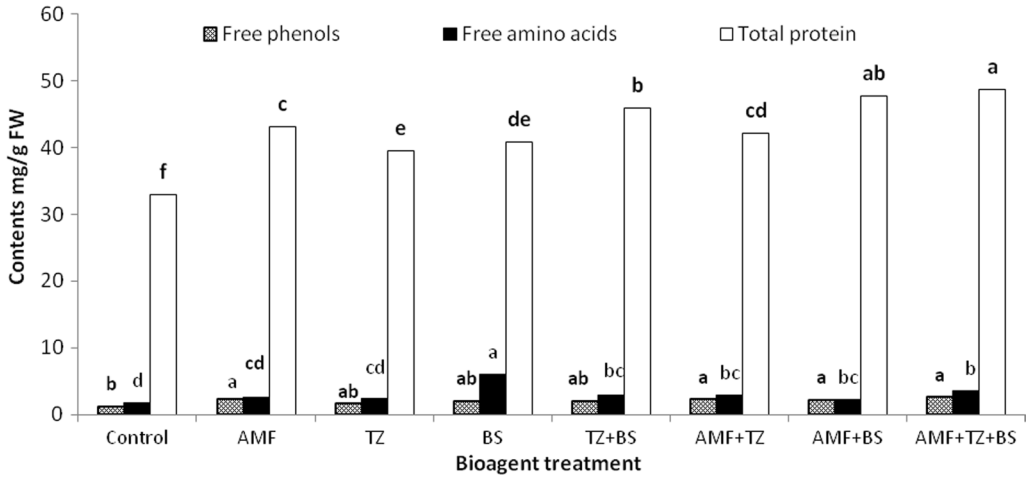

Fig. 2 Effect of AMF and PGPRs on the contents of free phenolic compounds,total free amino acids and total protein. Bars with different letters are significantly different (LSD, $P > 0.05$). Arbuscular Mycorrhizal fungi (AMF), *Trichoderma harzianum (*TZ*)*, *Bacillus subtills* (BS), control (without treatment)

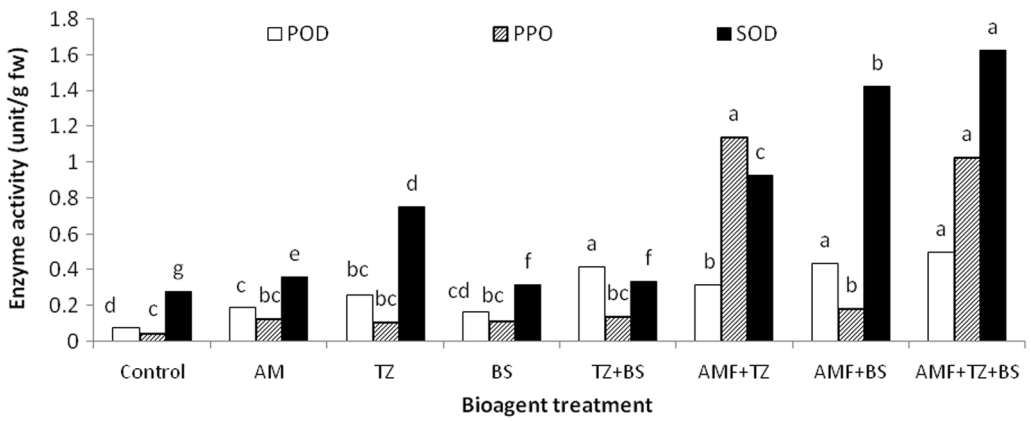

Fig. 3 Effect of AMF and PGPRs on peroxidase, polyphenol-oxidase and superoxide dismutase activity in squash plant. Bars with different letters are significantly different (LSD, $P > 0.05$). Arbuscular Mycorrhizal fungi (AMF), *Trichoderma harzianum (*TZ*)*, *Bacillus subtills* (BS), control (without treatment), (SOD), superoxide dismutase, (POD), peroxidase, (PPO), polyphenol-oxidase

AMF either in individual or in combined treatments (Fig. 3).

