Bulletin of the National Research Centre

RESEARCH Open Access

Effect of inoculum density of *Stromatinia cepivora* on the amount of white rot reduced by *Trichoderma* species in garlic

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Abstract

Background: White rot, a garlic disease caused by the soil-borne fungus *Stromatinia cepivora* (Berk.) Whetzel, is a serious problem of garlic productions in Egypt. This study examines the potential of controlling the disease biologically by using three *Trichoderma* species, i.e., *Trichoderma harzianum*, *Trichoderma koningii*, and *Trichoderma virens* employed either alone or in combination.

Results: In in vitro assays, three *Trichoderma* species, i.e., *Trichoderma harzianum*, *Trichoderma koningii*, and *Trichoderma virens* and tebuconazole, were compared for their ability to suppress *S. cepivora* isolate (Sc8). In greenhouse experiments, the chemical treatment was the most effective, with the lowest incidence of garlic white rot compared with the control. The antagonistic fungi tested either individually or in combination significantly reduced the incidence of white rot on garlic. In general, dual and triple combinations of the fungal isolates were more effective than these isolates used individually. The combination of the three *Trichoderma* species was the most effective treatment, decreasing disease incidence by 50.0% in 2016/2017 season and 40.0% in 2017/2018 season, respectively. The three *Trichoderma* species employed alone or in combinations and tebuconazole were evaluated under low and high disease pressures in field trials to determine which situation (s) provided the best control of garlic white rot. Under low (40 sclerotia/kg of soil) and high (600 sclerotia/kg of soil) inoculum density, the standard fungicide programme (dipping of garlic cloves in tebuconazole (1 ml of Folicur 25% l^{-1} of water) plus spraying garlic stem bases with the same concentration of tebuconazole) gave statistically significant disease control, decreasing disease incidence by 67.7 and 29.4% in 2016/2017 season and 72.6 and 31. 1% in 2017/2018 season, respectively. Under low disease pressure, significant control, equal to the fungicide treatment, was achieved with the trip combination of three *Trichoderma* species. However, *Trichoderma* species employed alone gave insignificant control of garlic white rot under high disease pressure. The triple combination of three *Trichoderma* species decreasing disease incidence by 65.6 and 15.5% in 2016/2017 season and 74.2 and 18.6% during 2017/2018 season, under low and high inoculum density, respectively. The activities of defense enzymes, i.e., peroxidase, polyphenoloxidase, and chitinase due to application of *Trichoderma* species, were enhanced in garlic plants either grown under low or high disease pressures. Reduction of white rot disease incidence was accompanied by increased growth parameters and bulbs yield of garlic plants grown under field conditions.

Conclusions: These results indicated that the performance of three *Trichoderma* species may be influenced as much by the absolute disease pressure. It was concluded that, at the low disease pressure site, the low level of inoculum and disease incidence enabled three *Trichoderma* species to bring about disease control.

Keywords: Garlic, White rot, Disease pressure, Three *Trichoderma* species

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Background

Garlic (*Allium sativum* L.) is a monocotyledonous plant that belongs to the family *Alliaceae*. It is the second most widely cultivated vegetable next to onion and widely produced for its medicinal and nutritional properties. The annual world garlic production is about 22.2 million tons (Palmero et al. 2013). In 2016, Egypt produced approximately 247,000 metric tons garlic from 10,473 ha (Anonymous 2017). Unfortunately, in Egypt and several other countries, garlic is liable to infection by several soil-borne fungi which affected on both quantity and quality of the cloves after harvest (Elshahawy et al. 2017a). White rot disease caused by the soil-borne pathogen *Stromatinia cepivora* (Berk.) Whetzel is one of the most important and destructive diseases of garlic and prevalent worldwide (Pinto et al. 2000; Coventry et al. 2002; Ulacio-Osorio et al. 2006;Tamire et al. 2007; Zeray and Mohammed 2013; Dilbo et al. 2015). The first indication of white rot infection is a change in leaf color from bright green to blue green. The outer leaves of the garlic then change to a yellowish color and begin to die back from the tips. Subsequently, the whole plant wilts and dies. Below ground, the roots are gradually destroyed, enabling the plant to be easily pulled from the soil. *S. cepivora* also causes a semi-watery decay of the scales which is associated with the growth of superficial white mycelium on the affected area (Abd El-Razik et al. 1973). Within several days, the mycelium darkens and is transformed into large numbers of sclerotia (Abd El-Razik et al. 1973). Plant decay and harvesting procedures result in the release of sclerotia into the soil. Sclerotia are the only reproductive structures of *S. cepivora* as no perfect stage has yet been described (Coley-Smith 1990; Crowe 1995) and no asexual spores are produced. The sclerotia are black, uniformly round, and are 200–500 μm in diameter (Coley-Smith 1960). A narrow, smooth, or pitted rind surrounds a medulla of compact interwoven hyphae and a gelatinous material is often present in the interhyphal spaces (Coley-Smith 1960). The protective nature of the sclerotial rind enables the sclerotia to remain viable in the soil for a substantial length of time reached to 20 years (Coley-Smith 1990). The sclerotia are stimulated to germinate only by *Allium*-specific root exudates, making the disease highly specific to *Allium* species. The disease has been found in every country where *Allium* species are cultivated (Stewart and McLean 2007), and to date no system of control has been shown to fully prevent the occurrence of the disease. As the disease became increasingly widespread and persistent, the control of garlic white rot by fungicides became almost exclusive. Successful control of white rot was obtained after the introduction of systemic fungicides. The dicarboximides, iprodione and vinclozolin, were highly effective in controlling white rot disease in New Zealand (Wood 1980). In the UK, iprodione was used as a seed and stem treatments (Entwistle and Munasinghe 1980). Unfortunately, in both the UK and New Zealand, the continued use of iprodione and vinclozolin had led to a decrease in effectiveness that was due to enhanced degradation of the chemicals by soil microorganisms (Entwistle 1983; Walker et al. 1986; Slade et al. 1992). However, concerns associated with environmental impacts of such fungicidal chemicals, the increasing development as well as registration costs, and the reduction of fungicide effectiveness were due to the resistance and enhanced microbial degradation, which called for alternative control measures include use of beneficial microorganisms (Entwistle 1992). A number of fungal species had been identified with potential as biological control agents of white rot disease (Utkhede and Rahe 1980; Abd-El-Moity and Shatla 1981; De Oliveira et al. 1984; Harrison and Stewart 1988; Kay and Stewart 1994; McLean and Stewart 2000; El-Khateeb 2004; McLean et al. 2005; McLean et al. 2012; Shalaby et al. 2013; Elshahawy et al. 2017b, c; Elshahawy et al. 2018). In previous study, the fungal isolates *Trichoderma harzianum*, *Trichoderma koningii*, and *Trichoderma virens* were confirmed as antagonists of *Stromatinia cepivora* isolate (Sc2), the most pathogenic isolate of onion and garlic white rot (Elshahawy et al. 2017b). We also found that in dual culture with *S. cepivora*, these isolates produced inhibition zones and colonized pathogen hyphae. When agar was amended with culture filtrates of each of *T. harzianum*, *T. koningii*, and *T. virens*, the growth of *S. cepivora* was distorted or unusual, indicating the production of antibiotics (Elshahawy et al. 2017b). This paper reports the results of tow field trials which compared the standard fungicide programme for garlic white rot with solid substrate formulations of *T. harzianum*, *T. koningii*, and *T. virens* employed either alone or in combination applied at sowing under varying disease pressures.

