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Inhibitory effect of antagonistic bio-agents and chitosan on the growth of tomato root rot pathogens *In vitro*

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Abstract Fungal and bacterial bio-agents and the by-product chitosan as fungicides alternatives were evaluated for their inhibitory effect against the growth of tomato root rot pathogenic fungi under laboratory conditions. The tested pathogenic fungi were *Fusarium oxysporum radicis-ycopersici*, *F. oxysporum lycopersici, F. solani, Rhizoctonia solani, Sclerotium rolfsii, Macrophomina phaseolinae*, *Pythium* sp. and *Phytophthora* sp., Meanwhile, isolates of the antagonistic microorganisms, *i.e*. *T. harzianum*, *T. viride*, *Bacillus subtilis*, and *P. fluorescens.* Mycelial growth of pathogenic fungi was significantly reduced by the inhibitor action produced by all antagonistic agents tested. The antagonistic fungi had a greater effect on the retardation of growth (75.5--100%) compared with the bacterial agents (57.7—83.3%). The inhibitor effect of the two tested chitosan (High and Low molecular weight) was increased as chitosan concentration is increased in growth medium to reach its maximum at the highest concentration (5 g/L). Complete inhibition of tested fungal growth was observed at the concentration of 4 g/L of LMW chitosan, while the highest fungal growth reduction was recorded in PDA-amended with HMW at the same concentration. On the light of the present study, it could be suggested that the use of chitosan as natural safe materialsalone or in combination with bio agents is considered one of low cost and effective applicable methods for controlling such soil-borne plant pathogens causing plant diseases.

Keywords: bio-agents, by-product, chitosan, fungal growth, pathogenic fungi, root rot, tomato

Introduction

Tomato plants (*Solanum lycopersicum* L.) considered one of the most important vegetable crops in Egypt and other countries in the world. Root rot disease caused *by Fusarium oxysporium*, *Rhizoctonia solani* Kuhu; *Fusarium solani* (Mart) Sacc. and *Sclerotium rolfsii* Sacc. are the most destructive disease of tomato (Benhamou *et al*., 1994; El-Mougy, 1995). Controlling such diseases mainly depend on fungicides treatments (Rauf, 2000). However, fungicidal

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applications cause hazards to human health and increase environmental pollution. Therefore, alternative treatments for control of plant diseases are needed. An investigation of root rot disease ant it's pathogens is considered particularly important in light of its wide prevalence in Egypt, particularly in sandy soils. Thus far, due to scientific and practical difficulties, there is no economic way to control root rot disease in many crops. The growing concern over the use of pesticides with respect to human health and environment has brought increasing interest in the use of alternatives characterized by the lake of negative effect on the environment. Additionally, resistance of pathogens to pesticides has rendered certain pesticides ineffective, creating a need for new ones with other modes of action.

The application of biological controls using antagonistic microorganisms has proved to be successful for controlling various plant diseases in many countries (Sivan, 1987) recorded that *Bacillus* sp. gave a highly antagonistic effect against some pathogenic fungi. Also, *Trichoderma* spp. are well documented as effective biological control agents of plant diseases caused by soil-borne fungi (Sivan and Chet, 1986; Whipps and Lumsden, 2001; McLean *et al*., 2004). As for antagonistic bacteria, Kim *et al*. (1997) found that seed treatment with *Bacillus* spp. actively controlled three fungal root diseases of wheat, and *Pseudomonas cepacia* or *P. fluorescens* applied to pea seeds acted as a biological control agent against Pythium damping-off and Aphanomyces root rot and was able to reduce disease incidence (Parke *et al*., 1991; King and Parke, 1993). In addition, *Bacillus cereus* has proven to have beneficial effects on crop health including enhancement of soybean yield, suppression of damping-off of tomato (Smith *et al*., 1999) and alfalfa (Kazmar *et al*. 2000). Extensive laboratory testing demonstrated a powerful suppression of damping-off of alfalfa by diverse strains of *B. cereus*, which confirmed preliminary testing under field conditions (Handelsman *et al*., 1990; Kazmar *et al*., 2000). Considerable research has been performed to investigate antagonistic microbes for use in seed treatments as reported by Callan *et al*. (1990), Baird *et al*. (1994), Howell and Stepanovic (1995) and Mathre *et al*. (1995). Also, El-Mougy and Abdel-Kader (2008) reported the inhibitory effect of antagonistic fungi and bacteria against the linear growth of root rot pathogenic fungi *in vitro.* The tested inhibitor factor in this study was the antagonistic agents applied as either growth culture discs or bio-primed faba bean seeds. They added that the inhibitor effect of *Trichoderma viride*, *T. harzianum*, *Bacillus subtilis* and *Pseudomonas fluorescens* was significantly higher than *T. hamatum* and *B. cereus*, respectively.