Effects on the contents of macro- and micronutrients

The lowest percentage values of leaves N, P, K macronutrients contents in squash leaves were obtained from untreated plants being 20.32, 1.76 and 11.7 g/kg dry weight. The highest content of leaf N concentrations were obtained when plants inoculated with TZ only at 26.24 g/kg dry weight followed by its combination with AMF and BS at 25.74 g/kg dry weight. As for P content, the highest content was recorded in AMF + TZ + BS treatment at 3.87 g/kg dry weight. The highest values of K content were in AM + TZ + BS treatment at 19.86 g/kg dry weight followed by AMF + TZ treatment at 17.81 g/kg dry weight. As for micronutrients, the highest values of both Fe and Zn contents were recorded in the combined treatment of AMF + TZ + BS at 1.91and 1.394 g/kg dry weight, respectively (Table 3). The highest value of leaf Mn content was recorded in AM + B.S treatment at 118.3 g/kg dry weight.

Effects of AMF and PGPRs on DSI of root rot caused by *F. solani*

In the first season trial (2020), AMF + TZ + BS, AMF + TZ, AMF + BS and TZ + BS treatments showed

Table 3 Effect of AMF and PGPRs on the contents of macro- and micronutrients in the leaves of squash plants

Treatment	Macronutrients content (g/kg dw)			Micronutrients content (g/kg dw)		
	N	P	K	Fe	Zn	Mn
Control	20.32e	1.76d	11.70e	0.171b	0.626e	58.10g
AMF	23.17 cd	2.42bcd	14.11d	0.283b	0.804de	80.40d
TZ	26.24a	2.73bcd	13.92d	0.194b	0.984bcd	64.80f
BS	22.30d	2.11cd	15.26cd	0.230b	0.871cd	77.20e
TZ + BS	23.82c	2.80abcd	16.03c	1.563a	1.049bc	85.00c
AMF + TZ	24.56bc	3.46ab	17.81b	0.474b	0.926bcd	84.77c
AMF + BS	25.26ab	3.08abc	16.64bc	1.69a	0.804de	118.3a
AMF + TZ + BS	25.74ab	3.87a	19.86a	1.91a	1.394a	92.50b
LSD 0.05	1.39	1.12	1.70	0.678	0.182	0.411

Means in a column followed by the same letters are not significantly different (LSD, $P < 0.05$). Arbuscular Mycorrhizal fungi (AMF), *Trichoderma harzianum (TZ)*, *Bacillus subtills* (BS), control (without treatment), dw (dry weight)

the lowest (DSI) of 8.87, 9.33, 12.43 and 13.33% which were significantly lower than that of control treatment (37.32%). In the second season trial (2021), the same trend was also recorded of DSI of squash Fusarium root rot (Fig. 4). Statistically, there were significant differences in the DSI among the tested treatments and control in the first and second season trials.

Effects on the plant growth parameters of squash plants

The tested PGPRs and AMF either individually or in combined treatments caused a significant increase in the plant growth parameters over that of control treatment (Table 4). In case of the First season, AM + TZ + BS gave the longest plant length (78.89 cm) with the subsequent greatest number of leaves per plant (36.17 leaves/plant) and total plant fresh weight (694.63 g). However AM + BS, gave the highest values of dry weight (128.3 g/plant). As for the second seasons, AM + TZ + BS also yielded the greatest number of leaves/plant and total plant fresh and dry weight at 39.25 leaves/plant, 723.9 g/plant and 118.86 g/plant, respectively. However AM + TZ gave the longest plant length at 75.29 cm. Indeed, there were significant differences among all PGPRs and AMF compared to control (Table 4).

Effect of AMF and PGPRs on the fruit yield

Generally, combined treatments of AMF + TZ + BS, AMF + BS and AMF + TZ showed the greatest squash yield followed by TZ + BS treatments with the subsequent greatest weight of fruits/plant in the two seasons of study (Table 5). In first season trial, AMF + TZ + BS, AMF + BS, AMF + TZ, TZ + BS, BS, T.Z and AMF showed the greatest yield at 8.783, 8.716, 8.623, 8.505, 8.043, 7.867 and 7.515 ton/feddan, respectively. AMF + TZ + BS recorded greatest fruits yield/plant at 2.56 kg/plant followed by AMF + B.S at 2.37 kg/plant.