Methods

Garlic white rot pathogen

One isolate of *S. cepivora* (Sc8) was obtained from the author's collection. This isolate was found to be of high virulence against garlic based on pathogenicity tests conducted in previous studies (Elshahawy et al. 2018).

Trichoderma species

Three identified fungal antagonists including *Trichoderma harzianum*, *Trichoderma koningii*, and *Trichoderma virens* were obtained from Plant Pathology Department, NRC. Identification of *Trichoderma* species were re-confirmed according to a taxonomic key for the genus *Trichoderma* (Rifai 1969; Watanabe 1994).

Testing the antagonistic activity of *Trichoderma* species

Each of the *T. harzianum*, *T. koningii*, and *T. virens* was tested for antagonism for *S. cepivora* (Sc8) using the dual

culture techniques (Bell et al. 1982). PDA plates were inoculated on one side with a 5-mm mycelial disk from a 7-day-old culture of the test fungus. The opposite side was inoculated with a disc of *S. cepivora* (Sc8) and the plates were incubated at 18 ± 2 °C in the dark. Four replicate plates were inoculated for each test fungus. After 14 days, the following parameters were measured: (i) average colony radius and percentages of growth reduction and (ii) average number of sclerotia per $cm^2$and percentages of reduction. Tebuconazole (commercialized as Folicur®, 25% a.i. Bayer Group Science, Germany) was used in the above experiments for comparison. The chemical treatment was applied at the recommended rate 1.0 ml Folicur/L.

Inhibitor effect of cultural filtrate to sclerotial germination

Sclerotia were collected from *S. cepivora* (Sc8) cultures (60-day-old) and soaked in test tubes containing the culture filtrate of each of the *Trichoderma* species to be tested for 12 h at room temperature. At end of the soaking period, sclerotia were washed with sterile distilled water and 30 sclerotia from each treatment were transferred individually under aseptic conditions to Petri dishes containing PDA. Water-soaked sclerotia were used as the control treatment. Four replicates (dishes) were used for each treatment. Petri dishes bearing sclerotia were incubated at 18–20 °C for 7 days and percentages of germinating sclerotia were determined. Tebuconazole was used at the rate of 1.0 ml Folicur/L for comparison.

Greenhouse experiments

Preparation of Trichoderma species inocula

T. harzianum, *T. koningii*, and *T. virens* used in this study were grown on sterilized (121 °C for 60 min) wheat bran as a carrier preparation (Mahdizadehnaraghi et al. 2015). Carboxymethyl cellulose (1%) was used as an adhesive at the rate of 1:10 (*v*/*w*). The pH was adjusted to neutral by adding $CaCO_3$at the rate of 15 g/kg. Then, 3% mannitol was added as osmoticant at the rate of 8.5 ml for 100 g formulation. Spore suspension (10^6 spores/ml) of each of *T. harzianum*, *T. koningii*, and *T. virens* were incorporated into sterilized wheat bran under aseptic conditions at the rate of 50 ml of suspension per 100 g and thoroughly mixed with a sterilized spoon. Subsequently, each of *Trichoderma* spp. spore suspension (10^6 spores/ml) was individually incorporated into sterilized wheat bran carrier under aseptic conditions at the rate of 50 ml of suspension per 100 g and thoroughly mixed with the help of sterilized spoon. The materials (35% moisture content) were packed in polythene bags, sealed, and stored at room temperature. These formulations used alone or their dual and triple combinations (equal volumes of each) were applied in the greenhouse and field experiments.

Preparations of garlic white rot pathogen inocula

Fungal mass of garlic isolate *S. cepivora* (Sc8) for soil infestation in greenhouse experiments was obtained by growing the isolate on a sand-barley medium (Abd El-Moity 1976). This medium was prepared by mixing 50 g barley grains, 50 g sand, and 40 ml water; then the mixture in glass bottles (500 ml capacity) with cotton plugs was sterilized at 121 °C for 30 min. The autoclaved medium was inoculated with a 5 mm disk of *S. cepivora* (Sc8) and incubated at 18 ± 2 °C for 5 weeks.

