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Chitosan and Three *Trichoderma* spp. to Control Fusarium Crown and Root Rot of Tomato in Jeddah, Kingdom Saudi Arabia

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Pathogenicity test using five isolates of *Fusarium oxysporum* f.sp. *radicis - lycopersici* (FORL) previously isolated from diseased samples of tomato showing typical symptoms of Fusarium crown and root rot in winter plantations of tomato in Jeddah district, Saudi Arabia revealed that they were all able to cause damping off symptoms in tomato plants. In this respect FORL (isolate No.2) was the most aggressive one in inducing the disease in tomato plants.

The inhibitory effect of chitosan against FORL growth under laboratory conditions was indicated. The inhibitory effect was increased as the concentration of chitosan increased from 0.38 to 6.00 mg/ml (pH 5.5). Chitosan also reduced the conidial germination. Complete inhibition was achieved when chitosan concentrations 3.00 and 6.00 mg/ml were used indicating that chitosan had a fungicidal effect. In greenhouse experiments, tomato seeds and transplants treated with chitosan at 3.00 and 6.00 mg/ml improved the stand of tomato plants grown in soil infested with FORL and significantly reduced both damping off and Fusarium crown root rot (FCRR) incidence. The highest height, fresh and dry weights of the shoot system and fruit yield were obtained in tomato plants grown from transplants treated with chitosan at 6.00 mg/ml concentration.

In vitro studies revealed that all the tested *Trichoderma* spp. isolates have sharply decreased the mycelial growth of the pathogenic fungus. Data of greenhouse experiments indicated that using any of the three *Trichoderma* spp. tested caused a significant reduction of FCRR disease incidence in comparison with the check treatment. Tomato transplants treated with *T. harzianum* before planting in soil artificially infested with FORL resulted in the lowest percentage of disease incidence.

Key words: Antifungal activity, chitosan, disease control, *Fusarium oxysporum* f.sp. *radicis-lycopersici*, pathogenicity, tomato and *Trichoderma harzianum*.

Fusarium crown and root rot (FCRR) of tomato caused by *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) has been recorded as the most prevalent soil-borne disease of this crop in different regions of the world, resulting in yield losses of up to 75% (Gotta and Tamietti, 1990; Dwivedi, 1991; Rattink, 1993; McGovern *et al.*, 1998 and Kuckareck *et al.*, 2000). The disease causes a significant threat to tomato transplant production and to both field and greenhouse fruit production wherever it

Trichoderma sp. were isolated from the rhizosphere of infected tomato plants and identified according to their morphological characters as described by Gilman (1957) and Barnett and Hunter (1972). All cultures were maintained on PDA slants and stored in a refrigerator at 10°C.

Pathological studies:

Pathogenic ability of five isolates of FORL, previously isolated and identified from naturally infected plants, to induce crown and root rot in tomato plants was evaluated under greenhouse conditions.

FORL isolates were separately grown on autoclaved barley meal sand medium (75 g, washed dried barley grains; 100 g, washed dried coarse sand and 65 ml, tap water per bottle) in 500 ml glass bottles. Inoculation was carried out using uniform agar discs (5-mm-diam.) bearing 7 days old fungal growth of any of the tested isolates. The bottles were incubated at 25±1°C for 15 days to obtain sufficient growth of the fungal isolates.

Formalin disinfested pots (25-cm-diam.) were filled with autoclaved sand loam soil (1:2 w/w). The potted soil was then artificially infested with the desired inoculum prepared at the rate of 5% (w/w), then watered two times during one week before planting. In check treatments, equal amounts of the uninoculated substrate were added in pots. Surface sterilized seeds of tomato cv. Castle Rock (Atlas Comp., USA) were sown in the infested soil at the rate of 10 seeds/pot. A set of four replicate pots was used for each of *Fusarium* isolates. Disease symptoms were noticed and the percentage of pre- and post-emergence was recorded 15 and 45 days after sowing. Reisolation from both ungerminated seeds of tomato at pre-emergence stage as well as infected tomato plants at post-emergence stage was carried out and the obtained fungi were compared with that used in soil infestation.

Effect of chitosan on the linear growth and spore germination of FORL:

Crab-shell chitosan was obtained from Pretreatment and Finishing of Cellulose Fibers Dept. NRC, Cairo, Egypt. Purified chitosan was dissolved in 0.25 N HCl by heating with constant agitation for 24 hr. The solution was adjusted to pH 5.5 by adding sodium hydroxide 1N, then 1ml of Tween 80 was added (El-Ghaouth *et al.*, 1991).