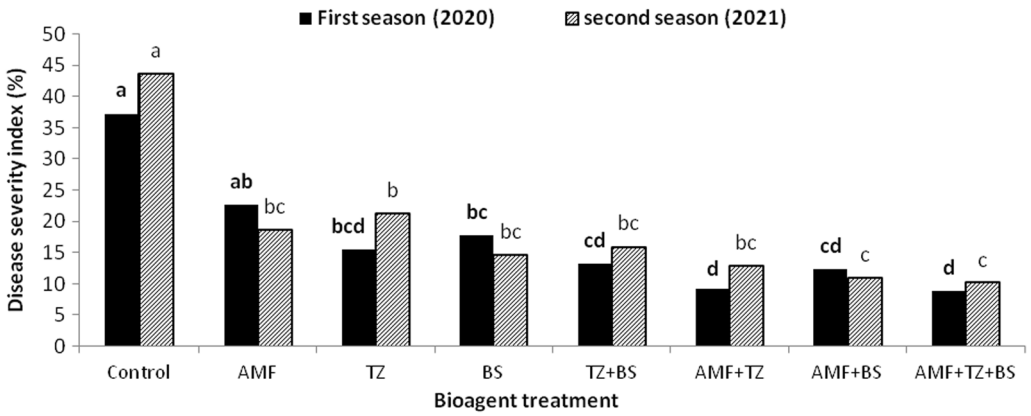

Fig. 4 Effect of AMF and PGPRs on DSI of squash root rot caused by *Fusarium solani* under field conditions during 2020 and 2021 seasons. Bars with different letters are significantly different (LSD, $P > 0.05$). Arbuscular Mycorrhizal fungi (AMF), *Trichoderma harzianum (TZ)*, *Bacillus subtills* (BS), control (without treatment)

Table 4 Effect of AMF and PGPRs in individual or in combined treatments on the plant growth parameters of squash plants under field conditions during 2020 and 2021 seasons

Treatment	First season (2020)				Second season (2021)			
	Length of plant (cm)	Leaves number	Fresh weight(g)	Dry weight(g)	Length of plant(cm)	Leaves number	Fresh weight(g)	Dry weight(g)
Control	49.05d	19.33d	386.36f	78.8c	39.25d	20.75e	362.16f	56.08b
AM	50.49d	29.83b	456.81e	104.22abc	58.15c	28.58d	535.94d	110.48a
TZ	56.06cd	28.5b	401.74f	98.2bc	63.2bc	32.83bc	426.74e	99.6a
BS	60.4bc	23.25c	506.81d	89.16bc	59.51c	30.17 cd	548.48 cd	107.82a
TZ + BS	63.78bc	30.33b	615.6c	115.6ab	65.9abc	29.83 cd	586.bc	100.93a
AM + TZ	64.3b	27.83b	640.6bc	101.27bc	75.29a	31.58bcd	610.07b	118.34a
AM + BS	61.96bc	31.08b	684.4ab	128.3a	62.44c	34.5b	717.54a	116.45a
AM + TZ + BS	78.89a	36.17a	694.63a	114.23ab	72.98ab	39.25a	723.9a	118.86a
LSD 0.05	8.07	3.89	47.21	26.44	9.98	3.91	44.55	28.42

Means in a column followed by the same letters are not significantly different (LSD, $P < 0.05$). Arbuscular Mycorrhizal fungi (AMF), *Trichoderma harzianum (*TZ*)*, *Bacillus subtills* (BS), control (without treatment)

Table 5 Effect of AMF and PGPRs on the fruits yield/plant (kg) and fruits yield/feddan (ton) under field conditions during 2020 and 2021 seasons

Treatment	First season (2020)		Second season (2021)	
	Fruit yield/ plant (kg)	Fruit yield/ feddan (ton)	Fruit yield/ plant (kg)	Fruit yield/ feddan (ton)
Control	1.25c	7.12 g	1.17d	7.235d
AM	1.87b	7.515f	2.07bc	7.827c
TZ	2.03b	7.867e	1.86c	8.074bc
BS	1.96b	8.043d	2.09bc	7.916c
TZ + BS	2.08ab	8.505c	2.13bc	8.24b
AM + TZ	2.04b	8.623b	2.15bc	8.643a
AM + BS	2.37ab	8.716ab	2.29ab	8.611a
AM + TZ + BS	2.56a	8.783a	2.48a	8.912a
LSD 0.05	0.52	0.107	0.32	0.316