Effects of *Trichoderma* species on garlic white rot disease development

The effects of the three *Trichoderma* species, i.e., *T. harzianum*, *T. koningii*, and *T. virens* and their combinations (equal volumes of each) were investigated on the development of white rot disease on garlic in soil artificially infested with *S. cepivora* (Sc8). The experiment was carried out in pots under greenhouse conditions (the minimum and the maximum temperatures were 5–10 °C and 20–25 °C, respectively) using susceptible cultivar of garlic as in El-Sheshtawi et al. (2009). The experiments were conducted with a completely randomized design (CRD) with nine treatments (seven of the three selected Trichoderma species and their combinations of *T. harzianum* (Th), *T. koningii* (Tk) and *T. virens* (Tv), Th + Tk, Th + Tv, Tk + Tv, Th + Tk + Tv, bioformulated on wheat bran previously described, chemical fungicide, and infected control) each with four replicates. Each replicate consisted of a sterilized plastic pot (30 cm diameter) containing 5 kg of autoclaved loamy clay soil pre-infested with *S. cepivora* (Sc8) at the rate of 2% (*w*/*w*) 2 weeks before sowing. The formulated *Trichoderma* species were added (at the rate of 1% *w*/*w*) to the infested soil one week before planting. The chemical treatment was applied according to the standard fungicide programme (garlic cloves dipped in 1.0 ml Folicur plus foliar spray). The chemical treatment was applied according to the standard fungicide programme. In this programme, garlic cloves were dipped and sprayed (two time intervals 6 weeks in between) with 1 ml/L Folicur. Before chemical treatment, cloves were superficially disinfected by dipping in sodium hypochlorite solution (0.25%) for 2 min and rinsed after surface-sterilization with sterile distilled water. Five surface disinfected garlic cloves (cv. Sides 40) were sown in each pot. Nitrogen fertilizer as urea (46% N) was added at the rate of 10 g/pot 30 days after planting and plants were irrigated when necessary. The percentages of disease incidence were calculated after 100 days after planting as follows: disease incidence (%) = 100 × No. of infected plants/No. of total plants (Zewide et al. 2007).

Field experiments

Selection of trials location

Field trials were conducted in El-Deer village, El-Qalubia governorate, whereas white rot disease was of high commercial interest. In this region, several fields with a well-established history of white rot disease were selected preliminarily for inoculum density determinations according to the procedure of Utkhede and Rahe (1979). After that, two field sites were chosen. One of them was characterized by their low sclerotial density and had 40 sclerotia per 1 kg soil. The second was characterized by high sclerotial density and had an average of 600 sclerotia per 1 kg soil.

Effects of Trichoderma species on garlic white rot disease development

Two field trials were used to estimate the efficiency of the three selected *Trichoderma* species and their combinations for controlling white rot disease of garlic plants. The low sclerotial density trial had an average of 40 sclerotia per 1 kg soil and the high sclerotial density second trial had 600 sclerotia per 1 kg soil. For each trial, the experiments were conducted with a completely randomized design (CRD) with nine treatments (seven of the three selected *Trichoderma* species and their combinations of *T. harzianum* (Th), *T. koningii* (Tk) and *T. virens* (Tv), Th + Tk, Th + Tv, Tk + Tv, Th + Tk + Tv, bioformulated on wheat bran previously described, chemical fungicide, and control) each with four replicates plots. The plot area was 3.0 × 3.5 m (10.5 m^2) each plot included six rows (each 3.0 m length and 50 cm width). Each treatment preparation freshly prepared was incorporated to soil at the rate of 300 g formulation/m length of the row. A cavity 15-cm in depth was made in the surface of each row. Then the powder preparation of each treatment was added to this cavity and then recovered with the soil and immediately irrigated. One week after incorporation, surface-disinfected garlic cloves (cv. Sides 40) were planted in each row. Garlic cloves that had been uniformly sized were hand planted 3-in deep in rows spaced 10 cm × 10 cm within each row. Based on garlic production regimes, the plots were planted with garlic cloves on 15-September of 2016 of 2016/2017 growing season and the experiment was repeated in 2017/2018 growing season. The chemical treatment was applied by dipping garlic cloves before sowing in fungicide formulation (1.0 ml Folicur/L) for 5 min. One month later, stem bases of garlic plants were sprayed (tow times) with the same concentration of Folicur at 6-week intervals. Irrigation and fertilization for garlic were conducted with commercial production in the area. White rot disease evaluations were conducted periodically during the growing season based on top symptoms of white rot, and were confirmed by gently removing some soil from around the base of some plants. At harvest, bulbs with symptoms of white rot were assessed by pulling and observing all garlic bulbs in each plot. The percentage of infected plants as well as white rot reduction (%) was calculated according to Hovius and McDonald (2002) as follows:

$$\text{White rot infection (\%)} \quad \frac{\text{No of infected plants with white rot}}{\text{Total no.of plants}} \times 100$$

$$\text{White rot reduction (\%)} \quad \frac{\text{White rot (\%) in control-White rot (\%) in treatment}}{\text{White rot (\%) in control}} \times 100$$

Effect of Trichoderma species on enzymatic activities in garlic plants

The effect of *T. harzianum*, *T. koningii*, *T. virens* alone, and their dual and triple combinations on the activities of the defense enzymes of peroxidase, polyphenoloxidase, and chitinase of garlic plants was estimated at 100 days after planting. To extract the enzyme, garlic-leave samples (g) were homogenized with 0.2 M Tris HCl buffer (pH 7.8) at 0 °C containing 14 m M B-mercaptoethanol at the rate of 1/3 *w*/*v*. The extracts were obtained by filtering off the debris with a clean cloth and centrifuging at 3000 rpm for 15 min. The supernatants were recovered and kept in a tube in an ice bath until assayed. The supernatant was used to determine the activity of enzymes using UV spectrophotometer. Peroxidase activity was assayed with guaiacol as the hydrogen donor as described by Hammerschmidt et al. (1984) and peroxidase activity was expressed as the increase in absorbance at 470 nm/g fresh weight/minute according to the method described by Lee (1973). Polyphenoloxidase enzyme activity was determined by measuring the rate of quinone formation as a result of oxidizing 3,4-dihydroxyphenylalanine (DOPA) and polyphenoloxidase activity was expressed as the increase in absorbance at 475 nm/g fresh weight/minute according to the method described by Bashan et al. (1985). The determination of chitinase enzyme was carried out using colloidal chitin as substrate and dinitrosalicylic acid (DNS) as reagent to measure reducing sugars according the method described by Monreal and Reese (1969). Chitinase activity was expressed as mM *N*-acetylglucose amine equivalent released/gram fresh weight/60 min at 450 nm.