F. oxysporum f.sp. *radicis-lycopersici* (FORL) isolate No.2 (a highly pathogenic isolate) was performed on PDA medium supplemented with chitosan at different concentrations, *i.e.* 0.00, 0.38, 0.75, 1.50, 3.00 and 6.00 mg/ml. Chitosan concentrations were added to PDA medium then autoclaved at 121°C for 15 min. The stock medium of PDA of each concentration was poured into 9-cm-plates. After solidification, plates were inoculated at the centre with 5 mm mycelial disks, cut from the periphery of 7-day-old culture. Plates were incubated at 25±1°C for 7 days. Four replicate plates were prepared for each treatment. Chitosan free plates served as a check. The two diameters of the developed colonies were measured when the fungus growth covered plate in the check treatment and the percentage of reduction in linear growth in each treatment was calculated.

infested with FORL (isolate No.2) at the rate of 5% (w/w). Five replicate pots were used for each particular treatment. Percentage of FCRR incidence was determined 4 weeks after transplanting.

Statistical analysis:

Most data obtained were subjected to analysis of variance according to procedures described by Snedecor and Cochran (1980).

Results and Discussion

Pathological studies:

Pathogenicity test for the five isolates of FORL revealed that they were all able to cause damping off symptoms in tomato plants (Table 1).In this respect , FORL (isolate 2) was the most aggressive, followed by isolates (4 and 3), respectively. Meanwhile, isolate (5) was the weakest in this concern. These results are in a accordance with those reported by other investigators (Jarvis, 1988; Benhamou, 1992; Rattink, 1993; Lafontaine and Benhamou, 1996 and McGovern *et al.*, 1998) who stated that different isolates of *F. oxysporum* differed in their aggressiveness on tomato plants. All fungal isolates were reisolated from the infected plants and were found to be identical with the original isolates used in soil infestation. Plants grown as check treatment did not show any disease symptoms.

Table 1. Pathogenic ability of different isolates of FORL on tomato plants (cv. Castle Rock) under greenhouse conditions

Tested FORL isolate	Damping off (%)	
	Pre-emergence	Post-emergence
1	13.5	27.4
2	13.2	47.5
3	16.5	28.8
4	11.2	31.6
5	15.2	27.2
Check	00.0	00.0

Effect of chitosan on the linear growth and spore germination of FORL:

Data presented in Table (2) show the effect of chitosan on the linear growth and spore germination of FORL (isolate 2). Addition of chitosan to the agar medium led to clear inhibition of FORL. Data also indicate that the antifungal activity of chitosan against FORL growth was increased as the concentration of chitosan was increased. Mycelial growth was completely inhibited by chitosan at 3 and 6 mg/ml medium during 7 days of incubation period.

On the other hand, chitosan at 0.38, 0.75, and 1.50mg/ml medium caused significant reduction in mycelial growth of the fungus in comparison with the check treatment (Table 2). Similar results were also reported by other investigators

Table 3. Effect of chitosan on damping off FCRR caused by FORL (seed experiments)

Treatment	Damping off (%)	
	Pre-emergence	Post-emergence
Untreated seeds sown in infested soil	17.5	48.6
Seeds treated with chitosan at 3 mg/ml and sown in infested soil	13.4	35.2
Seeds treated with chitosan at 6 mg/ml and sown in infested soil	6.2	12.6
Untreated seeds sown in uninfested soil (Check)	0.0	0.0
L.S.D. at 5%	3.2	4.8

As a result of treating transplants with chitosan at any concentration before planting, great reduction in percentage of FCRR incidence was obtained (Table 4). This effect was more pronounced when chitosan was used at 6 mg/ml, as the corresponding percentage of FCRR incidence was 10% versus 52.5% in the plants grown from untreated transplants planted in infested soil .The obtained results confirmed the previous studies which suggested that application of chitosan resulted in great reduction in the severity of some root diseases caused by phytopathogenic fungi (Mitchell and Alexander, 1961; El-Ghaouth *et al.*, 1992; Benhamou *et al.*, 1998 and Ragab *et al.*, 2001).

Data (Table 4) also show that the two doses of chitosan tested much improved the stand of tomato plants compared with untreated seeds (Table 3) or untreated seedlings grown in soil infested with *F. oxysporum* f.sp. *radicis-lycopersici*. This might be due to the fungicidal property and/or its ability to enhance defense mechanism in plant cells (El-Ghaouth *et al.*, 1992). It appears that increase in chitinase and β-1, 3 glucanase activities reinforced the microbial defense mechanism of the plant and was beneficial in creating resistance to fungi (Rayan, 1987 and 1988 and Chang *et al.*, 1992).

