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ORIGINAL ARTICLE

Biological and fungicidal antagonism of *Sclerotium cepivorum* for controlling onion white rot disease

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Abstract The action of some microbial isolates and Topsin-M against the most pathogenic isolate (Sc_2) of *Sclerotium cepivorum* causing onion white rot was tested. *Bacillus subtilis* B_4, *B. subtilis* B_5, *Trichoderma koningii* and *Trichoderma harzianum* were the most antagonistic isolates of the causal fungus. Mycelia of sclerotial germination of *S. cepivorum* (Sc_2) were completely inhibited in vitro by application of 2.0 g L^{-1} Topsin-M. In pots, disease incidence was decreased to 8.33 % by the use of Topsin-M followed by *T. koningii* (29.17 %) compared with 95.83 % for the control, i.e., a remarkable reduction in severity was obtained. Under field conditions, disease incidence was decreased to 2.78 % by Topsin-M and to 11.11 % by *T. harzianum*. Both agents caused a sharp reduction in disease severity, reaching 1.39 % and 9.72 %, respectively, with 11.80 % being achieved by *T. koningii* and *B. subtilis* B_5. A close link between the biological action and enhancement of the enzyme activities of polyphenol oxidase and peroxidase with ability of onion to resist *S. cepivorum* was found, indicating induction of systemic acquired resistance. Accordingly, chlorophyll, root and foliage lengths, foliar, bulb dry matter and bulb productivity were also enhanced upon application of this biological control strategy.

Keywords Biological control · *Sclerotium cepivorum* · Defensive enzymes

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Introduction

Allium white rot, a fungal disease caused by *Sclerotium cepivorum* Berk., is the predominant disease of onion crops worldwide. The fungus produces long-lived round survival structures (sclerotia). Efficiency of disease control depends on the method used and on the time of application, but no one method offers complete control (Melero-Vara et al. 2000). Several methods have been suggested, including chemical fungicide applications (Stewart et al. 1994), soil fumigants (Entwistle 1990), soil solarisation (McLean et al. 2001) and biological control (Clarkson et al. 2004). Chemical fungicides are still the superior strategy used effectively against different causal pathogenic isolates. Topsin-M 70 % is used widely for suppressing growth of *Cephalosporium maydis* (Singh and Siradhana 1988), *Sclerotium rolfsii* (Danhiber et al. 1991), *Rhizoctonia solani, Alternaria* spp., *Fusarium* spp. and *Macrophomina phaseolina* (Ali et al. 1995) under field conditions.

Some bacterial and fungal antagonists were formerly applied by many investigators against *S. cepivorum*. Abd El-Razik et al. (1985) studied the antagonistic effects of 23 fungal and 28 bacterial isolates against *S. cepivorum*. *Trichoderma harzianum* and *Bacillus subtilis* were found to have the greatest inhibitory action against the pathogen. Mousa et al. (1987) tested antagonism of 19 *B. subtilis* and 9 *T. harzianum* isolates against *S. cepivorum* under laboratory conditions. They found that the bacterial isolates were powerful in their antagonistic potency against all the pathogenic fungus isolates. Culture filtrate of *T. harzianum* also proved to have strong antifungal activity against linear growth of

both *S. cepivorum* and *Fusarium oxysporum* f. sp. *cepae* (Tohamy et al. 1993), although activity against the former was stronger. Clarkson et al. (2001) investigated degradability of sclerotia of *S. cepivorum* by *Chaetomium minitans*, *Trichoderma virens* and *T. harzianum* in vitro and in vivo trials. Cotes and Monte (2001) found that *Trichoderma koningii* was more effective when used as a seed treatment; adding a suspension of the antagonist twice into soil obtained more than 60 % protection against *S. cepivorum* in onion plants.

Activities of both peroxidase and polyphenol oxidase are well known to be associated with the defensive system of host plants, indicating induction of systemic acquired resistance (SAR) under pathogenic conditions (Hatcher 1995; Saravanakumar et al. 2007). Assay of defensive enzymes by these latter authors found that PGPR bio-formulations induced greater production of enzymes in PGPR-treated plants than in control plants. In her study to control onion white rot disease, El-Khateeb (2004) stated that onion transplants treated before planting with *Trichoderma lignorum*, *T. harzianum*, *B. subtilis* and *B. pumilis* led to high foliage fresh and dry weights in addition to a notable increase in bulb crop yield compared to that obtained using the chemical fungicide Folicure. On the other hand, untreated field plots produced lower yields in terms of onion bulb productivity.

To reduce the harmful effects of fungicides on environmental systems, use of effective and safer methods not only for controlling plant diseases, but also for enhancing plant growth and productivity are required. Therefore, the present work aimed to suppress the causal agent of onion white rot disease biologically instead of using a chemical strategy.

Materials and methods

This study was carried out at the Agricultural Botany Department, Faculty of Agriculture, Kafrelsheikh University as well as within fields of El-Gharbia and Kafr El-Sheikh Governorates, Egypt during the 2010 and 2011 growing seasons.

Media

The fungal isolates were pre-cultured and maintained on potato dextrose agar (PDA) medium (1 L; pH 6.5–6.8) contained 200 g potato, 20 g dextrose and 15 g agar; Tsao 1970). Nutrient agar (NA) medium (1 L; pH 6.3–6.8) contained 5 g peptone, 3 g beef extract and 15 g agar was used for bacteria cultivation; Vincent 1970). The shaking flask culture media potato dextrose broth (PDB) and nutrient broth (NB) (as PDA and NA but without agar) were used for fungal and bacterial antagonistic inocula, respectively. An enrichment barley grains (BG) culture medium (glass bottles of 500 mL capacity containing 100 g barley grains and 50 mL water) was also used for the fungal pathogen as described in the methods of Abd El-Moity (1976). All media were autoclaved at 121 °C for 30 min before use.