Effect of Trichoderma species on plant growth and bulb yield

Effects of *T. harzianum* (Th), *T. koningii* (Tk), and *T. virens* (Tv) on plant growth were studied on onion and garlic grown under field conditions. The isolates alone and their dual and triple combinations and chemical fungicide were applied as described in field experiments. Four replicates were used per treatment. Negative control plots were treated with wheat bran free of antagonistic fungi. At 100 days after planting, some vegetative

growth parameters: average plant height (cm), average number of leaves/plant, and average plant biomass (g), of each crop was estimated. At the end of the experiment (180 days for garlic after planting), fresh weight of garlic plants (bulbs with the tops of the plants) within each plot were weighed. Efficacy of treatments was calculated using the following formula: efficacy (%) = fresh weight of plants in control − fresh weight of plants in treatment / fresh weight of plants in control × 100.

Statistical analysis

Data were entered into SPSS software version 14.0 and analyzed statistically by the analysis of variance (ANOVA) test and the means were compared by Duncan's multiple range test at $P < 0.05$. Data collected from field experiments were analyzed separately for each growing season. Data for percentage germinated sclerotia and percent data on disease incidence were statistically analyzed after arcsine square-root transformation; however, untransformed data are presented.

Results

Laboratory experiments

Antagonistic activity

Three antagonistic fungi, i.e., *T. harzianum*, *T. koningii*, *T. virens*, and tebuconazole, were compared for their ability to suppress *S. cepivora* (Sc8). Data presented in Table 1 indicated that the chemical treatment of tebuconazole inhibited both of the growth and sclerotial formation of *S. cepivora* (Sc8). All *Trichoderma* species isolates significantly inhibited the growth of *S. cepivora* (Sc8) in dual culture compared to control (Fig. 1). The inhibition in the growth and sclerotial formation of *S. cepivora* caused by *T. harzianum*, *T. koningii*, and *T. virens* was 90.4; 76.3, 91.2; 51.9, and 86.8; 60.6%, respectively.

Inhibitor effect of cultural filtrate to sclerotial germination

The three *Trichoderma* species cultural filtrates decreased *S. cepivora* sclerotial germination after soaking for 12 h (Table 2). *T. koningii* filtrate caused the greatest reduction (79.3%) in sclerotial germination, followed by *T. harzianum* filtrate (70.5%). *T. virens* culture filtrate caused the least reduction (67.9%). In the same trend, the chemical treatment with tebuconazole inhibited the sclerotial germination of *S. cepivora* completely after soaking for 12 h.

Greenhouse experiments

Data presented in Table 3 showed that the treatments significantly reduced the incidence of garlic plant during 2016/2017and 2017/2018 seasons. *Trichoderma* species (either individually or in combination) significantly reduced the incidence of white rot on garlic. In general, dual and triple combinations of *Trichoderma* isolates were more effective than these isolates used individually. The combination of all three *Trichoderma* species was the most effective treatment. The rates of decreasing disease incidence were 50.0 and 60.0% during 2016/2017and 2017/2018 seasons.

Field experiments

Results of the two growing seasons followed the same trends. The amount of white rot was related to inoculum density. The mean disease incidence of *S. cepivora* infection among garlic plants in soil containing 40 sclerotia/kg of soil were significantly less than in those containing 600 sclerotia/kg of soil. In general, the *Trichoderma* isolates were more effective in reducing white rot disease in the trial with low inoculum density than in high inoculum density. In the low inoculum density trial, the chemical treatment was the most effective, with the lowest disease incidence (7.75% in 2016/2017 season and 13.0% in 2017/2018 season), compared with 24.0 and 47.5% for the control, respectively as shown in Table 4. In general, the dual and triple combinations of the tested *Trichoderma* isolates were more effective than these isolates used individually. The combination of all three *Trichoderma* isolates was the most effective treatment, decreasing disease incidence by 65.6% and 74.2% during 2016/2017and 2017/2018 seasons. In the high inoculum

Table 1 Growth area (cm^2) and number of sclerotia/cm^2 of *Stromatinia cepivora* isolate (Sc8) in dual culture with *Trichoderma* species as well as in tebuconazole and control

Treatment	Growth area (cm^2)[a]	Reduction (%)	No. of sclerotia in (cm^2)	Reduction (%)
T. harzianum	06.75 ± 0.14 c[a]	90.4	19.00 ± 1.00 d	76.3
T. koningii	06.13 ± 0.13 c	91.2	38.50 ± 0.50 b	51.9
T. virens	09.25 ± 0.48 b	86.8	31.50 ± 0.87 c	60.6
Tebuconazole[b]	00.00 ± 0.00 d	100.0	00.00 ± 0.00 e	100.0
Control	70.0 ± 0.00 a	–	80.0 ± 0.00 a	–

Values are mean of four replicates for each treatment as well as the control
Means ± standard error within a column followed by the same letter are not significantly different by Duncan multiple range test at $P < 0.05$
[a]Growth area (cm^2) of *S. cepivora* was calculated using a planimeter
[b]The fungicide tebuconazole was used at the recommended dose of 1 ml Folicur/L

