Unconventional alternatives for control of tomato root rot caused by *Rhizoctonia solani* under greenhouse conditions

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Abstract: This study was done to assess the antifungal effect of some biocontrol agents effective microorganisms (EMs1), *Pseudomonas fluorescens*, and *Bacillus pumilus*, titanium dioxide (TiO_2) nanoparticles, black cumin and wheat germ oils as well as the recommended fungicide (flutolanil) against root rot of tomato. Moreover, gas chromatography-mass spectrometry (GC-MS) examination was completed to identify the bioactive compounds in plant oils (dark cumin and wheat germ). Also the impact of these medicines on some biochemical and growth parameters of tomato was examined. Flutolanil was the best treatment followed by dark cumin, TiO_2, EMs1, *Pseudomonas fluorescens*, *Bacillus pumilus* and wheat germ oil, individually in both test seasons. The outcomes demonstrated a marked increase in each biochemical character (chlorophyll substance, peroxidase and polyphenoloxidase) and plant development (height and fresh and dry weight) under all the tried treatments in comparison to the controls.

Key words: bioagents, nanoparticles, plant oils, *Rhizoctonia solani*, rot root, tomato

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is a very important vegetable crop because of its financial significance as well as for its nutritive qualities (Giovannucci 1999). It is basically present worldwide either as an open field or protected crop. As in other countries, it stands out as one of the most essential vegetables in Egypt and is utilized for food and commercial reasons (El-Mougy 1995). Tomato plants are infected by a few soil-borne contagious pathogens, for example, *Fusarium* spp., *Rhizoctonia solani*, and *Sclerotium rolfsii* which cause marked diseases such as root rot and -wilt that reduce harvest yield and quality (Saad 2006; Abdel-Monaim 2010).

Conventional fungicides are presently being utilized to control plant infection. But unconventional methods for the control of plant pathogens are in demand due to negative public observations about the utilization of chemicals, the resistance of fungicides among contagious pathogens, and the expense of obtaining new chemicals.

For these reasons, new fungicides and other alternatives against resistant strains of fungal pathogens (Kanhed *et al*. 2014) must be found. The use of plant-origin products as disease control agents has been studied because they are safer, less harmful to the environment and have wider public acceptance (Lee *et al.* 2007). In general, botanical products are potentially active against several plant pathogens with no phytotoxic effects on treated plants (Pandey *et al.* 1982; Chuang *et al.* 2007). Consequently, they can be utilized to suppress fungal pathogens and reduce disease severity. There is a growing interest in developing safer antifungal agents from plants, for example, essential oils and extracts to control fungal pathogens (Prasad *et al*. 2004; Chuang *et al*. 2007).

It has been proposed that antagonistic microorganisms can potentially be used to control plant pathogens with no harm to the host plant. Antagonistic microorganisms are considered to be potentially cost-effective by decreasing the population of plant pathogens in soil (Wright *et al*. 2003). Biological control of plant pathogens with antagonistic microorganisms has been the subject of various surveys and a few books. Promising antagonists that might be valuable for controlling soil-borne plant pathogens were additionally reported (Selvarajan and Jeyarajan 1996).

Likewise, one of the solutions of fungi resistance would be nanotechnology which upgrades the antimicrobial effect of materials by changing them to nano size. The enhanced antimicrobial action of nanoparticles contrasted with their salts because of their unique properties i.e. huge surface range to volume proportion (Kanhed *et al*. 2014).

This study was carried out to assess the efficacy of certain biocontrol agents (*Pseudomonas fluorescens, Bacillus pumilus* and effective microorganisms), titanium dioxide (TiO_2) nanoparticles, black cumin and wheat germ oils as well as the recommended fungicide (flutolanil) against *R. solani*, the causative fungus of root rot of tomato under greenhouse conditions in two developing seasons, to identify the bioactive compounds in plant oils (black cumin and wheat germ oils) by gas chromatography-mass spectrometry (GC-MS) examination and finally to investi-

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gate the impact of these treatments on some biochemicals (chlorophyll content, peroxidase and polyphenoloxidase) and developmental (plant height as well as fresh and dry weight) characteristics of tomato plants.