Means in a column followed by the same letters are not significantly different (LSD, $P < 0.05$). Arbuscular Mycorrhizal fungi (AM), *Trichoderma harzianum (*TZ*)*, *Bacillus subtills* (BS), control (without treatment)

On contrast, control treatment achieved the lowest squash fruit yield of 7.12 ton/feddan and 1.25 kg fruit/plant. Indeed, significant differences existed among tested treatments in weight of fruit yield/feddan and fruit yield/plant (Table 5). The same trend of fruit yield was observed in the second season, AMF + TZ + BS, AMF + TZ and AMF + BS, also received 8.912, 8.643 and 18.611 ton/feddan, respectively with subsequent the highest fruits yield/plant. Significant differences existed among tested treatments in the total fruits yield/feddan and fruits yield/plant (Table 5).

Discussion

The use of plant growth promoting rhiz-microorganisms (PGPRs) against soil borne pathogenic fungi may offer an opportunity for an environmentally and friendly control method that could minimize the environmental hazards and improve the biological control of phytopathogens (El-Gamal et al. 2016). In vitro experiment, all tested PGPRs reduced the mycelial growth of *F. solani* as compared to control. *T. harzianum* was the most effective PGPR in reducing *F. solani* mycelial growth. These findings were harmony with (Abdel-Naby 2010) who found that PGPRs *(T. viride, B. subtilis, P. fluorescens* and *Actinomyces* spp.) exerted different effect reducing mycelia growth of *F. solani* the causal agent of root rot in squash. Also, Nawar (2007) found that *T. harzianum* is successful bioagents to control and inhibit the mycelial growth of *F. solani* in squash.

The 4 squash cultivars showed different varietal reaction to *F. solani*. Similarly, Hernández et al. (2017) recorded different reactions of 14 squash cultivars to *F. solani* f. sp. *cucurbitae*. Also, submitting genotypes of *Cucurbita* spp. to different isolates of *F. solani* f. sp. *cucurbitae* under greenhouse conditions showed different reaction among the genotypes (Dos Santos et al. 2020). Differences among cultivars in their resistance/tolerance might be attributable to the differences in the genetic structure that affected the morphological characters and their chemical components as well as environmental factors that might affect and alter the reaction of any cultivar to a given pathogen (Dos Santos et al. 2020).

The treatment of squash seeds with AMF and bioagents (*T. harzianum* and *B. subtilis*) individually or in combinations significantly elevated the total chlorophyll and

carotenoids as compared to control. The increment in chlorophyll contents reflects the health condition of plant and enhancing plant resistance to disease. The highest SPAD values as an indicator of chlorophyll content were recorded in plants inoculated with *Trichoderma* in squash (Formisano et al. 2021).The highest SPAD and chlorophyll fluorescence in zucchimi was recorded in combined treatment of AMF and *T. atroviride* (Colla et al. 2015). Carotenoid levels were collaborated in photosynthesis, protect plants against oxidative damage and prospector of volatile that attracts pollinators (Felemban et al. 2019). Root colonization by *Trichoderma* induced gene and chloroplast components up- regulation in plants, which led to improve photosynthetic process (Harman et al. 2021). Also, carotenoids accumulated in roots and shoots of mycorrhized plants (Wang et al. 2018).

The tested bioagents caused elevation in the free phenols, total free amino acid and total protein. Also, Abdel-Naby (2010) found that the PGPR treatments elevated the phenolic compounds in squash plants. Also, AM fungi and *T. koningii* increased secondary metabolism and caused phenolic compounds accumulation in pepper plants (Bonini et al. 2020). Plants subjected to PGPRs presented high levels of phenolic compounds, which are essential for lignin, biosynthesis and plant defense against pathogens resulting in resistant plants (Bhattacharya et al. 2010). AMF also caused enhancement of water relations and photosynthetic activity; secondary metabolism, nutrient availability, amino acids, phenolics, organic acids and proteins into the rhizosphere (Rouphael et al. 2015).

The inoculation of AMF and PGPRs either as individual or as combined inoculation increased POD, PPO and SOD activity. Similarly, PGPR (*T. viride, B. subtilis, P. fluorescens* and *Actinomyces* spp.) increased POD and PPO activities in squash (Abdel-Naby 2010). *Trichoderma* significantly increased activities of resistance enzymes, including SOD, POD, PPO and catalase (CAT) in cucumber seedlings (Li et al. 2019). Similarly, combined inoculation of AMF, PGPR and *Trichoderma* spp. on tomato significantly increased the antioxidant enzyme activity (Cai et al. 2021).