Pathogen

Screening of onion (*Allium cepa* L.) white rot disease was performed within naturally infested fields (clay; EC 4.2 dS m^{-1}; pH 6.4–6.7) in El-Gharbia and Kafr El-sheikh Governorates. Samples showing typical white rot symptoms were collected and divided into small pieces. Using 0.25 % sodium hypochlorite solution, pieces were surface-sterilised for 4 min, washed thoroughly with sterile distilled water, blotted between sterile filter papers and plated on PDA medium. Inoculated plates were incubated at 18–20 °C for 10–15 days and examined daily to observe growth of the causal mycelium. Formation of distinguishable sclerotia was also noted and the cultures were purified using the hyphal tip technique according to Booth (1977). Pure cultures were maintained on PDA slants and kept in a refrigerator at 4 °C as stock cultures for further tests.

Eight isolates of the causal fungus were subjected to pathogenicity testing using onion transplants under sterilised conditions. Pathogenicity tests were carried out in pots under outdoor conditions using the most common cultivar (Giza 20) planted in the Nile Delta of Egypt. Pots (30 cm in diameter) were sterilised by immersing in 5 % formalin solution for 15 min and left until complete evaporation of formalin. Clay soils were washed several times in 0.1 *N* HCl solution and further washed with distilled water before filling the pots. Inocula of the eight isolates were prepared according to the methods of Abd El-Moity (1976) using sterilised BG medium. After sterilisation, bottles were inoculated with 5-mm diameter discs of the fungal isolates using 10-day-old cultures and incubated at 18–20 °C for 25 days. Inoculum of each isolate was mixed thoroughly with the sterilised clay soils at rate of 2 % w/w. Each pot was filled with 5 kg infested clay soil. Pots filled only with sterile soil acted as a control. All pots were watered 2 weeks before planting to achieve very good growth and distribution of the pathogen. Four healthy onion transplants (60 days old) were sown in each pot. Four pots of each isolate were used as replicates. At 100 days after transplanting, plants were uprooted to estimate pathogenicity with severity based on the 0–100 scale reported by Abd El-Moity (1976) and Shatla et al. (1980) as follows:

- 0 Healthy plants
- 25 Slight severe (yellowing of the leaves, reduced root system)
- 50 Moderate severe (yellowing and die-back of leaves, root system badly decayed)
- 75 Severe (complete yellowing of the plant, die-back of the leaves, semi watery soft rot of scales and roots)

100 Highly severe (completely dead plants, extensive decayed bulbs and roots).

According to Walker (1952) and Alexopoulos and Mims (1979), the cultural, microscopic and phytopathological properties of the eight isolates tested were found to belong to *Sclerotium cepivorum*. Based on its high pathogenicity, only one isolate of *S. cepivorum* was selected for further study.

Antagonists

The antagonistic microorganisms were isolated from rhizosphere soil samples of healthy onion plants growing close to diseased ones using the soil dilution plate method (Johnson and Curl 1972). Soils adhering with the healthy plants were removed from the roots, collected, air dried and mixed very well together. 10 g of the homogenised soils were suspended with sterilised distilled water to reach 100 mL volumes in conical flasks. After thoroughly shaking (150 rpm at 28–30 °C) for 10 min, series dilutions were prepared. The last dilutions were used to inoculate plates containing PDA and NA media for fungi and bacteria, respectively. 100 μL of soil suspension were spread on the surfaces of Petri-plates using sterilised Drigalski glass triangle. Plates were incubated at 28–30 °C for 3 days. Growth of incubated plates was examined and then purified using single colony method for bacteria and hyphal tip technique for fungal isolates. Pure cultures were kept in a refrigerator at 4 °C as stock cultures for further tests. Antagonistic activities of both bacterial and fungal isolates against *S. cepivorum* were compared to a chemical fungicide commonly used at onion cultivations.

(1) Bacterial antagonists:
Petri-dishes (9 cm in diameter) of PDA-medium (15 mL dish^{-1}) were inoculated in their centre with agar discs (5 mm) bearing mycelium of 7-day-old cultures of *S. cepivorum* (Sc_2). The periphery of each plate was inoculated with standard amounts of 4 bacterial isolates using sterile tooth picks. Plates inoculated with the pathogen without antagonists were used as control. Experiments were represented by three replicates. Plates were incubated at 18–20 °C until full growth of the control treatment. The diameter of the inhibition zone surrounding each antagonistic isolate was recorded, and the relative power of antibiosis (RPA) of each isolate was estimated according to the formula described by (Ibrahim et al. 1987):

$$RPA = Z/C$$

Where:

Z Diameter of inhibition zone.
C Diameter of spotted antagonistic isolate.

Two of the most antagonistic bacterial isolates (higher RPA ratio) were subjected to identification. According to the methods described in *Bergey's Manual of Determinative Bacteriology* (Holt et al. 1994; Parry et al. 1983), both isolates were identified as *Bacillus subtilis*.