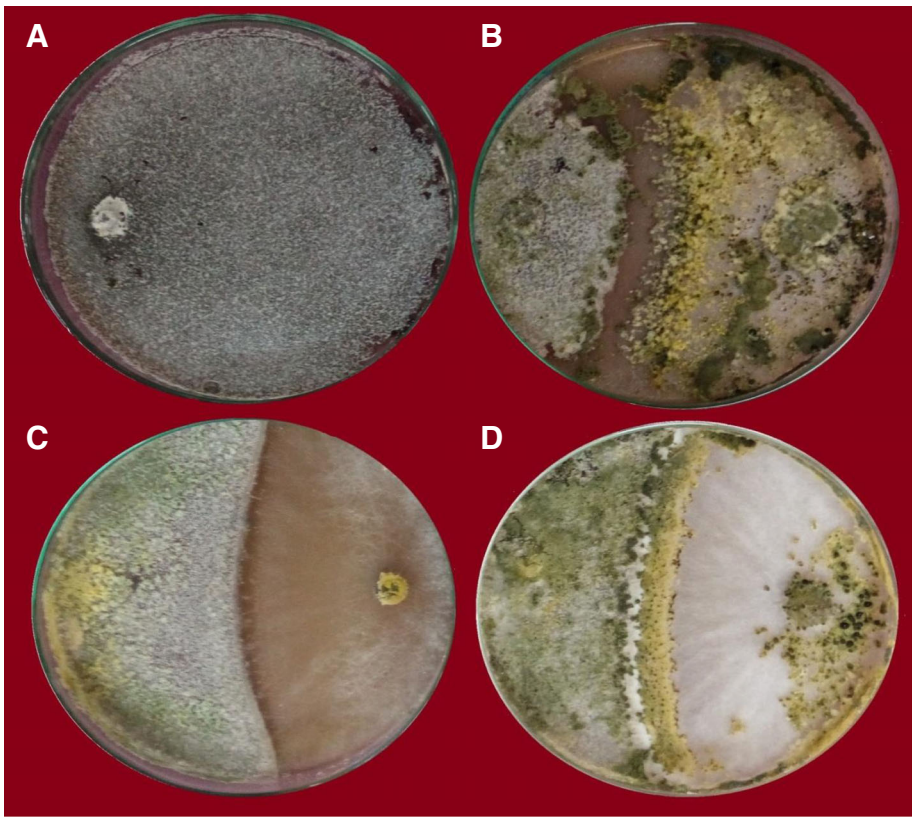

Fig. 1 Antagonistic effect of *Trichoderma* species on *Stromatinia cepivora* growth. **a** *Stromatinia cepivora* alone (control), **b** *Stromatinia cepivora* in the presence of *T. harzianum*, **c** *S. cepivora* in the presence of *T. koningii*, and **d** *S. cepivora* in the presence of *T. virens*

density trial, no *Trichoderma* isolates employed alone gave significant control of garlic white rot. In general, the dual and triple combinations of the tested *Trichoderma* isolates give significant white rot control than these isolates used individually. The combination of all three *Trichoderma* isolates was the most effective treatment, decreasing disease incidence by 15.5% in 2016/2017 season and 18.6% in 2017/2018 season.

Table 2 Sclerotial germination (%) of *S. cepivora* isolate (Sc8) after soaking in cultural filtrates of *Trichoderma* strains as well as chemical fungicide in vitro

Treatment	Sclerotial germination (%)	Reduction (%)
T. harzianum	28.5 ± 0.96 b[a]	70.5
T. koningii	20.0 ± 0.71 c	79.3
T. virens	31.0 ± 1.22 b	67.9
Tebuconazole[b]	00.0 ± 0.00 d	100.0
Water (negative control)	96.7 ± 1.36 a	–

Values are mean of four replicates for each treatment as well as the control
[a]Means ± standard errors within a column followed by the same letter are not significantly different by Duncan multiple range test at $P < 0.05$
[b]The fungicide tebuconazole was used at the recommended dose of 1 ml Folicur/L

Effects on enzymatic activities in garlic plants

Results of the two trails followed the same trends. The tested antagonistic fungi used either individually or in combinations were pronounced in induction of defense enzyme in comparison with the control. In general, the dual and triple combinations were more effective than using these isolates individually (Table 5). The triple combination of all three *Trichoderma* species was the most effective treatment, induced high activation of peroxidase, polyphenoloxidase, and chitinase by 75.0; 72.3, 61.5; 63.7, and 62.3; 70.8% increase over control, under low and high inoculum density, respectively.

Effect of the Trichoderma isolates on plant growth and bulb yield in the field

The *Trichoderma* isolates and their combinations significantly affected plant growth of garlic cv. Sides 40 during two field trials (Table 6). Results of the two trials followed the same trends but the amount of growth improvements was related to inoculum density. The mean growth parameters among plants in soil containing 40 sclerotia/kg were significantly greater than in soil containing 600 sclerotia/kg of soil. In general, the *Trichoderma* isolates were more effective in improving onion and garlic growth in

Table 3 Effects on white rot incidence (%) in garlic under greenhouse conditions

Treatment	White rot incidence (%) and reduction (%)			
	2016/2017 season		2017/2018 season	
	Incidence (%)	Reduction (%)	Incidence (%)	Reduction (%)
T. harzianum (Th)	80.0 ± 0.00 b[a]	20.0	85.0 ± 5.00 b	15.0
T. koningii (Tk)	80.0 ± 0.00 b	20.0	75.0 ± 5.00 bc	25.0
T. virens (Tv)	80.0 ± 0.00 b	20.0	75.0 ± 5.00 bc	25.0
(Th) + (Tk)	70.0 ± 5.77 b	30.0	75.0 ± 5.00 bc	25.0
(Th) + (Tv)	70.0 ± 5.77 b	30.0	70.0 ± 5.77 cd	30.0
(Tk) + (Tv)	70.0 ± 5.77 b	30.0	70.0 ± 5.77 cd	30.0
(Th) + (Tk) + (Tv)	50.0 ± 5.77 c	50.0	60.0 ± 0.00 d	40.0
Tebuconazole	20.0 ± 0.00 d	80.0	30.0 ± 5.77 e	70.0
Control	100.0 ± 0.00 a	–	100.0 ± 0.00 a	–