Materials and Methods

Treatments used

Effective microorganisms (EMs) formulation was obtained from the Egyptian Ministry of Agriculture and Land Reclamation, Giza, Egypt. This formulation contains 60 types of useful microorganisms developed in unique medium and delivered to Egypt under supervision of the Japanese Effective Microorganisms Research Organization (EMRO) Scientific Organization. *Pseudomonas fluorescens* and *B. pumilus*, as bio-agents were obtained from the Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt. The tried fungicide that was utilized as a part of this study was Moncut (flutolanil) 25% WP. This fungicide was applied at its prescribed field rate. Titanium dioxide nanoparticles were obtained from Egypt Nanotech Company Limited, Dreamland, El-Wahaat Road, sixth October, Giza, Egypt Black cumin and wheat germ oils were purchased from Elcaptain Company for Extracting Natural Oils, Plants and Cosmetics, Al Azhar, Cairo, Egypt.

Preparation of bio-formulations

The talc-based formulation of biocontrol agents (*P. fluorescens* and *B. pumilus*) was set up with a modified method by Commare *et al*. (2002). The formulations were inoculated into the King's B medium and incubated in a shaker at 150 rpm for 48 h at room temperature (28±2°C). One kilogram of talc powder was put on a sterilized metal plate and the pH was changed by adding $CaCO_3$ at the rate of 15 g · kg^{-1}. Ten grams of carboxymethyl cellulose (CMC) were added to every 1 kg of talc and blended well. The blend was autoclaved for 30 min. The 400 ml of 48 h developed suspension containing 9×10^8 cfu · ml^{-1} were blended with transporter cellulose blend under aseptic conditions. After drying (to about 35% moisture content) for overnight, it was put into polypropylene bags, closed and stored at room temperature (28±2°C).

Isolation, identification and culturing of *Rhizoctonia solani*

Samples of tomato plants showing root rot were collected from various fields. The samples with their rhizosphere soil were placed in polyethylene bags and taken to the laboratory for further treating. To remove all dirt particles in the lower parts of stems and roots faucet water was utilized for washing it, and then cut into little pieces (approx. 2 cm). The pieces were surface sanitized in 3% sodium hypochlorite for 3 min and washed a few times utilizing reestablished cleaned refined water. They were then dried between two disinfected channel papers. The disinfected pieces were then put onto Potato Dextrose Agar (PDA) medium, and incubated at 27–30°C for 3–5 days. The hyphal tip method was utilized to purify the developing fungal colonies on the PDA medium (Dhingra and Sinclair 1995). Identification of the fungus was done according to the morphological and microscopical characters and compared with the descriptions given by Alexopoulos *et al.* (1996) and Ainsworth *et al*. (1971). Identification was confirmed at the Mycology Research and Survey of Plant Diseases Section, Plant Pathology Research Institute, Agriculture Research Center, Giza, Egypt. Cultures of identified *R. solani* were grown independently on hull rice medium. Five hundred ml glass bottles each contained 100 g hull rice, 200 g sand and 100 ml water. They were autoclaved for 20 min at 1.5 atm. From this point on, the flasks of medium were inoculated independently by adding three mycelia disks (6 mm in breadth) taken from a developing colony of the fungal isolate on PDA. The glass-bottles with hull rice culture were incubated at 20–28±2°C for 7 days.

Application of used treatments under greenhouse conditions

Sandy-clay soil was sterilized using 5% formalin solution and then put into 25 cm width pots, with 4 kg soil per pot. Artificial inoculation was done in pots one week before transplanting at the rate of 3% utilizing the hull rice culture (w/w) and kept wet until transplanting. Roots of tomato seedlings (cv. Super Strain B), 25 days old, were treated by immersion for 2 h in a solution of various treatments followed by foliar spray after transplanting (EL--Mohamedy *et al*. 2014). Roots of tomato seedlings were treated with *P. fluorescens* and *B. pumilus*, by immersing them in a spore suspension of 1×10^8 cfu · ml^{-1} while tomato was immersed in EMs formulation at a level of 5 ml · l^{-1} water (Derbalah *et al*. 2013). Titanium dioxide nanoparticles were utilized at a level of 0.5 g · l^{-1} water (Derbalah *et al*. 2013). Black cumin and wheat germ oils were used at a level of 5% with the addition of 0.01% of Tween 80 emulsifier (Hamza *et al*. 2015) to improve oil solubility. After that, the tomato seedlings were transplanted into soil inoculated with *R. solani* in the pots with five plants to every pot. Each treatment was represented by five replicates and each replicate consisted of five plants. For control treatment roots of tomato seedlings were dipped in pure water for 2 h. Pots were kept in a greenhouse and watered when required. Some crop parameters (plant height and fruit yield) of tomato plants were measured in the second season. Disease incidence of post-rise damping-off and plant survival were recorded 45 days after sowing as shown in the following equations (Khalifa 1987).

$$\% \text{ Post-emergence damping-off} = \frac{\text{No. of dead seedlings}}{\text{No. of sown seeds}} \times 100,$$

$$\% \text{ Efficacy} = \frac{A - B}{A} \times 100,$$

where:
A = number of dead seedlings in control; B = number of dead seedlings in treatment.