(2) Fungal antagonists:
Screening of fungal antagonists was performed using the dual culture technique. Agar discs (5 mm in diameter) bearing mycelium of 7-day-old cultures of one of the isolated fungal antagonists and *S. cepivorum* (Sc_2) were placed on the opposite sides of Petri-dishes containing 15 mL PDA-medium. Plates containing *S. cepivorum* (Sc_2) alone were used as controls. Three replicates were used. Plates were incubated at 18–20 °C until full growth of the control. Degree of antagonism was scored based on the scale of 1–5 classes described by Bell et al. (1982):

Class 1 The antagonist covered the entire medium surface and completely over grew the pathogen.
Class 2 The antagonist over grew at least two-thirds of the medium surface.
Class 3 The antagonist and the pathogen each colonised approximately one-half of the medium surfaces (more than one-third and less than two-thirds) and neither organism appeared to dominant the other.
Class 4 The pathogen colonised at least two-thirds of the medium surface and appeared to withstand encroachment by antagonist.
Class 5 The pathogen occupied the entire medium surface and completely over grew the antagonist.

Based on their antagonistic efficacy, two of the fungal antagonists were subjected to identification tests according to the methods stated by Gilman (1957), Domsch et al. (1980) and Watanabe (1994). The two isolates were identified as *Trichoderma koningii* and *Trichoderma harzianum*. Identification was also confirmed against morphological, cultural and microscopic properties described on the website: http://nt.arsgrin.gov/taxadescriptions/keys/rptdescriptiononlyList.cfm?whichOne=trichoderma

(3) Chemical fungicide:
A specific chemical fungicide {Topsin-M [dimethyl 4,4-(O-phenylene) bis (3-thioallophanate) 70 %]} used commonly in disease control programs of soil-borne phytopathogens of different crops under field conditions was also tested against *S. cepivorum* (Sc_2) in this study. It is known as Thiophanate-methyl 70 % WP and the recommended dose is 1 g L^{-1}. To investigate its effect, different concentrations of Topsin-M

(0.5, 1.0, 2.0, 3.0 and 5.0 g L^{-1}) were tested. A known weight of Topsin-M was first dissolved in 10 mL distilled water before adding to flasks containing 90 mL still-warm sterilised PDA medium under aseptic conditions using a membrane filter syringe of 0.2 μm. After thorough mixing by shaking by hand, the medium was poured into 9 cm in diameter Petri-dishes (15 mL $dish^{-1}$). As replicates, three dishes of each treatment were inoculated centrally with 5 mm agar discs bearing mycelium of 7-day-old cultures of *S. cepivorum* (Sc_2), then incubated at 18–20 °C. PDA-medium free from fungicide served as a control treatment. Growth was observed daily and the maximum linear growth was measured at the time of full growth in the control treatment. Net growth data of each concentration were calculated and percentages of inhibition (I %) were tabulated according to similar formula suggested by Topps and Wain (1957):

$$I\% = [(A - B)/A] \times 100$$

Where:

I % Percentage of inhibition.
A Mean diameter growth in the control.
B Mean diameter growth in a given treatment.

Antagonistic effect on germination of sclerotia

Due to their widespread occurrence and importance in the life cycle of *S. cepivorum*, germination of sclerotia was also investigated. Regarding bacterial antagonists, filtrates of 7-day-old cultures (0.8×10^{8} CFU mL^{-1}) of both *B. subtilis* isolates grown on NB medium incubated at 28–30 °C in a shaking incubator (150 rpm) were used. For fungal antagonists, filtrates of PDA-medium cultures (7 days old) of both *Trichoderma* species were also used. Fungal cultures were also shake incubated at 28–30 °C, 150 rpm and their spore contents adjusted to 0.5×10^{7} spore mL^{-1} each using a counting chamber (haemocytometer specialised microscope slide). To obtain filtrates, both bacterial and fungal cultures were filtered using cheese cloth and centrifuged for 15 min at 5,000 rpm (Heraeus Biofuge Pico, UK) to separate the biomass. The resulting filtrates were kept in a refrigerator at 4 °C as stock substances for the planned experiments. As with the biological antagonists, 0.2 g Topsin-M was well dissolved in 100 mL distilled water, filtered and centrifuged to obtain the most effective concentration (2 g L^{-1}). Thus, prepared filtrates of all the control agents used were prepared ready for the proposed tests with germination of sclerotia.

Sclerotia of 30-day-old cultures of *S. cepivorum* (Sc_2) were collected from the edges of Petri-dishes then soaked in test tubes containing the filtrate to be tested for 12 h at room temperature. At end of the dipping period, sclerotia were washed by sterile distilled water and ten sclerotia from each treatment were transferred individually under aseptic conditions to Petri-dishes containing a thin layer of PDA medium. Water-soaked sclerotia were used for control treatment. Three replicates (dishes) were used for each treatment. Petri dishes bearing sclerotia were incubated at 18–20 °C for 6 days and percentages of germinating sclerotia were determined.

Experimental preparations and treatments

Performance of the tested antagonists was investigated under both infested pot and open field conditions. At 60 days after transplanting, the activities of two enzymes known to act during plant defence against pathogen infection [polyphenol oxidase (PPO) and peroxidase (PO)] were determined, in addition to certain plant growth and yield parameters. At the end of the growing season (100 days after transplanting), disease incidence and disease severity were estimated.