Chitosan is a partly de-acetylated form of chitin, and consists of polymers of *b*-1,4-glucosamine subunits, with molecular weight up to 400 kDa. It is

environmentally safe and non-toxic to higher organisms (Kumar, 2000). Chitosan and its derivatives display antibiotic activity against microorganisms, both bacteria (Rabea *et al*., 2003; Liu *et al*., 2004; Tikhonov *et al*., 2006) and fungi (Bell *et al*., 1998; Saniewska, 2001; Parke *et al*., 2002; Rabea *et al.*, 2003; Tikhonov *et al*., 2006). Chitosan is not toxic to plants, but it can enhance plant resistance in seeds (Benhamou *et al*., 1994; Lafontaine and Benhamou, 1996), fruits (Benhamou, 2004) or leaves (Trotel-Aziz *et al*., 2006) and reduce disease caused by fungal pathogens. Furthermore, Saniewska (2001) recorded that the inhibitory effect of crab-shell chitosan, medium and high molecular weight, toward *Alternaria alternata*, *Botrytis tulipae*, *Fusarium oxysporum* f. sp. *callistephi, Fusarium oxysporum* f. sp. *tulipae*, *Phoma narcissi* and *Phoma poolensis* was evaluated *in vitro* and *in vivo*. He conclude that chitosan evidently inhibited *in vitro* growth of all tested pathogens, with a marked effect at higher concentrations above 200 mg/cm^3.

Present research focuses on fungicides alternative measures that are safe to human and environment. The inhibitor effect of some antagonistic fungi and bacteria as well as chitosan at different concentrations against the growth of tomato root rot pathogens was evaluated under *in vitro* conditions. An alternative to pesticide application is that, it may be possible to utilize against a broad spectrum of disease-causing pathogenic microorganisms.

Materials and methods

Tested materials

Pathogens and antagonists

Virulent pathogenic fungal isolates were isolated from collected tomato plants showing root rot symptoms grown at various locations either in open fields or plastic houses throughout Egypt. The diseased tomato samples were subjected to isolation trails. The isolated fungi were identified according to morphological and microscopically characters described by (Gilman, 1957; Barnett and Hunter, 1972). The isolated fungi were *Fusarium oxysporum radicis-ycopersici*, *F. oxysporum lycopersici, F. solani, Rhizoctonia solani*, *Sclerotium rolfsii*, *Macrophomina phaseolinae*, *Pythium* sp., *Phytophthora* sp., .These isolated fungi were tested for their pathogenic ability to induce root rot incidence to tomato seedlings in pot experiment under greenhouse conditions (unpublished data). The pathogenic fungi were subjected to the laboratory tests in the present work.

The antagonistic microorganisms, *i.e. T. harzianum*, *T. viride*, *Bacillus subtilis*, and *P. fluorescens*, obtained from the Plant Pathology Department of the National Research Centre, Giza, Egypt were used in the present study.

These microorganisms were isolated from the rhizosphere of various healthy and root rot infected vegetables crops, grown in the Delta and Middle Egypt regions, and proved their high antagonistic ability during previous work at the same department. Fungal and bacterial cultures were maintained on potato dextrose agar (PDA) and nutrient agar slant media at 5 ± 1^0C as stock cultures until use. All isolates were refreshed by growing at the optimum growth conditions at the beginning of the present experiments.