Values are mean of five replicates for each treatment as well as the control
[a]Means ± standard errors within a column followed by the same letter are not significantly different by Duncan multiple range test at $P < 0.05$

the trial with low inoculum density compared with the trial with high inoculum density. In the low inoculums density trial, the triple combinations of the tested *Trichoderma* isolates were the most effective. The increase rates of plant growth parameters (plant height, number of leaves/plant biomass) were 27.1, 33.0, and 42.1%, respectively compared with the control (Table 6). The effects on garlic grown in soil with high inoculum density followed the same trend (Table 6). The effects of soil application with *Trichoderma* isolates and their combinations on garlic bulb yield at two

Table 4 Effects on white rot disease incidence (%) in garlic under field conditions

Treatment	White rot incidence (%) and reduction (%)			
	Incidence (%)	Reduction (%)	Incidence (%)	Reduction (%)
	Trial I (40 sclerotia/kg soil)		Trial II (600 sclerotia/kg soil)	
2016/2017 growing season				
T. harzianum (Th)	20.75 ± 0.25 b[a]	13.5	76.50 ± 0.50 ab	1.3
T. koningii (Tk)	18.25 ± 0.63 c	23.9	76.00 ± 0.41 ab	1.9
T. virens (Tv)	17.75 ± 0.25 c	26.0	76.25 ± 0.25 ab	1.6
(Th) + (Tk)	13.50 ± 0.29 d	43.8	72.75 ± 0.25 c	6.1
(Th) + (Tv)	13.00 ± 0.41 d	45.8	74.25 ± 0.48 bc	4.2
(Tk) + (Tv)	12.75 ± 0.48 d	46.9	71.25 ± 2.75 c	8.1
(Th) + (Tk) + (Tv)	8.25 ± 0.25 e	65.6	65.50 ± 0.50 d	15.5
Tebuconazole	7.75 ± 0.25 e	67.7	54.75 ± 0.25 e	29.4
Control	24.00 ± 1.35 a	–	77.50 ± 0.50 a	–
2017/2018 growing season				
T. harzianum (Th)	24.00 ± 0.82 b	49.5	85.00 ± 0.41 a	1.2
T. koningii (Tk)	22.50 ± 0.29 b	52.6	85.00 ± 0.41 a	1.2
T. virens (Tv)	22.25 ± 0.48 b	53.2	85.00 ± 0.41 a	1.2
(Th) + (Tk)	17.25 ± 0.48 c	63.7	78.25 ± 0.48 b	9.0
(Th) + (Tv)	16.00 ± 0.71 c	66.3	79.50 ± 0.29 b	7.6
(Tk) + (Tv)	17.00 ± 1.08 c	64.2	78.50 ± 0.29 b	8.7
(Th) + (Tk) + (Tv)	12.25 ± 0.63 d	74.2	70.00 ± 0.41 c	18.6
Tebuconazole	13.00 ± 0.58 d	72.6	59.25 ± 0.48 d	31.1
Control	47.50 ± 1.19 a	–	86.00 ± 0.71 a	–

Values are mean of four replicates for each treatment as well as the control
[a]Means ± standard errors within a column followed by the same letter are not significantly different by Duncan multiple range test at $P < 0.05$

Table 5 Effects on peroxidase, polyphenoloxidase, and chitinase enzymes activities of garlic plants under field conditions at 100 days after planting

Treatment	Enzyme activities in garlic leaves[a]		
	Peroxidase	Polyphenoloxidase	Chitinase
Trial I (40 sclerotia/kg soil)			
T. harzianum (Th)	0.313 ± 0.005 de	0.435 ± 0.004 d	1.754 ± 0.014 c
T. koningii (Tk)	0.312 ± 0.006 de	0.445 ± 0.004 cd	1.593 ± 0.021 d
T. virens (Tv)	0.331 ± 0.008 cd	0.439 ± 0.003 cd	1.515 ± 0.017 d
(Th) + (Tk)	0.378 ± 0.005 b	0.542 ± 0.009 b	1.909 ± 0.033 b
(Th) + (Tv)	0.379 ± 0.006 b	0.547 ± 0.003 b	1.921 ± 0.036 b
(Tk) + (Tv)	0.339 ± 0.007 c	0.531 ± 0.005 b	1.900 ± 0.025 b
(Th) + (Tk) + (Tv)	0.472 ± 0.004 a	0.587 ± 0.002 a	2.026 ± 0.046 a
Tebuconazole	0.295 ± 0.019 e	0.454 ± 0.014 c	1.386 ± 0.052 e
Control	0.118 ± 0.002 f	0.226 ± 0.004 e	0.763 ± 0.022 f
Trial II (600 sclerotia/kg soil)			
T. harzianum (Th)	0.339 ± 0.009 d	0.566 ± 0.011 e	1.806 ± 0.028 d
T. koningii (Tk)	0.372 ± 0.001 c	0.583 ± 0.005 d	1.933 ± 0.012 c
T. virens (Tv)	0.376 ± 0.005 c	0.566 ± 0.005 e	1.918 ± 0.024 c
(Th) + (Tk)	0.419 ± 0.004 b	0.616 ± 0.004 c	2.198 ± 0.018 b
(Th) + (Tv)	0.434 ± 0.013 b	0.632 ± 0.006 b	2.193 ± 0.020 b
(Tk) + (Tv)	0.432 ± 0.003 b	0.635 ± 0.004 b	2.172 ± 0.001 b
(Th) + (Tk) + (Tv)	0.502 ± 0.004 a	0.659 ± 0.005 a	2.692 ± 0.024 a
Tebuconazole	0.329 ± 0.003 d	0.436 ± 0.003 f	1.457 ± 0.064 e
Control	0.139 ± 0.004 e	0.239 ± 0.005 g	0.786 ± 0.017 f

Values are mean of eight replicates for each treatment as well as the control
Means ± standard errors within a column followed by the same letter are not significantly different by Duncan multiple range test at $P < 0.05$
[a]Peroxidase activity was expressed as the increase in absorbance at 470 nm/g fresh weight/minute. Polyphenoloxidase activity was expressed as the increase in absorbance at 475 nm/g fresh weight/minute. Chitinase activity was expressed as mM N-acetyl glucose amine equivalent released/gram fresh weight/60 min at 540 nm. Values are means of four replicates

sites followed the same trend but the bulb yield was greater in soil with low inoculum density than with high inoculum density as shown in (Table 7).