Biochemical changes

Determination of chlorophyll

Five leaf disks were collected from cucumber leaves (five leaves from every plant and three plants for each replicate of every treatment). Chlorophyll was extracted from cucumber leaf disks by utilizing 5 ml N,N-dimethyl formamid for 48 h at room temperature (Moran and Porath 1980). Samples were measured at wavelengths of 645 and 663 nm for chlorophyll *a* and *b*, separately and chlorophyll content was estimated using the following equations as reported by Arnon (1949):

$$\text{Chlorophyll } a = 12.64(A663) - 2.49(A645)$$

$$\text{Chlorophyll } b = 5.6(A663) + 23.26(A645)$$

Sample preparation and enzyme extraction

The leaf specimens were ground with 0.1 M sodium phosphate buffer at pH 7.1 (2 ml buffer · g^{-1} tissue) in a mortar. These crushed tissues were strained through four layers of cheese cloth and the filtrates were centrifuged at 3,000 rpm for 20 min at 6°C. The supernatant liquid was utilized for assay of polyphenoloxidase and peroxidase using UV-vis Spectrophotometer, Shimadzu 3700 Model, Shimadzu Limited Company, Japan.

Polyphenoloxidase measure

Polyphenoloxidase (PPO) activity was estimated according to Galeazzi *et al*. (1981). In this manner, 100 µl of extract were incubated with 2 ml of 0.05 M phosphate support (pH 7.0) and 0.5 ml of 0.5 M catechol at 24°C for 2 min. The absorbance of the resulting mix was measured at 398 nm using spectrophotometer. The PPO was estimated using the following equation:

$$U398 = 0.01\Delta OD398,$$

where: U398 = the unit of enzyme at 398 wave length; ΔOD = the change of optical density per mg of protein per min.

Peroxidase measure

Peroxidase (POX) activity was assessed using guaiacol as the substrate. The reaction mix was comprised of 0.1 ml of plant concentrate and 2 ml of guaiacol (8 mM, in 100 mM sodium phosphate support, pH 6.4) and incubated for 30 min at 30°C. The absorbance at 460 nm was measured after the addition of 1 ml H_2O_2 (24 mM). The activity of POX was calculated using the following equation:

$$U460 = 0.01\Delta OD460,$$

where: U460 = the unit of chemical at 460 wave length; ΔOD = the change of optical density per mg of protein per min (Ippolito *et al*. 2000).

Black cumin and wheat germ oil analysis

For wheat germ oil analysis, Agilent 6890N GC was interfaced with a VG Analytical 70–250s double-focusing mass spectrometer with a carrier gas of Helium. The instrument conditions were: ionization voltage 70 eV, ion source 250°C. The gas chromatography was fitted with a 30 m × 0.32 mm fused capillary silica column coated with DB-5 (Yusuf and Bewaji 2011). Black cumin oil was analyzed by GC-MS as described by Adams (1995). Oven temperatures rose from 40 to 240°C at a rate of 4°C · · min^{-1}, transfer line temperature 260°C, injector temperature 250°C, carrier gas helium with a linear velocity of 31.5 cm · s^{-1}, split ratio 1/60, flow rate 1.1 ml · min^{-1}, ionization energy 70 eV; mass range 40–350 amu.

The identification of selected oil compounds was carried out by a comparison of their mass-spectra and retention times with those of a computer library or with original compounds.

Statistical examination

The analysis of variance test (ANOVA) using SPSS and the Duncan's multiple ranges test (Duncan 1955) were used to analyze the obtained data.

Results

Potential effect of examined treatments against tomato root rot

The outcomes demonstrated that all treatments suppressed the severity of tomato root rot compare to untreated control. The information displayed in Table 1 demonstrates that flutolanil was the best treatment against root rot followed by wheat germ oil, TiO_2 nanoparticles, *B. pumilus*, *P. fluorescens*, EMs1, and black cumin oil, separately in both growing seasons.