Preparation of the tested antagonists

Antagonistic bacterial isolates of *B. subtilis* B_4 and *B. subtilis* B_5 (each at 0.8×10^{8} CFU mL^{-1}) were prepared by growing them on NB medium at 28–30 °C for 10 days in a shaking incubator (160 rpm). Antagonistic fungi *T. koningii* and *T. harzianum* (each at 0.5×10^{7} spore mL^{-1}) were grown on PDB-medium and incubated at 28–30 °C for 10 days. For the chemical fungicide, 2.00 g original powder of Topsin-M was well dissolved in a total volumes of 1 L distilled water using a magnetic stirrer.

Treatment of onion transplants

Giza 20 onion transplants (60 days old) were immersed for 12 h at room temperature in each of the antagonistic control agents. One month after transplanting, a booster dose (5 mL) of each antagonist was added around plants in both pots and open field trials.

Pot experiments

Under outdoor conditions, pots (30 cm in diameter) were filled with infested 8 kg clay soil (field capacity, FC 41 %). Soil was infested with the pathogenic isolate (Sc_2) of *S. cepivorum* using a method similar to that applied for pathogenicity test via enrichment BG-medium (Abd El-Moity 1976). Two weeks after soil infestation, immersed onion transplants were planted using eight transplants per pot. Three pots were used for each treatment as replicates. Transplants dipped in distilled water and planted in infested pots served as controls. The experimental units were watered when needed and recommended agricultural practices were also carried out.

Open field experiments

A field naturally infested (FC 42 %) with *S. cepivorum* located at Negrig region, El-Gharbia Governorate was used for controlling white rot disease of onion plants. Plots of 1.5 m long and 75 cm wide (an area of 1.125 m^2) were each planted with 30 onion transplants immersed in the tested control agents. Each treatment was represented by three plots. Transplants dipped in distilled water represented the control treatment. Irrigation and fertilisation were conducted as generally recommended for onion production regimes.

Determinations

Disease index parameters

At 100 days after planting, plants were uprooted and examined for disease index parameters. Percentages of white rot disease incidence were calculated based on the formula suggested by Crowe et al. (1994):

$$\text{Disease incidence} = \frac{\text{No. of infected plants}}{\text{No. of total plants}} \times 100$$

Percentages of disease severity were also estimated based on the 0–100 scale suggested earlier by Abd El-Moity (1976) and Shatla et al. (1980), which was also applied in the pathogenicity test (see above).

Enzyme activities

The effect of the studied antagonists on the activities of the defence enzymes PPO and PO in the shoots was estimated 60 days after planting. For this purpose, crude enzyme extract was prepared according to the methods described by Maxwell and Bateman (1967). Briefly, tube-leaf samples were cleaned, weighed and triturated in a ceramic mortar in the presence of 0.1 M sodium phosphate as buffer solution (pH 7.1). Samples of 1 g fresh weight in 2 mL buffer solution were filtered through cheese cloth and centrifuged at 3,000 rpm at 6 °C for 20 min.

PPO enzyme activity was assayed according to the colorimetric procedures adopted by Matta and Dimond (1963). The reaction mixture contained 1.0 mL enzyme extract, 1.0 mL 0.2 M sodium phosphate buffer at pH 7.0, 1.0 mL 10^{-3}M catechol $[C_6H_4(OH)_2]$ and 3.0 mL distilled water brought to a final volume of 6.0 mL. Using a UV–VIS spectrophotometer (Jenway, 6105), absorbance was measured at an optical density (OD) of 495 nm and ODs were recorded at 0, 30, 60, 90 and 120 s intervals. PPO enzyme activities were carried out in triplicate and expressed as changes in OD $s^{-1}g^{-1}$ fresh weight. A sample containing all the chemical reagents except the enzyme extract served as a blank, which was used to reset or calibrate the device.

PO enzyme activity was assayed according to the methods of Srivastava (1987), by measuring the oxidation of pyrogallol to pyrogallin in presence of H_2O_2. A mixture of 0.5 mL 0.1 M sodium phosphate, 0.3 mL enzyme extract, 0.3 mL 0.05 M pyrogallol (C_6 H_3 COH_3), 0.1 mL 10 %H_2O_2 (v/v) was diluted to a final volume of 3 mL using distilled water. At 425 nm, absorbance of the samples was measured and ODs recorded after 0, 30, 60, 90 and 120 s using a spectrophotometer (Jenway 6105 UV–VIS). Enzyme activity of PO was measured in three replicates. Reading OD $s^{-1}g^{-1}$ fresh weight was used as an indication of activity. A sample containing all the chemical reagents except the enzyme extract served as a blank, which used to reset or calibrate the device.

Plant growth and yield parameters

Chlorophyll (*a* and *b*) contents of plant leaves 60 days after planting were estimated according to Moran (1982). A known area (one disk 0.5 cm in diameter) equal 0.1963 cm^2 leaf was taken and the chlorophyll content extracted by immersing in 5 mL N,N-dimethylformamide in the dark. Absorbance was measured at 647 and 664 nm using a spectrophotometer (Jenway 6105 UV–VIS). Readings were used to calculate chlorophyll *a* and *b* and total chlorophyll per $\mu g mL^{-1}$ based on the following equations:

$$\text{Chl. a} = 12.46(\text{A}664) - 2.49(\text{A}647)\mu g\, mL^{-1}$$

$$\text{Chl. b} = -5.6(\text{A}664) + 23.26(\text{A}647)\mu g\, mL^{-1}$$

Thus, chlorophyll content relative to leaf area per µg $(cm^2)^{-1}$ was recalculated. Additionally, root and foliage lengths (cm) were also measured. To determinate dry matter, weighed fresh samples of both bulbs and leaves were dried separately at 60 °C, with time to constant weight ranging from 4 to 7 days. Specimens were allowed to cool before weighing again. The difference between the two weights in grams was recorded as moisture and percentage dry matter was calculated. Yield productivity of onion bulbs (weight) per kilogram was also recorded for each treatment.