Discussion

In vitro, all *Trichoderma* species isolates significantly inhibited the growth of *S. cepivora* (Sc8) in dual culture compared to control (Fig. 1). Obtained results are in harmony with those obtained by Francisco et al. (2011). *Trichoderma* spp. exert their biocontrol action against fungal phytopathogens either indirectly by competing for nutrients and space or indirectly by mechanisms such as antibiosis and mycoparasitism (Peyghami 2001; Benítez et al. 2004). These results were in agreement with those of Kay and Stewart (1994) and McLean and Stewart (2000), who found strong antagonistic effects of *Trichoderma* spp. against most pathogenic fungi. They reported that *Trichoderma* depends on one or more of the following mechanisms: competition for nutrients or space, mycoparasitism or antibiosis, and/or antibiotic excretion. Peyghami (2001) described the mechanism by which *T. viride* and *T. harzianum* affect the pathogen *S. cepivora* via hyphal contact, deformation, and lysis. The three *Trichoderma* species cultural filtrates decreased *S. cepivora* sclerotial germination after soaking for 12 h (Table 2). The present data are in accordance with those obtained by Shalaby et al. (2013), who reported that the percentage of germinated sclerotia soaking in filtrates of *T. harzianum* and *T. koningii* were 46.67 and 56.67%, compared with 93.3% for control, respectively. This suggests that filtrates of the *Trichoderma* isolates may contain lytic enzymes or antibiotics, as found by others. McLean and Stewart (2000) and Clarkson et al. (2002) suggested that chitinase, glucanase, and protease produced by antagonistic fungi dissolve pathogen cell walls in certain locations, thus causing substantial damage. Additionally, secretion of antibiotics into filtrates also affects sclerotial germination and growth of soil-borne pathogenic fungi (EL-Kazzaz et al. 2002).

In greenhouse and field experiments, the dual and triple combinations of the tested *Trichoderma* isolates were more effective than these isolates used individually. Such results are in agreement with the earlier findings of Abd El-Razik et al. (1988). These results also agree with those reported recently by Elshahawy et al. (2017b). Francisco et al. (2011) found that 41 *Trichoderma* isolates showed excellent levels of antagonism toward *S. cepivorum* either by competition for nutrients, antibiosis by volatile compounds, or effect of filtering toxins. Shalaby et al. (2013) reported that onion white rot disease incidence was decreased to 29.17; 13.89% and 37.50; 11.11%, by the use of *T. koningii* and *T. harzianum*, under pots and field experiment, respectively.

Under field conditions, a positive correlation was found between the biocontrol activates of *Trichoderma* species isolates and enhancement of peroxidase, polyphenoloxidase, and chitinase enzymes in garlic to resist infection with *S. cepivora*. The reduction in garlic white rot disease incidence and severity may be due to an increase in the defense-related enzymes such as peroxidase, polyphenoloxidase, and chitinase. These results are in agreement with those obtained by Sharma et al. (2012). Shalaby et al. (2013) who reported that a close link was found between the biological action of *T. koningii* and *T. harzianum* and enhancement of the enzyme activities of polyphenol oxidase and peroxidase with ability of onion to resist *S. cepivora*. The oxidative enzymes play an important role in induced resistance by the oxidation of phenols to oxidized toxic products (quinine) which limit fungal activity. Peroxidases catalyze a number of reactions that fortify plant cell walls. These reactions include the incorporation of phenolics into cell

Table 6 Effects on plant height, number of leaves/plant, and plant biomass of garlic plants under field conditions at 100 days after planting

Treatment	Garlic plants grown in field[a]		
	Plant height (cm)	Number of leaves/plant	Plant biomass (g)
Trial I (40 sclerotia/kg soil)			
T. harzianum (Th)	69.0 ± 2.17 b	7.9 ± 0.13 c	64.95 ± 2.36 cd
T. koningii (Tk)	68.4 ± 1.65 b	7.6 ± 0.18 cd	64.24 ± 2.58 d
T. virens (Tv)	68.7 ± 1.85 b	7.6 ± 0.18 cd	65.63 ± 2.79 bcd
(Th) + (Tk)	75.1 ± 3.68 a	8.1 ± 0.13 bc	70.85 ± 4.37 bcd
(Th) + (Tv)	75.4 ± 3.60 a	8.1 ± 0.23 bc	73.65 ± 4.98 b
(Tk) + (Tv)	75.7 ± 3.72 a	8.6 ± 0.18 b	73.29 ± 5.24 bc
(Th) + (Tk) + (Tv)	79.3 ± 3.61 a	9.4 ± 0.18 a	85.48 ± 8.66 a
Tebuconazole	61.4 ± 0.24 c	7.3 ± 0.16 d	54.30 ± 0.64 e
Control	57.8 ± 0.71 c	6.3 ± 0.25 e	49.53 ± 1.06 e
Trial II (600 sclerotia/kg soil)			
T. harzianum (Th)	55.8 ± 1.89 cd	7.1 ± 0.13 a	46.40 ± 1.29 c
T. koningii (Tk)	54.8 ± 2.24 d	7.3 ± 0.16 a	43.59 ± 2.13 d
T. virens (Tv)	55.7 ± 1.89 cd	7.1 ± 0.13 a	46.01 ± 1.79 c
(Th) + (Tk)	58.0 ± 2.40 bc	7.4 ± 0.18 a	50.40 ± 1.22 b
(Th) + (Tv)	59.3 ± 2.51 b	7.6 ± 0.18 a	50.41 ± 1.59 b
(Tk) + (Tv)	59.9 ± 2.87 b	7.5 ± 0.19 a	51.23 ± 1.70 b
(Th) + (Tk) + (Tv)	62.6 ± 2.81 a	7.6 ± 0.18 a	54.61 ± 2.41 a
Tebuconazole	45.6 ± 0.76 e	6.0 ± 0.19 b	41.78 ± 0.65 d
Control	39.6 ± 0.76 f	5.5 ± 0.19 c	32.40 ± 0.72 e