Chemical constitutes of the plant oils

Compounds identified from black cumin and wheat germ oils are outlined in Tables 2 and 3. Thirteen compounds were found in black cumin oil (Table 2). On the other hand, twenty compounds were found in wheat germ oil (Table 3). The compounds were identified as aldehydes, esters, alcohols and unsaturated fatty acids.

Impact of the experimental treatments on some biochemical characters in tomato

There was a marked raise in each biochemical parameter (chlorophyll content, POX and PPO) in treated tomato plants in comparison to control. The information delineates in Figures 1 and 2 show chlorophyll content, POX and PPO in tomato plants treated with flutolanil, wheat germ oil, black cumin oil, EMs1, *B. pumilus*, TiO_2, *P. fluorescens*, respectively.

Table 1. Effect of the various treatments on the severity of root rot of tomato in two seasons

Treatments	% Post-emergence damping-off	
	first season 2012–2013	second season 2013–2014
Pseudomonas fluorescens	37.47±2.63 bc	32.81±0.54 cd
Bacillus pumilus	34.12±2.10 c	30.41±2.02 d
Titanium dioxide (TiO_2)	28.58±1.19 d	25.31±2.83 e
Effective microorganisms	39.15±3.10 c	35.22±0.94 bc
Black cumin oil	43.33±1.08 b	38.80±1.36 b
Wheat germ oil	19.51±2.07 e	15.15±1.17 f
Flutolanil	7.9±0.45 f	5.67±0.56 g
Control	91.07±1.4 a	88.31±4.58 a

The small letters indicate the significance and non-significance between means using Duncan multiple range test

Table 2. Components of black cumin oil analysed by gas chromatography-mass spectrometry (GC-MS)

No.	Components	% Area
1	Myristic acid	13.17
2	Myristoleic acid	1.83
3	Palmitic acid	20.59
4	*n*-Hexadecanoic acid	2.48
5	Oleic acid	13.20
6	Linoleic acid	1.92
7	Thymoquinone	2.17
8	Dihydrothymoquinone	2.92
9	*ρ*-Cymene	3.50
10	Carvacrol	14.46
11	*α*-Thujene	2.67
12	Thymol	4.12
13	*α*-Pinene	16.94

Table 3. Components of wheat germ oil analysed by gas chromatography-mass spectrometry (GC-MS)

No.	Components	% Area
1	Eugenol	10.41
3	Caryophyllene	13.83
4	1-Eicosene	7.53
5	Manool	0.40
6	Octadec-9-enoic acid	10.71
7	Tetracosan-1-ol	10.57
8	Trans-m-propenyl guaiacol	7.40
9	Hexadecyl acetate	0.57
10	Pentacosane	3.25
11	Nonacosane	1.61
12	Humulene-1,2-epoxide	1.62
13	(-)-Spathulenol	1.86
14	Cedrenol	1.41
15	Geranyl-*α*-terpinene	1.64
16	*α*-Bisabolol	1.66
18	Hexadecanoic acid, ethyl ester	10.68
19	9-cis-Retinal	1.96
20	cis-13-Eicosenoic acid	2.67

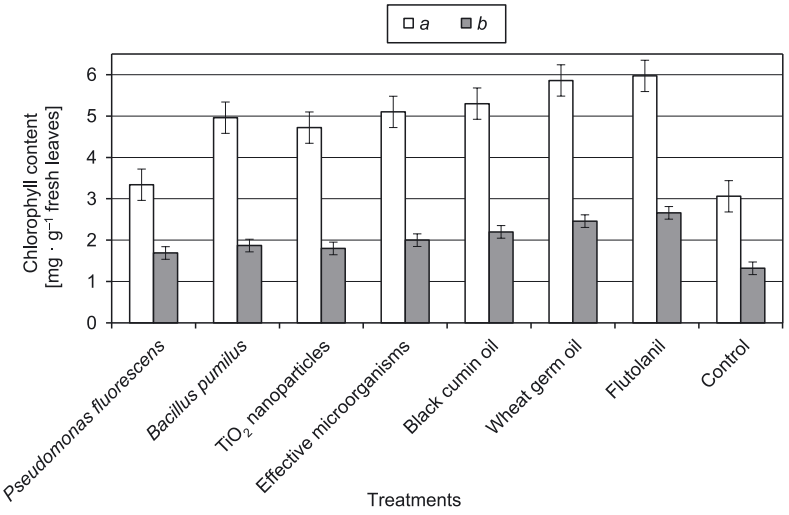

Fig. 1. Effect of the examined treatments on chlorophyll content (*a* and *b*) in tomato plants