Statistical analysis

A complete randomised block was applied to the trials in this study. Data were subjected to statistical analysis of variance by ANOVA test in SPSS, 11 software statistical packages (Gomez and Gomez 1984). Duncan's multiple range tests were performed for comparing means (Duncan 1955).

Results and discussion

Pathogen

Screening trials of the fields infested with onion white rot disease resulted in eight isolates of *S. cepivorum*. Pathogenicity of these isolates showed varied degrees against onion transplants cv. Giza 20 (Table 1), ranging from the most aggressive (100 %) in case of isolate Sc_2 to the lowest degree (75 %) for isolate Sc_1. Due to its aggressive pathogenicity, Sc_2 was selected as the main isolate for the further trials. Strong pathogenic variations between *S. cepivorum* isolates were also confirmed by Marei (1988), Moreno and Acevedo (2002) and Sanchez-Pale et al. (2002).

Biological antagonists

Several microorganisms were isolated from the rhizosphere of healthy onion plants growing near infected plants. As shown in Fig. 1, 12 bacterial isolates were found to have significant antifungal activities against *S. cepivorum* (Sc_2). The relative power of antibiosis (RPA) of these isolates was tabulated (Table 2). Isolates B_5 and B_4 (0.8×10^8 CFU mL^{-1}) proved to have the highest antagonistic effect against *S. cepivorum* (Sc_2), with the highest RPA values (2.80 and 2.75, respectively) recorded. Based on their cultural, morphological and some biochemical properties, B_4 and B_5 were both identified as belonging to *B. subtilis* (Table 3).

Regarding the basis of biological control, the principal antagonistic mechanism of both bacterial isolates against *S. cepivorum* suggests production of effective antibiotics causing damage to the fungal cells (Kowall et al. 1998). Surfactin, fengycin, mycosubtilin and baciflomycin are among the antibiotics produced by *B. subtilis*. These compounds are amphilic, membrane-active surfactants and peptide antibiotics with specific antimicrobial potential. Peptide antibiotics represent the predominant class and exhibit highly rigid, hydrophobic and/or cyclic structures with unusual constituents like D-amino acids and are generally resistant to hydrolysis by peptidases and proteases (Stein 2005). Additionally, the antagonistic efficacy of two *B. subtilis* isolates against five soybean root rot pathogens of *Rhizoctonia solani*, *Colletotricum truncatum*, *Sclerotinia sclerotiorum*, *Macrophomina phaseolina* and *Phomopsis* sp. was also reported by Araujo et al. (2005).

For the fungi, eight isolates were found to belong to the genus *Trichoderma* and to have antifungal activities against *S. cepivorum* (Sc_2). Antagonistic data were represented by the scale of 1–5 classes adopted by Bell et al. (1982) in Table 4. Both *Trichoderma* isolates T_1 and T_2 were ranked in class 1, in which growth of the pathogen was totally suppressed, while T_3 and T_7 showed moderate effects (class 2). The lowest antagonistic actions were seen with isolates T_4, T_5, T_6 and T_8, which ranked in class 3. Based on their properties (colonies, conidiophores, shape of phialids, angle of primary and secondary branches, conidia, etc.), the isolates were identified as *T. koningii* (T_1) and *T. harzianum* (T_2), respectively. 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. cepivorum* via hyphal contact, deformation and lysis. Mycoparasitism is the main mechanism noted for different *Trichoderma* species against *S. cepivorum* (Margni et al. 2002). El-Kazzaz et al. (2002) found that *Trichoderma* can secrete antibiotics and toxins such as trichothecin and a sesquiterpine, trichodermin, which have a direct effect on other organisms. Against *S. cepivorum*, hyphae of *Trichoderma* spp. either grow along the host hyphae or coil around it and secrete different lytic enzymes such as chitinase, glucanase and pectinase. These enzymes dissolve the cell wall of the pathogen's mycelium firstly in certain locations followed by hyphal penetration, which has evolved as mycoparasitism (Clarkson et al. 2001; McLean et al. 2001).

Table 1 Pathogenicity degree (%) of eight *Sclerotium cepivorum* isolates against onion transplants (Giza 20)

Isolate	Pathogenicity degree (%)*
Sc_1	75.00 b
Sc_2	100.00 c
Sc_3	96.88 c
Sc_4	95.33 c
Sc_5	96.88 c
Sc_6	92.20 c
Sc_7	93.75 c
Sc_8	96.88 c
Non-infested (Control)	0.00 a

*Numbers in the same column means followed by the same letter are not significantly different according to DMRT at 0.05 levels

Chemical fungicide

Due to the use of sequential doses of Topsin-M, radial growth of *S. cepivorum* was measured in vitro. Based on the data obtained, inhibition percentages were calculated. Table 5 illustrates that the tested fungicide has a high antifungal value (100 %) from 2.0 g L^{-1} of Topsin-M upwards, by which mycelium growth was completely prevented. It is worth noting that the maximum inhibitory effect of the fungicide was stayed constant even after increasing its concentration. The antagonistic effect of Topsin-M might be due to disorder and striking changes in the cell wall of