Regarding the morphogenesis of the plant survival as represented by the height and fresh and dry weights of the shoot system, data presented in Table (4) reveal that these values were increased in the plants treated with chitosan and increased by increasing the concentration. The highest length, fresh and dry weights of the shoot system were observed in tomato plants treated with chitosan at 6 mg/ml. This was clear since the average height of plant was increased from 27.00 cm for plants grown from untreated transplants planted in infested soil to 43.09 and 49.50 cm for plants grown from transplants treated with chitosan at 3 and 6 mg/ml, respectively.

Concerning tomato fruit weight, data show that chitosan application significantly increased the average fruit weight of plants grown in infested soil than the corresponding figures of check.

b. Greenhouse experiments:

Data of greenhouse experiments shown in Table (6) clearly indicate that using any of the three *Trichoderma* spp. tested caused a noticeable significant reduction in percentage of FCRR disease incidence, ranging between 31.0 to 59.8% in comparison with check treatment. It is obvious from data in Table (6) that tomato transplants which their roots treated with *T. harzianum* before planting in soil artificially infested with FORL resulted in the lowest percentage of disease incidence of FCRR ,being 22.6% with significant difference with the other tested treatments. These results are in a harmony with those obtained by other investigators (Sivan *et al.*, 1987; McGovern, 1994 and Datnoff *et al.*, 1995) who obtained good results in controlling FCRR in tomato using *T. harzianum.*

Table 6. Evaluation of biological treatments for controlling FCRR disease of tomato under greenhouse conditions

Trichoderma spp. used in treating transplants	FCRR incidence (%)	Reduction (%) in FCRR incidence
T. harzianum	22.6	59.8
T. viride	36.2	35.6
Trichoderma sp.	38.8	31.0
Check *	56.2	-----
L.S.D. at 5%	8.7	-----

* Untreated transplants planted in soil infested with the pathogen

In conclusion, this study demonstrates the potential of using chitosan, a nontoxic compound to suppress FCRR of tomato caused by FORL when used for treating seeds or transplants before planting. This potential value appears to be attributable to the combination of the antifungal and eliciting properties of chitosan. These unique properties may very well make chitosan an ideal antifungal agent against greenhouse diseases considering its nontoxic effect on the environment. Study also verifies the effectiveness of using three *Trichoderma* spp. in controlling the disease under greenhouse conditions.

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استخدام الكيتوزان وثلاثة أنواع من التريكودرما لمقاومة مرض عفن التاج والجذور الفيوزاريومي في نباتات الطماطم في جدة بالمملكة العربية السعودية

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أوضحت تجارب العدوى الصناعية باستخدام خمس عزلات من الفطر فيوزاريم اوكسيسبورم راديسيس ليكوبيرسيكي التي سبق عزلها من نباتات طماطم مريضة مظهرة لأعراض نموذجية لمرض عفن التاج والجذور الفيوزاريومي وذلك من زراعات الطماطم الشتوية بمنطقة جدة – المملكة العربية السعودية ، قدرتها على إحداث أعراض مرض موت البادرات في نباتات الطماطم وكانت العزلة رقم (2) أكثر العزلات مرضية.

ثبت معمليا الفعل التثبيطي للكيتوزان على نمو الفطر فيوزاريم أوكسيسبورم راديسيس ليكوبيرسيكي وقد إزداد هذا الفعل التثبيطي عند زيادة تركيزه من 0.38 –6.0 مجم/ مل بيئة ذات حموضة 5.5 . كما أحدث الكيتوزان إنخفاضا في نسبة إنبات الجراثيم الكونيدية للفطر المختبر، وصل إلى درجة التثبيط التام للنمو عند التركيزين 3 و 6 مجم/ مل بيئة. .وفي تجارب الصوب ، أدت معاملة بذور وشتلات الطماطم بالكيتوزان قبل الزراعة إلى زيادة نسبة النباتات القائمة النامية في تربة ملوثة بالفطر الممرض بالإضافة إلى حدوث نقص معنوي في الاصابة بالمرض. وقد تم الحصول على أعلى أطوال للنباتات بالإضافة إلى اعلى وزن رطب وجاف للمجموع الخضري ووزن ثمار من نباتات نتجت من شتلات عوملت جذورها قبل الزراعة بالكيتوزان بتركيز 6مجم/ مل ماء.