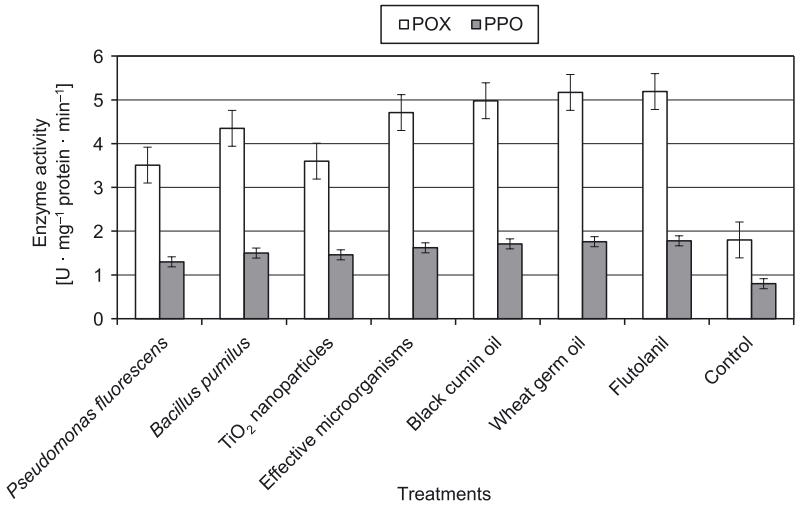

Fig. 2. Effect of the examined treatments on peroxidase (POX) and polyphenoloxidase (PPO) activity in treated tomato plants

Table 4. Effect of the examined treatments on some growth parameters (plant height as well as fresh and dry weight) of tomato plants in season 2013–2014

Treatments	Plant height [cm]	Fresh weight/plant [g]	Dry weight/plant [g]
Pseudomonas fluorescens	76.33±1.52 cd	55.33±1.5 b	16.03±0.60 c
Bacillus pumilus	68.00±2.0 e	43.00±30 d	14.57±0.30 d
TiO_2 nanoparticles	73.00±1.0 d	49.67±1.52 c	15.17±0.15 cd
Effective microorganisms	87.33±2.51 b	58.00±1.0 b	17.93±1.35 b
Black cumin oil	93.33±1.52 a	65.33±3.51 a	19.53±1.35 a
Wheat germ oil	79.33±1.52 c	56.33±1.52 b	17.73±0.3 b
Flutolanil	78.33±2.88 c	57.33±1.52 b	16.07±0.55 c
Control	51.67±2.88 f	32.33±2.51 e	9.93±0.56 e

The small letters indicate the significance and non-significance between means using Duncan multiple range test

Impact of the experimental treatments on some growth parameters of tomato

All the growth parameters (plant height and fresh and dry weight) were increased in treated tomato plants in comparison to the controls. The information in Table 4 demonstrates that the highest growth was recorded in tomato plants treated with flutolanil followed by dark cumin oil, TiO_2, Ems1, *B. pumilus*, *P. fluorescens* and wheat germ oil, respectively.

Discussion

Wheat germ and black cumin oils markedly reduced the severity of tomato root rot in tomato plants. The outcomes are in agreement with past studies on an extensive variety of plant pathogens including bacteria and fungi (Bowers and Locke 2000, 2004; Momol *et al.* 2000; Oka *et al.* 2000). Another study showed that *Eucalyptus unigera* oil suppressed mycelial development with 100% inhibition in all of the researched soil-borne fungi (*P. ultimum* and *R. solani*) after three days of treatments (Huv *et al.* 2000). Moreover, the oil concentrate of *Nigella sativa* showed *in vitro* and *in vivo* antimicrobial effects against *Candida albicans* (Khan *et al.* 2003; Mashhadian and Rakhshandeh 2005). The volatile oil of *N. sativa* was found to have incredible antifungal activity, especially against *Aspergillus* species (Amrouche *et al.* 2011). The antifungal properties of wheat germ and black cumin oils can be ascribed to the existence of some compounds, for example, carvacrol, α-terpinyl acetate, cymene, thymol, pinene and linalool which are known for their antimicrobial action (Knobloch *et al.* 1985; Juven *et al.* 1994; Harborne and Williams 1995).