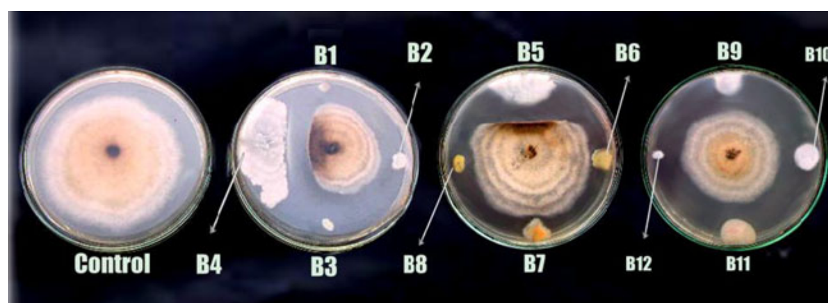

Fig. 1 Degrees of antagonistic effect of 12 bacterial isolates against *Sclerotium cepivorum* (Sc_2) on potato dextrose agar (PDA) medium showing highest antagonistic action with B_4 and B_5

hyphae, phialides and conidiophores (Abd El-Ghany and Tayel 2009). These alterations in the wall were not detected in untreated hyphae. Disorganisation of the cytoplasm was also recorded. Additionally, vacuoles disappeared completely under the influence of the fungicides (Amer and El-Shennawy 2003). Based on the regulation of enzyme activity as explained by Schlegel (1992), the cells may harbour a sensitive system to adjust activity of these enzymes, in addition to regulation of its levels.

Germination test of sclerotia

Table 6 shows that germination of sclerotia was totally prevented upon soaking in Topsin-M. On the other hand, *B. subtilis* B_5 and *T. harzianum* were equal in their effect, with germination rates of 46.67 % each. *B. subtilis* B_4 and *T. koningii* both led to the highest germination percentage, reached 56.67 % each, indicating a weak effect on sclerotia compared with 93.33 % for the control. Damage effects preventing germination of sclerotia completely might be due to strong suppression of their enzymatic regulation by Topsin-M. This is in agreement with Nesci et al. (2003), who stated that fungicides inhibit the functions of several enzymes via oxidised compounds and/or by more nonspecific interactions with proteins. For biological antagonists, different lytic enzymes are probably secreted, by which the sclerotial walls become damaged and lose their ability to self-control. This view was supported by Clarkson et al. (2001) and McLean et al. (2001), who suggested that chitinase, glucanase and pectinase dissolve pathogen cell walls in certain locations, thus causing substantial damage. Additionally, secretion of some antibiotics into the culture filtrates might also affect sclerotial germination and growth of soil-borne pathogenic fungi (Howell and Stipanovic 1995 and El-Kazzaz et al. 2002).

In vivo trials

In pots and in natural infested field trials, biological antagonists and chemical fungicide were also tested using the seedling dip treatment method. Disease index parameters (incidence and severity) were estimated in both cultivation types. Under open field conditions, PPO and PO enzyme activities, growth and yield parameters were therefore determined.

Disease index parameters

The action of the tested control agents of onion white rot disease was determined in both pots and open field treatments

Table 2 Relative power of antibiosis (RPA) values of 12 bacterial antagonistic isolates against *S. cepivorum* (Sc_2) on potato dextrose agar (PDA) medium

Code no.	RPA*
B_1	1.57 bc
B_2	1.60 bc
B_3	1.17 b
B_4	2.75 d
B_5	2.80 d
B_6	2.00 c
B_7	2.00 c
B_8	0.60 a
B_9	0.43 a
B_{10}	0.80 ab
B_{11}	0.50 a
B_{12}	2.33 cd

*Numbers in the same column means followed by the same letter are not significantly different according to DMRT at 0.05 levels

Table 3 Morphological, cultural and some biochemical characteristics of the antagonistic bacterial isolates coded as B_4 and B_5 identified as *Bacillus subtilis*

Characteristic	B_4 and B_5
Shape of cell	Rods
Formation of endospore	+
Motility	+
Gram reaction	+
Anaerobic growth	−
Growth on 7 % NaCl	+
Starch hydrolysis	+
Casein hydrolysis	+
Gelatin hydrolysis	+
Nitrate reduction	+
Indole production	−
Catalase reaction	+
Glucose fermentation	−
Lactose fermentation	−

Table 4 Antagonistic degree of eight *Trichoderma* isolates against *S. cepivorum* (Sc_2) on PDA medium

Trichoderma isolate	Degree of antagonism[a]
T_1	1
T_2	1
T_3	2
T_4	3
T_5	3
T_6	3
T_7	2
T_8	3

[a]The 1–5 scale classes described by Bell et al. (1982)

Table 6 Germination percentages of *S. cepivorum* (Sc_2) sclerotia soaked overnight (12 h) in filtrates of the most antagonistic doses of the tested control agents

Treatment	Rank	Germination of sclerotia (%)*
Control	4	93.33 c
B. subtilis B_4	3	56.67 b
B. subtilis B_5	2	46.67 b
T. koningii	3	56.67 b
T. harzianum	2	46.67 b
Topsin-M	1	00.00 a

*Numbers in the same column means followed by the same letter are not significantly different according to DMRT at 0.05 levels

(Table 7). In pots, Topsin-M was the superior control agent, with the lowest percentage of disease incidence (8.33 %) compared with 95.83 % for the control. *T. koningii* was the most effective biological antagonist, reducing the incidence of onion white rot disease to 29.17 %. Disease severity was also reduced to 8.33 % and 21.88 % by Topsin-M and *T. koningii*, respectively. The other biological isolates showed lower magnitudes. Under open field conditions, disease incidence was reduced strongly to 2.78 % by Topsin-M, followed by 11.11 % and 13.39 % for *T. harzianum* and *T. koningii*, respectively. Accordingly, disease severity declined sharply to 1.39 % and 9.72 % via superiority of Topsin-M and *T. harzianum*, respectively, in comparison with 11.80 % for *T. koningii* and *B. subtilis* B_5 each.