By-Product (Chitosan)

Two chitosan samples from shrimp shells (2-Amino-2-deoxy-(1→4)-*β*-D-glucopyranan Poly-(1,4-*β*-D-glucopyranosamine) with Low molecular weight (LMW ~150000 Dalton) and High molecular weight (HMW ~600000 Dalton) produced by Sigma-Aldrich Chemicals Company were used in present work.

Growth media

PDA and nutrient broth medium (Difco Laboratories, Detroit, MI) were used for growing fungal and bacterial isolates tested in the present work.

Effect of antagonistic microorganisms on fungal growth

The inhibitory effect of the abovementioned fungal and bacterial antagonistic agents against the linear growth of the tomato pathogenic fungi was evaluated using the modified dual culture technique (Ferreira *et al*., 1991). Abundant fungal and bacterial growth was first prepared. Ten mL of each individual bacterial isolate was grown for 48 h on nutrient broth medium and poured into flasks containing sterilized PDA medium. Before solidifying, each flask was rotated gently to ensure equal distribution of bacterial growth, and then poured into 9-cm-diameter Petri dishes. Inoculated plates were incubated for 48 h at 28 ± 1^oC. For fungal growth, a 5-mm disk of each tested fungi was transferred to the centre of a PDA plate then incubated at 25 ± 1^oC. The incubation period was 5 and 7 days for antagonistic and pathogenic fungi, respectively. *In vitro* antagonistic studies of bio-control microorganisms and pathogenic fungi were performed on PDA medium in 9-cm-diameter Petri dishes. A 5-mm disk of each antagonistic fungal or bacterial growth culture was placed onto the PDA, 10mm from the edge of the Petri dish. Another disk of the same diameter of each pathogenic fungal growth culture was placed on the

opposite side of the dish at the same distance. The control treatment was inoculated with a culture disk of either a pathogenic or antagonistic culture alone at the same conditions. Both experimental and control dishes were assigned to a completely randomized design, with five replicates per treatment. All inoculated Petri dishes were incubated at 28±1^{o}C and the fungal growth diameter away from and towards the antagonist agent was measured after the pathogenic fungal growth in the control treatment had reached the edge of the Petri dish. This test was repeated three times and the inhibition was calculated as the percentage reduction in colony diameter growth compared with the control.

Effect of chitosan on fungal growth

An *in vitro* experiment to evaluate the effect of chitosan on fungal growth was carried out on PDA plates amended with either Low molecular weight and High molecular weight of chitosan at different concentrations (1, 2, 3, 4 1 and 5 g/L) followed the method described by Laflamme *et al.* (1999). Un-amended PDA plates served as controls. Fungi used in this experiment included pathogenic fungal isolates: *F. oxysporum radicis-ycopersici* (isolate ForlQ4), *F. oxysporum lycopersici* (isolate FolG14), *F. solani* (isolate FsG1); *R. solani* (isolate RsG1), *S. rolfsii* (isolate SrM2), *M. phaseolinae* (isolate MpB1), *Pythium* spp (isolate Py H2) and *Phytophthora* spp (isolate Ph MPC). Five plates per each chitosan concentration as well as controls were inoculated in the centre with a plug (5 mm diameter) from the edge of a 10–15 day-old-colony of each fungus to be tested. Growing fungal colonies were measured daily for each plate, until controls reached the edge of the plate. For each tested fungus average percentage of growth reduction was calculated as the growth in each plate amended with chitosan at each concentration in respect to that of the corresponding control plate. The experiments were performed twice.

Statistical analyses

All experiments were set up in a complete randomized design. One-way ANOVA was used to analyze differences between antagonistic inhibitor effect and linear growth of pathogenic fungi *in vitro.* A general linear model option of the analysis system SAS (SAS Institute Inc. 1996) was used to perform the ANOVA. Duncan's multiple range test at $P < 0.05$ level was used for means separation (Winer, 1971).