Even though the inhibitory action of plant oils is not completely understood it has been reported that unsaturated fatty acids and their derivatives permeate the lipid membrane and dissociate into the more alkaline interior and cause metabolic interruption (Russel and Diaz-Gonzalez 1998). Moreover, the high inhibition created by the fatty acids could be because of their cytolytic action of being a solvent of cellulose, a constituent of the cell membrane of fungi. In addition to fatty acids, for example, oleic acid has potential antifungal properties ascribed to long chain unsaturation (Agoramoorthy *et al.* 2007). These plant oils can possibly be produced in an environmentally friendly way and considered to be an alternative to fungicides in an integrated management of plant diseases.

Titanium dioxide nanoparticles potentially reduce the severity of root rot of tomato in comparison to the control, with no antagonistic impact on the treated tomato plants. This might be due to their affect on cell membranes that lead to a spillage of intracellular contents and finally the death of sporangia or zoospores (Jin *et al*. 2009). This mechanism may be induced through the generation of oxygen species such as $OH^{\cdot}$, O_2^{-}, and H_2O_2 generated on the surface TiO_2 (Sunada *et al*. 1998). The created oxygen species can infiltrate the cell membrane and push the microbes to death (Fang *et al*. 2006). The production rate of H_2O_2 depends highly on the surface area of TiO_2, which results in more oxygen species at the surface and the higher antimicrobial action of nanoparticles (Ohira *et al*. 2008). In our study, TiO_2 at its application rate did not induce phytotoxicity and this was affirmed by the incredible rise in plant growth as shown in Table 4. This was in agreement with reports by Owolade and Ogunleti (2008).

The severity of root rot was significantly reduced in tomato plants treated with experimental bio-agents (EMs1, *B. pumilus* and *P. fluorescens*) in this study. This may be because these biocontrol agents have the ability to rapidly use seed and root exudates, colonize and increase in the rhizosphere and spermosphere and in the inside of the plant, release a wide range of bioactive metabolites (i.e. antibiotics, siderophores, volatiles, and growth-promoting substances), that attack soil-borne fungi such as *R. solani*; and adapt to environmental stresses (Pertot *et al*. 2008).

It is also possible that the potential effect of the examined bio-agents against root rot might be because of nutrient competition, hyperparasitism, and/or antibiosis (Falk *et al.* 1995). Mycoparasitism is viewed as one system of plant pathogens control (Harman 2000), whereby a species or strain of microorganism straightforwardly assaults and feeds on other parasites (Kendrick 1992). The generation of anti-microbes or chemicals that can suppress the development or reduce the competitive strength of other organisms is another control action (Dunlop *et al.* 1989; Howell *et al.* 1993; Harman 2000). Control may also be achieved through competition for space and resources with highly competitive bio-control agents (BCAs) quickly colonizing plant surfaces, creating an effective 'living barrier' to subsequent pathogen invasion (Cook 1988). Another mechanism is the mobilization of nutrients in the soil, a process that makes compounds in the soil more available for plant uptake, resulting in increased general health and disease resistance (Harman 2000). Bio-control agents may also induce changes in the plant that increase disease resistance similar to the phenomena of induced and systemic acquired resistance (Handelsman and Stabb 1996; Harman 2000).

The increase in chlorophyll content in treated tomato plants compared to the control might be because the examined treatments can minimize the leaf zone influenced by *R. solani* and serve to postpone the loss of green leaf region due to the pathogen. This study reports the marked rise of defense enzymes as a result of the induction of resistance in plants (Stangarlin *et al.* 2011) in response to the treatment with elicitor agents, protecting against subsequent infection by pathogens.

Thus the control of root rot of tomato by the tried medications might be because of the induction of tomato resistance against rot root by the activation of defense enzymes (POX and PPO) which through oxidation produced quinines that are known as antimicrobial agents and lethal to the pathogen. This is in addition to that the lignin which together with cellulose and different polysaccharides found in the cell wall of the plants, work as a physical barrier to the pathogen infection.

The increase in tomato growth under various treatments compare to control might be because of the reduction in disease severity as a result of the utilization of tried treatments as foliar sprays which affect plant growth and also its yield (El-Mougy *et al*. 2013).

Conclusions

The tested treatments are potentially effective for controlling root rot of tomato as an alternative to chemical fungicides. This physical antifungal action of zinc oxide nanoparticles can overcome the resistance issue of fungi to fungicides. Plant oils and bio-control agents are considered to be effective natural alternatives to fungicides for control of root rot of tomato. Further studies are needed to assess the safety of the tested treatments on the public health and it efficacy under field conditions.

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