The efficiency of the bio-control agents in comparison with Topsin-M against *S. cepivorum* was tested both in pots and in natural infested open field experiments. As expected, the results indicated that Topsin-M was the superior agent for suppressing incidence and severity of onion white rot disease under pot and field conditions. Hendy et al. (1994) tested the efficiency of certain fungicides against a wide range of fungal disease attacking different crops. They found that soil drench with Topsin-M was very effective for reducing damping-off, root-rot and wilt diseases on some vegetables. Topsin-M was found to be more aggressive against a wide range of soybean pathogenic fungi (Amer and EI-Shennawy 2003). On the other hand, results also indicated that the biological antagonists of *T. koningii* and *T. harzianum* proved effective and environmentally safe control agents as an alternative to chemical fungicides in pots and open fields, respectively. The antagonistic action of *Trichoderma* strains against *Sclerotinia sclerotiorum* and *S. cepivorum* was emphasised more recently by Castillo et al. (2011). They concluded that 41 *Trichoderma* isolates showed excellent levels of antagonism toward *S. cepivorum* and *Sclerotinia sclerotiorum* either by competition for nutrients, antibiosis by volatile compounds or effect of filtering toxins. *Trichoderma* species colonise and penetrate plant root tissues and initiate a series of morphological and biochemical changes in the plant, considered to be part of the plant defence response, which in the end leads to induced systemic resistance (ISR) in the entire plant.

Table 5 Inhibitory effect of Topsin-M on the linear growth of *S. cepivorum* (Sc_2)

Fungicide	Concentration (g L^{-1})	Net diameter of linear growth (cm)	Inhibition (%)
Control	0.00	8.50	0.00
Topsin-M	0.50	0.80	90.59
	1.00	0.50	94.12
	2.00	0.00	100.00
	3.00	0.00	100.00
	5.00	0.00	100.00

Enzyme activities

As defensive responses to phytopathogens, the enzyme activities of both PPO and PO were also determined in onion

Table 7 Effect of the tested control agents on disease index parameters of onion white rot in pots and natural infested field trials

Treatment	Disease index parameters %*			
	Pots trials		Field trials	
	Disease incidence	Disease severity	Disease incidence	Disease severity
Control (untreated)	95.83 e	90.63 e	80.55 d	73.15 e
B. subtilis B_4	41.67 d	28.13 d	16.67 abc	15.97 d
B. subtilis B_5	33.33 cd	25.00 cd	25.00 c	11.80 cd
T. koningii	29.17 bcd	21.88 cd	13.89 abc	11.80 cd
T. harzianum	37.50 cd	29.17 d	11.11 abc	9.72 bcd
Topsin-M	8.33 a	8.33 ab	2.78 a	1.39 a

*Numbers in the same column means followed by the same letter are not significantly different according to DMRT at 0.05 levels

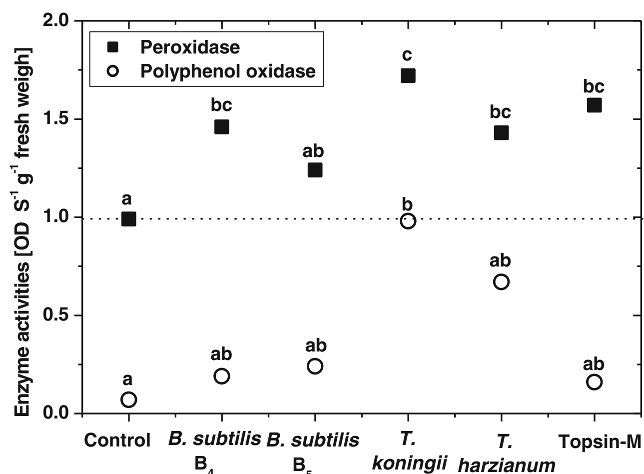

Fig. 2 Enzyme activities of peroxidase (PO) and polyphenol oxidase (PPO) in onion plants under natural infested field conditions. The same Duncan's letters indicate no significant difference according to DMRT at 0.05 levels. For PO, SE=0.183 and F-value=3.975. For PPO, SE=0.352 and F-value=2.079

plants under natural infested field conditions. Both enzyme activities were assayed over 2 min (every 30 s). For both enzymes, activities seemed constant during this time course. Therefore, average data were plotted in Fig. 2. For both enzymes, all treatments were pronounced in comparison with the control, but it was noted that activation levels of PPO were lower in comparison with those of PO. *T. koningii* followed by *T. harzianum* and Topsin-M induced high activation of both PO and PPO. On the other hand, low activity of PPO was induced by Topsin-M, which did not fit with its superiority in reducing white rot disease. The data indicated that phenols were oxidized by *T. koningii* and *T. harzianum* more than by Topsin-M in controlling *S. cepivorum*, as well indicated by induction of SAR in the onion plants. SAR is a phenomenon originally suggested by Hatcher (1995) to describe the systemic induction of resistance against a broad spectrum of phytopathogens. It was supported by Scalbert (1991), who found that the more highly oxidized phenols are the greater the inhibitory effect on the pathogen. Although Topsin-M strongly suppressed disease, the lowest activation of PPO was seen, indicating no SAR coinciding with the antifungal effects. Similarly, high induction of PPO observed in tea plants treated with *Pseudomonas fluorescens* led to accumulation of higher phenolic compounds, which may play an important role in defence mechanisms in plants against pathogens (Sivakumar and Sharma 2003). These consequences are also in agreement with the findings of Saravanakumar et al. (2007), who found a remarkable increase in PPO and PO activities in tea plants treated with *P. fluorescens* in comparison with untreated plants.