Clearly inoculation of AMF and PGPRs elevated the contents of macro- and micronutrients in the leaves of squash plants. Similarly, the inoculation with AMF led to enhancement of p uptake in squash plants (Al-Hmoud and Al-Momany 2017). The use of *T. harzianum* led to an increase in P content in squash plants (Formisano et al. 2021). Combined treatment of *T. atroviride* and *G. intraradices* increased the content of P, Fe, Zn and Mg in zucchini plants (Colla et al. 2015). Also, inoculation of AMF and *Bacillus* sp. induced secondary metabolism and reduced fertilizer application by 50% of the recommended NPK fertilization without compromising crop growth and yield in pepper (Nanjundappa et al. 2019).

DSI of squash root rot caused by *F. solani* under field conditions was significantly affected by treatments of AMF and PGPRs. Clearly, several commercial microbial products of various species as *Trichoderma* spp. and *Pseudomonas* spp. were found to reduce the severity of *Fusarium* crown and foot rot of zucchini squash (Roberti et al. 2012). *Trichoderma* was known to enhance the tolerance of Cucurbitaceae families to infection caused by fungi including *Fusarium* spp. (Woo et al. 2006). AMF exhibited bioprotective functions against diseases by enhancing chitinolytic activity, photosynthesis and production of phytoalexins, metabolites production, lignification and exclusion of pathogen (Sharma et al. 2017).

The tested PGPRs and AMF either as individual or as combined treatments caused a significant increase in plant growth parameters. These results are in harmony with those reported by Formisano et al. (2021) who found that treated zucchini squash plants with *T. harzianum* T22 resulted in significant increase in fresh biomass and root dry weight. Also, the highest shoot, root dry in zucchini was observed in combined treatment of AMF and *T. atroviride* followed by a single inoculation of them (Colla et al. 2015). Similarly, interaction of *B. subtilis* and *T. harzianum* with AMF *Glomus mosseae* showed increase in growth and yield of cucumber plants (Aboud et al. 2014).

Combined inoculation of AMF + TZ + BS, AMF + BS and AMF + TZ showed the greatest squash yield followed by TZ + BS treatments with the subsequent greatest weight of fruits/plant in the two studied season. The current results are in agreement with those of Colla et al. (2015) who found that treatment of field-grown zucchini plants with AMF and *T. atroviride* induced early and total yields compared to un-inoculated. Similarly, Co- inoculation applied of *B. subtilis* and *T. harzianum* with AMF, induced total yields in cucumber (Aboud et al. 2014) and might induce systemic resistance to soil borne pathogens and promote growth of plants either separately or in combination (Rana et al. 2020).

Conclusions

Based on laboratory, glasshouse and field experiments, the tested bioagents (*Trichoderma harzianum album, T. koningii*, *B. subtilis* and *P. fluorescens*) proved to have great potential to control *Fusarium* root rot in squash. Squash seed inoculation with PGPR or AMF enhanced the tolerance of squash plant to root rot via plant vigor, growth parameters, induced plant defense and antagonistic effect. Indeed, combined inoculation resulted in significant increase in measured parameters with subsequent increase in fruit yield that indicated the possibility

of using seed inoculation with microbial control agents as potential candidate to control root rot in squash.

Abbreviations
AMF: Arbuscular mycorrhizal fungi; TZ: *Trichoderma harzianum*; BS: *Bacillus subtills*; dw: Dry weight; fw: Fresh weight; SOD: Superoxide dismutase; POD: Peroxidase; PPO: Polyphenol-oxidase; DSI: Disease severity index; N: Nitrogen; K: Potassium; P: Phosphorous.

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Authors' contributions
EEE contributed in all experiments, data analysis and interpretation and writing the manuscript. AE performed the determination of macro, micro-elements, enzymatic and non enzymatic compounds and data analysis, interpretation of data and revising the MS. All authors read and approved the final manuscript." and ensure that this is the case.

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Declarations

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Competing interests
The author(s) declare(s) no conflicts of interest.

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