Results and discussioins

Effect of antagonistic microorganisms on fungal growth

The inhibitory effect of antagonistic fungi and bacteria was tested against some pathogenic fungi *in vitro*. Percentages of the reduction in growth of pathogenic fungi in response to antagonistic agents are presented in Table (1). The presented data show that the growth of pathogenic fungi was significantly reduced by the inhibitor action produced by all antagonistic agents tested. The antagonistic fungi had a greater effect on the retardation of growth (75.5--100%) compared with the bacterial agents (57.7—83.3%). The inhibitor effect of *T. harzianum* was observed to be higher than *T. viride* against all tested pathogenic fungi. Data also reveal that the antagonistic bacteria also showed significant differences among the tested isolates. The higher inhibitory effect on pathogenic fungal growth was recorded for *B. subtilis* (64.4-83.3%) comparing with *P. fluorescens* (57.7-77.7%). Similar results were reported by many investigators (Andersen *et al.,* 2003; Carisse *et al.,* 2003; Leclère *et al.,* 2005).

They reported the inhibitory effect of antagonistic fungal and bacterial microorganisms such as *Trichoderma* spp., *B. subtilis* and *P. fluorescens* that cause a growth reduction of *P. ultimum* under *in vitro* conditions. The inhibition in growth of the pathogen could be attributed to antibiosis, hyperparasitism (We *et al*., 1986) or production of chitinase and *ß*-1,3 glucanase enzymes which degrade the cell wall leading to lysis of mycelium of the pathogen (Ahmed and Baker 1987). In this regards, biological control of plant diseases, especially soil-borne plant pathogens, has been the subject of much research in the last two decades. Therefore, biological control of plant pathogens is becoming an important component of plant disease management practices. In the present study, the evaluated fungal and bacterial antagonists demonstrated an inhibitor effect against root rot pathogens under *in vitro* conditions. These results are also confirmed by several researchers (Bell *et al*., 1982; Abdel-Kader, 1997; El-Mougy, 2001). Microorganisms can play an enormously important role in plant disease control. As naturally occurring resident antagonists, they can be managed or exploited to achieve the desired results. Biological control with introduced microorganisms presents challenges not encountered with naturally occurring parasitic organisms. When used, natural enemies do not depend on the target pest as a host, which is the case with most antagonists of plant pathogens. Recent research on the use of introduced antagonists has to be considered in two ways: (i) antagonists, like the pathogen, should be adapted to the host plant to be protected, in addition to their ability to inhibit or compete with the target pathogen; and (ii) antagonists that can be applied directly and precisely to the infection court need not be able to spread or even persist in the

environment. These two considerations for biological control sparked the current and much more successful effort with plant associated microorganisms as agents introduced for biological control of plant pathogens (Mathre *et al*., 1999).

Table 1. Growth reduction of pathogenic tomato root rot fungi in response to the inhibitor effect of antagonistic agents *in vitro*

Pathogenic[3] Fungal isolate	*T. harzianum*		*T. viride*		*B. subtilis*		*P. fluorescens*	
	L[1] (mm)	R[2] (%)	L (mm)	R (%)	L (mm)	R (%)	L (mm)	R (%)
Forl-Q4	15 d	83.3	22 c	75.5	28 bc	68.8	32 b	64.4
Fol-G14	12 d	86.6	17 d	81.1	22 c	75.5	26 c	71.1
Fs-G1	8 de	91.1	10 de	88.8	16 d	82.2	22 c	75.5
Rs-G1	0 f	100	5 f	94.4	15 d	83.3	20 c	77.7
Sr-M2	18 d	80.0	22 c	75.5	30 b	66.6	38 b	57.7
Mp-B1	15 d	83.3	20 c	77.7	32 b	64.4	35 b	61.1
Py-H2PC	9 de	90.0	14 d	84.4	20 c	77.7	20 c	77.7
Ph -MPC	5 f	94.4	10 de	88.8	20 c	77.7	24 c	73.3
Control	90 a	0.0	90 a	0.0	90 a	0.0	90 a	0.0

[1] Fungal linear growth (mm)

[2] Fungal growth reduction (%)