Means ± standard errors within a column followed by the same letter are not significantly different by Duncan multiple range test at $P < 0.05$
[a]Values are mean of eight replicates for each treatment as well as the control

walls and lignifications and suberization of plant cell walls. On the other hand, the chitinase enzymes play roles in plant defense against fungi by hydrolyze their cell wall. The amount of them significantly increase and play main role of defense reaction against fungal pathogen by degrading cell wall, because chitin is a major structural component of the cell walls of many pathogenic fungi. *Trichoderma* application improves plant growth and bulb yield of garlic plants grown under field conditions. In general, the dual and triple *Trichoderma* isolates combinations were more effective than these isolates used individually (Table 7). These results are in agreement with those obtained by Metcalf et al. (2004). Growth promotion effect is one of the mechanisms of *Trichoderma* spp. exerted for control of phytopathgenic diseases (Benítez et al. 2004; Sharma et al. 2012; Gajera et al. 2013). The capacity of *Trichoderma* spp. to promote growth results from the production of phytohormones that promote growth characteristics of the plants. Hexon et al. (2009) described induced production of three auxin-related compounds (indole-3-acetic acid, indole-3-acetaldehyde, and indole-3-ethanol) causing development of *Arabidopsis* seedlings in response to inoculation with *T. virens* and *Trichoderma atroviride*. This was also supported by Harman et al. (2004) who found that *Trichoderma* spp. colonize root surfaces and penetrate the epidermis before producing or releasing a variety of compounds that induce localized or systemic resistance responses. Therefore, plants become protected from the pathogenic fungus, indicating induction of SAR in plants treated with the biological isolates.

Conclusion

White rot caused by *S. cepivora* is one of the most important diseases of garlic in Egypt. In field experiments, under low (40 sclerotia/kg of soil) and high (600 sclerotia/kg of soil) inoculum density, the standard fungicide programme (garlic cloves dipped in tebuconazole plus foliar spray) gave statistically significant disease control compared with the untreated control. Under low disease pressure, significant control, equal to the fungicide treatment, was achieved with the combination of the three *Trichoderma* spp. biocontrol agents. Reduction of white rot disease was accompanied by increased growth parameters and bulb yield of garlic plants grown under field conditions. However, no antagonistic *Trichoderma* spp. employed alone gave significant control

Table 7 Effect of selected *Trichoderma* strains and their combinations on garlic bulb yield under field conditions

Treatment	Garlic bulb yield (kg/plot) and efficiency of treatment (%)			
	Yield (kg/plot)	Efficiency (%)	Yield (kg/plot)	Efficiency (%)
	Trial I (40 sclerotia/kg soil)		Trial II (600 sclerotia/kg soil)	
2016/2017 growing season				
T. harzianum (Th)	26.85 ± 0.03 a	40.4	11.88 ± 0.03 a	20.6
T. koningii (Tk)	26.85 ± 0.03 a	40.4	11.80 ± 0.07 a	20.6
T. virens (Tv)	26.83 ± 0.03 a	40.4	11.85 ± 0.03 a	20.4
(Th) + (Tk)	27.28 ± 0.05 a	41.3	11.85 ± 0.03 a	20.4
(Th) + (Tv)	27.33 ± 0.05 a	41.5	11.83 ± 0.03 a	20.3
(Tk) + (Tv)	27.38 ± 0.03 a	41.6	11.88 ± 0.03 a	20.6
(Th) + (Tk) + (Tv)	27.50 ± 0.00 a	41.8	11.90 ± 0.00 a	20.8
Tebuconazole	20.45 ± 2.45 b	21.8	9.80 ± 0.11 b	3.8
Control	16.00 ± 0.17 c	–	9.43 ± 0.15 c	–
2017/2018 growing season				
T. harzianum (Th)	21.60 ± 0.07 c	35.5	8.78 ± 0.06 c	10.3
T. koningii (Tk)	21.78 ± 0.03 c	36.0	8.90 ± 0.04 c	11.5
T. virens (Tv)	21.85 ± 0.32 c	36.2	9.13 ± 0.23 bc	13.7
(Th) + (Tk)	22.65 ± 0.09 b	38.5	9.53 ± 0.11 ab	17.3
(Th) + (Tv)	22.60 ± 0.07 b	38.4	9.78 ± 0.11 a	19.4
(Tk) + (Tv)	22.70 ± 0.08 b	38.6	9.88 ± 0.09 a	20.2
(Th) + (Tk) + (Tv)	23.50 ± 0.22 a	40.7	9.95 ± 0.03 a	20.8
Tebuconazole	16.00 ± 0.18 d	12.9	8.15 ± 0.12 d	3.3
Control	13.93 ± 0.24 e	–	7.88 ± 0.36 d	–

Values are mean of four replicates for each treatment as well as the control
[a]Means ± standard errors within a column followed by the same letter are not significantly different by Duncan multiple range test at $P < 0.05$

of white rot under high disease pressure. It was concluded that, at the low disease pressure site, the low level of inoculum and disease incidence enabled the three biocontrol agents to bring about disease control that were related to improve garlic growth and production.

Acknowledgements
The authors thank The Affairs of Research Projects, National Research Centre, for funding this research program under Grant no. 11030137.

Funding
National Research Centre.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

Received: 16 December 2018 Accepted: 28 January 2019
Published online: 12 February 2019

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