أظهرت الدراسات المعملية أن كل عزلات التريكودرما المختبرة أحدثت نقصا حادا في النمو الميسليومي للفطر الممرض ، كما أوضحت النتائج المتحصل عليها من اختبار هذه العزلات تحت ظروف الصوبة احداثها إنخفاضا معنويا في نسبة الإصابة بالمرض تراوح بين 31–59% مقارنة بالتجربة المقارنة. وقد كانت أقل نسبة إصابة بالمرض مسجلة من النباتات التي عوملت جذور شتلاتها قبل الزراعة بتريكودرما هيرزيانم.

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Table 4. Effect of chitosan on Fusarium crown and root rot (FCRR) disease development and on some growth parameters of tomato plants

Treatment	FCRR (%)	Plant height (cm)	Fresh weight (g)	Dry weight (g)	Fruit weight / plant (g)
Untreated transplants planted in infested soil	52.5	27.00	14.2	3.54	135.4
Transplants treated with chitosan (3.0mg/ml) and planted in infested soil	20.0	43.09	39.65	11.00	362.62
Transplants treated with chitosan (6.0mg/ml) and planted in infested soil	10.0	49.50	40.54.	12.03	441.62
Untreated transplants planted in uninfested soil (check)	00.0	38.50.	25.83.	6.54	221.33
L.S.D. at 5%	9.6	4.48	1.66	2.02	25.12

Trichoderma spp. and their antagonistic ability:

a. In vitro experiment:

Microorganisms that can grow in the rhizosphere are ideal as biocontrol, since the rhizosphere provides the front – line defense for roots against attack by pathogens. The chance of selecting effective bioagents may be improved initially by first isolating microorganisms from the same environment in which they will be used.

In the present study, three different *Trichoderma* spp. were isolated from roots of diseased tomato plants showing typical symptoms of FCRR disease. Data in Table (5) clearly indicate that all the tested *Trichoderma* spp. sharply decreased the mycelial growth of the pathogenic fungus. Maximum reduction in mycelial growth of FORL (70.5%) occurred using *T. harzianum*, while *T. viride* and *Trichoderma* sp. caused 60.4 and 41.6% reduction in mycelial growth of the same pathogen, respectively. Chet (1984) found that Trichoderma apparently acts as mycoparasite which detects its host by sugar-lectin linkage and begins to excrete extracellular lytic enzyme such as ß-1,3 glucanase, chitinase, protease and/or lipase. The same author (1987) stated that the genus *Trichoderma* has a substantial ability to suppress a wide range of plant pathogenic fungi by various mechanisms including the production of cell – wall degrading enzymes.

Table 5. Antagonistic effect of three *Trichoderma* spp. on growth of FORL on PDA medium

Tested *Trichoderma* spp.	growth reduction (%) of pathogenic fungus
T. harzianum	70.5
T. viride	60.4
Trichoderma sp.	41.6

Table 2. Effect of chitosan on radial growth and spore germination of FORL

Chitosan mg/ml	Radial growth (mm)	Reduction (%) in mycelial growth	Spore germination (%)
Check (0.00)	90.00	0.00	100.00
0.38	69.00	23.33	63.42
0.75	64.00	28.89	45.49
1.50	51.75	42.50	30.47
3.00	0.00	100.00	0.00
6.00	0.00	100.00	0.00
L.S.D. at 5%	1.84	4.13	5.05

(Stossel and Leuba, 1984; Hirano and Nagao, 1989; El-Ghaouth *et al.*, 1991; Wang, 1992; Bautista *et al.*, 2003 and Rabea *et al.*, 2003). The mode of action of chitosan as fungicide might be explained by its interaction with the fungal DNA and/or RNA as stated by Hadwiger and Loschke (1981). Additionally, Leuba and Stossel (1986) indicated that the antifungal activity of chitosan is related to its ability to interfere with the function of plasma membrane of fungal cells.