[3] Pathogenic Fungal isolates: *F. oxysporum radicis-lycopersici* (isolate ForlQ4), *F. oxysporum lycopersici* (isolate FolG14), *F. solani* (isolate FsG1); *R. solani* (isolate RsG1), *S. rolfsii* (isolate SrM2), *M. phaseolinae* (isolate MpB1), *Pythium* spp (isolate Py H2) and *Phytophthora* spp (isolate Ph MPC), Figures with the same letters are not significant different (P =0.05)

Effect of chitosan on fungal growth

The effect of the two types of chitosan on fungal growth Plant pathogenic fungi show clear differences when growing in chitosan-amended PDA at different concentrations Table 2. The inhibitor effect of the two tested chitosan types was increased as chitosan concentration is increased in growth medium to reach its maximum at the highest concentration (5 g/L). Complete inhibition of tested fungal growth was observed at the concentration of 4 g/L of LMW chitosan, while the highest fungal growth reduction was recorded in PDA-amended with HMW at the same concentration. The presented results in Table (2) reveal that the tested HMW chitosan showed more inhibitor effect against mycelia growth of tested fungi than that of LMW.

According to Sigma aldrich company HMW have ≤10% water solubility, while LMW is water insoluble (http://www.sigmaaldrich.com/catalog/product/fluka/22743?lang=en&region=EG). In this regard, Liu *et al*. (2001) demonstrated that the antibacterial activity of chitosan intensified with increasing molecular weight. It seems that the antibacterial activity of chitosan depends on the amount of reactive amino groups. However, when too many reactive amino groups exist within the chitosan molecule, the chitosan's

capacity to attach to bacterial surfaces decreases. In addition, the antibacterial activity of chitosan increases with increasing degree of deacetylation (Liu *et al*., 2001; Chung *et al*., 2004).

Furthermore, both HMW and LMW chitosan had antimicrobial activity. Chitosan and its derivatives offer a great potential as natural biodegradable substances which have anti-microbial and eliciting activities (Bautista-Banˇos et al., 2006; Benhamou, 1996). The present study demonstrated that HMW and LMW chitosan were effective in inhibiting mycelial growth of all tested fungi. Chitosan have broad-spectrum antibacterial activities as fungicides in inhibiting spore germination, germ tube elongation and mycelial growth of fungal phytopathogens, such as Fusarium (Eweis *et al*., 2006; Xu *et al.,* 2007), Phytophthora capsici (Xu *et al*., 2007) and Sclerotium rolfsii (Eweis *et al.*, 2006). Also, Tikhonov *et al.* (2006) reported that low molecular weight chitosan (4.6 kDa) and N-/2(3)-(dodec-2-enyl)succinoyl/-derivatives of different degrees of substitution were tested for their antimicrobial activity against *Escherichia coli*, *Pseudomonas aureofaciens*, *Enterobacter agglomerans, Bacillus subtilis, Candida kruisei* and *Fusarium oxysporum* f. sp. *radicis lycopersici*. They added that the results indicated that the chitosans show high activities against all bacteria, yeast and filamentous fungus. In fact, they suppressed fungal colony growth and inhibited fungal spore germination at 0.01% (w/v) concentration.

Table 2. Reduction % in the linear growth of tomato fungal pathogens in response to different concentrations of Chitosan HMW and Chitosan LMW *in vitro*

Pathogenic	**Chitosan LMW [1] concentration**					**Chitosan HMW [2] concentration**				
Fungal isolate	1 g/L	2 g/L	3 g/L	4 g/L	5 g/L	1 g/L	2 g/L	3 g/L	4 g/L	5 g/L
Forl-Q4	24.4	38.8	41.1	72.2	91.1	63.3	91.1	100	100	100
Fol-G14	4.4	33.3	41.1	63.3	86.7	55.5	87.8	94.4	100	100
Fs-G1	22.5	42.2	52.2	80.0	94.4	68.8	100	100	100	100
Rs-G1	28.0	55.5	64.4	88.8	100	76.7	88.8	100	100	100
Sr-M2	11.1	30.0	58.8	77.7	88.8	11.1	27.8	66.6	88.8	100
Mp-B1	11.1	33.3	37.7	37.7	83.3	8.8j	24.4	68.8	100	100
Py-H2PC	6.6	11.1	24.2	37.7	88.8	11.1	66.7	88.8	100	100
Ph -MPC	0.0 k	11.1	11.1	54.4	77.7	11.1	61.1	83.3	100	100