Plant growth and yield parameters

Under open field conditions, the tested control agents were also evaluated by investigating their effects on some plant growth and yield parameters (Table 8). Chlorophyll, lengths and bulb dry matter data showed superiority of all treatments, in particular Topsin-M, compared with controls. This might be due to induction of the formation of some substances in the plants, by which onion plants become strong under pathogenic conditions. This notion was proposed by Amaresh and Bhatt (1998), who stated that a large portion of the nutrients and essential components required for photosynthesis were formed in the host plants to prevent the harmful effects of the pathogen.

Differences between treatments recorded for the dry matter of foliage and bulbs were also of low significance. In contrast, Topsin-M caused a lower reduction in shoot dry matter (6.03 %) even than the 7.12 % of the control. Here, rapid oxidation of chlorophyll due to Topsin-M occurred, so dry matter formed in the leaves was less than that in bulbs. It is worth noting that *B. subtilis* B_4, *B. subtilis* B_5, *T. koningii* and *T. harzianum* proved to be plant growth promoting agents in previous studies (Sharma 2011; Wahyudi et al. 2011). This was in agreement with Yobo et al. (2011), who stated that both *Bacillus* and *Trichoderma* species are well known for both their biological control and plant growth promoting properties. Treatment with these agents also

Table 8 Effect of the tested control agents on specific aspects of plant growth and yield parameters of onion plants (Giza 20) grown in a field naturally infested with *S. cepivorum* (Sc_2)*

Treatment	Chlorophyll (μg $(cm^2)^{-1}$)		Length (cm)		Dry matter (%)		Bulb yield (kg $plot^{-1}$)
	Chl. a	Chl. b	Root	Foliage	Foliage	Bulb	
Control (untreated)	33.91 a	7.68 a	8.23 a	38.43 a	7.12 ab	9.09 a	1.45 a
B. subtilis B_4	41.36 b	14.25 ab	12.07 cd	49.10 bcd	8.57 ab	9.93 ab	2.60 cde
B. subtilis B_5	46.32 bc	16.51 ab	11.57bcd	49.13 bcd	10.59 b	12.2 ab	2.39 bcd
T. koningii	44.96 bc	21.58 bc	12.83 c	40.33 ab	7.85 ab	9.94 ab	2.61 cde
T. harzianum	54.41 bc	29.95 cd	11.30bcd	50.77 cd	8.15 ab	11.43 ab	2.26 bc
Topsin-M	62.83 e	42.33 e	12.90 c	55.83 d	6.03 a	13.49 b	2.50 bcde

*Numbers in the same column means followed by the same letter are not significantly different according to DMRT at 0.05 levels

resulted in elongation of root system and foliage as well as dry matter of both leaves and bulbs. This was in agreement with numerous reports implicating these antagonistic bacterial isolates as plant growth promoting rhizobacteria (PGPR) (EL-Tahlawy 2006). Metabolities of two *B. subtilis* strains found to produce phytohormones belonging to indole acetic acids (IAA) caused stimulation of root hairs, lateral roots and promotion of different soybean growth parameters (Araujo et al. 2005). In addition, the capacity of *Trichoderma* spp. to promote growth was also observed. This capability probably 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 (*Arabidopsis thaliana*) seedlings in response to inoculation with *T. virens* and *T. atroviride*. This was also supported by Harman et al. (2004) who found that *Trichoderma* spp. colonise root surfaces and penetrate the epidermis before producing or releasing a variety of compounds that induce localised or systemic resistance responses. Therefore, plants become protected from the pathogenic fungus, indicating induction of SAR in plants treated with the biological isolates, but not those treated with the chemical fungicide.

Both *T. koningii* and *B. subtilis* B_4 played a role in enhancing bulb productivity, reaching 2.61 and 2.60 kg plot^{-1} (≈9.74 and ≈9.70 t feddan^{-1}), respectively, due to their plant growth promoting properties as previously reported by Yobo et al. (2011). These data seem are similar to those seen after treatment with Topsin-M (2.50 kg plot^{-1}). Accordingly, use of either *T. koningii* (fungal isolate) or *B. subtilis* B_4 (bacterial isolate) as alternative agents to chemical fungicides proved promising, not only to suppress *S. cepivorum* but also to promote plant defences, growth and onion productivity.

Conclusion

Chemical fungicides are still the major means of controlling plant diseases under field conditions. Despite being less efficient in controlling white rot disease, *B. subtilis* B_4, *B. subtilis* B_5, *T. koningii* and *T. harzianum* proved to be promising agents for enhancing growth and productivity of onion plants, but still require optimising of conditions to induce formation of their biomass and active ingredients.

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