[1] High Molecular Weight

[2] Low Molecular Weight

[3] Pathogenic Fungal isolates: *F. oxysporum radicis-ycopersici* (isolate ForlQ4), *F. oxysporum lycopersici* (isolate FolG14), *F. solani* (isolate FsG1); *R. solani* (isolate RsG1), *S. rolfsii* (isolate SrM2), *M. phaseolinae* (isolate MpB1), *Pythium* sp (isolate Py H2) and *Phytophthora* sp (isolate Ph MPC).

The fungicidal activity of chitosan has been well documented . Literature generally reports that the level of inhibition of fungi is highly correlated with chitosan concentration, indicating that chitosan performance is related to the application of an appropriate rate. It is believed that the polycationic nature of this compound is the key to its antifungal properties and that the length of the polymer chain enhances its antifungal activity (Hirano and Nagao, 1989). An additional explanation includes the possible effect that chitosan might have on the synthesis of certain fungal enzymes (El-Ghaouth *et al*., 1992). Recent studies have shown that chitosan is not only effective in halting the growth of the pathogen, but also induces marked morphological changes, structural alterations and molecular disorganization of the fungal cells (Benhamou, 1996; El-Ghaouth *et al*., 1999). There is strong evidence that mycelial growth can be inhibited or retarded when the growth media of fungi are amended with chitosan. For example, as chitosan concentration increased from 1% to 4%) the radial growth of *Sclerotinia sclerotiorum*, decreased (Cheah *et al.*, 1997). Other studies showed a linear decrease of growth of *Rhizoctonia solani* as the chitosan concentration gradually increased from 0.5 to 6.0 mg ml−1 (Wade and Lamondia, 1994). Mycelial growth of *Fusarium solani* f. sp. *phaseoli* and *F. solani* f. sp. *pisi* was inhibited at the minimum concentrations of 12 and 18 mgml−1, respectively (Hadwiger and Beckman, 1980; Kendra and Hadwiger, 1984). Other studies reported a complete growth inhibition of fungi such as *F. oxysporum*, *R. stolonifer*, *Penicillium digitatum* and *Colletotricum gloeosporioides* at concentrations of 3% (Bautista-Ban˜ os *et al*., 2003, 2004). The long-term fungicidal effect of chitosan can also be related to concentration and incubation time. For example inhibition of *F. oxysporum* f. sp. *radicis-lycopersici* grown at two of the lowest concentrations (1.0 and 2.0 mg ml-1) decreased with increased incubation time (Benhamou, 1992). Overall, sporulation of fungi treated with chitosan is generally reported to be lower than in untreated fungi. Moreover, in some reports no spores were observed after chitosan treatment.

The mechanism by which chitosan affects the growth of several phytopathogenic fungi has not been fully elucidated, but several hypotheses have been postulated. Because of its polycationic nature, it is believed that chitosan interferes with negatively charged residues of macromolecules exposed on the fungal cell surface. This interaction leads to the leakage of intracellular electrolytes and proteinaceous constituents (Leuba and Stossel, 1986). Other mechanisms mentioned in the literature are the interaction of diffused hydrolysis products with microbial DNA, which leads to the inhibition of mRNA and protein synthesis (Hadwiger *et al*., 1986) and the chelation of metals, spore elements and essential nutrients (Cuero *et al*., 1991).

In the present study HMW and LMW chitosan were investigated for their inhibitory effect against mycelia growth of various pathogenic fungi on agar media. According to the results obtained, both chitosan samples had the ability to inhibit mycelia growth depends on their molecular weight and applied concentration. In conclusion, our results showed that HMW chitosan exhibited higher antifungal activity than LMW chitosan against fungi tested. Therefore, it could be suggested that chitosan might be used commercially as easily, safely and applicable fungicides alternatives for controlling such soil-borne plant pathogens

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