Also, addition of chitosan to the agar medium led to significant inhibition of spore germination (Table 2). Similar inhibitory effects of chitosan were recorded by Benhamou *et al.* (1994) and El-Ghaouth *et al.* (1997). Complete inhibition was achieved at concentrations of 3 and 6 mg/ml, indicating that chitosan showed fungicidal activity rather than fungistatic. In this regard it is worthy to note that other reports showed that the minimum concentration of chitosan for the antimicrobial activity was 7 mg/ml against *Fusarium solani* (Hadwiger *et al.*, 1988). Rabea *et al.* (2003) reported that chitosan was found to have fungicidal effect against several fungi. The minimum inhibitory concentrations reported for specific target organisms ranged from 0.0018% to 1.00% and are influenced by a multitude of factors such as pH of the growth medium, the degree of polymerization of chitosan and the presence or absence of interfering substances such as lipids and proteins.

Effect of chitosan on crown and root rot disease of tomato under greenhouse conditions:

Data (Table 2) of the *in vitro* effect of chitosan indicated that chitosan at concentrations of 3 and 6 mg/ml were the most effective in preventing the growth and spore germination of FORL. These concentrations were tested to control the pathogen and the data obtained are shown in Tables (3 and 4). Data of seed treatments (Table 3) indicate that treating tomato seeds with any concentration used of chitosan has significantly reduced percentage of damping off FCRR in comparison with check treatment. Increasing dosage of chitosan from 3 to 6 mg/ml caused the lowest percentage of FCRR incidence, being 6.2 and 12.6% in pre- and post-emergence FCRR, respectively. Data also show that no infection was observed in the uninfested control treatment.

To clarify the effect of the previously mentioned concentrations of chitosan on spore germination of *F. oxysporum* f.sp. *radicis-lycopersici* (isolate 2), 10-day-old cultures were flooded with 10 ml of sterilized distilled water containing 0.1% Tween 80. Plates were then incubated at 25±1°C for 6 hours (Dhingra and Sinclair, 1985). Spores were harvested by scrapping them using a glass rod. The mixture of mycelium and spores was double filtered through cheesecloth and the resulting suspension was adjusted to 10 ml using sterilized distilled water. The average number of spores/ml in each treatment was determined using a haemocytometer. The average percentage of germinated spores in each treatment was determined.

Effect of chitosan on crown and root rot disease of tomato under greenhouse conditions:

Pot experiment was carried out to evaluate the role of chitosan on *F. oxysporum* f.sp. *radicis-lycopersici* (isolate 2) under greenhouse conditions using sand/loam soil. The soil was infested with the pathogenic isolate as mentioned before. Tomato seeds (cv. Castle Rock) were coated with 3% carboxymethyl cellulose then with chitosan at conc. 3 or 6mg /ml before sowing. In another treatment, roots of apparently healthy tomato transplants (cv. Castle Rock) were dipped in the same chitosan solution for 3 min before transplanting. Two transplants were planted in each pot and four replicate pots were used for each particular treatment.

Number of plants showing characteristic crown and root rot symptoms were recorded weekly for two months after planting. Also, plant height (cm), fresh and dry weight (g) measurements and also fruit weight (g) / plant in each pot were also recorded 90 days after transplanting.

Trichoderma spp. and their antagonistic ability:

a. In vitro experiments:

Screening of three isolates of *Trichoderma* spp. (*i.e. T. harzianum, T. viride* and *Trichoderma* sp.) previously isolated from roots of infected plants, for their antagonistic ability against FORL (isolate No. 2) was carried out using dual culture technique adopted by Ferreira *et al.* (1991). Four Petri dishes were used for each isolate tested. All plates were incubated at 25±1°C for 7 days then examined. The reduction percentage in growth of the pathogenic fungus due to the antagonistic effect of *Trichoderma* spp. was calculated using the following formula:

$$\text{Growth reduction (\%)} = \frac{\text{Growth in check - Growth in treatment}}{\text{Growth in check}} \times 100$$

b. Greenhouse experiments:

In greenhouse experiment, evaluation of biological treatments for controlling FCRR disease of tomato was carried out using healthy transplants (40- day- old) of tomato cv. Castle Rock grown in autoclaved soil. Roots of tomato transplants were immersed in spore suspension ($5x10^8$ spore/ ml) of any of the three tested *Trichoderma* spp. for 2 hours. Another set of transplants was immersed in sterilized distilled water and served as check treatment. Treated and untreated transplants were planted in plastic pots (25-cm-diam.) containing sand loamy soil artificially

occurs (Jarvis, 1988 and McGovern, *et al.*, 1993). Fusarium crown and root rot of tomato caused by FORL killed about 70-83 % of tomato young plants causing root rot and basal stem decay and eventually death (Kuckareck *et al.*, 2000).

Although, application of fungicides is far the most effective method to control tomato wilt, crown and root rot diseases, it is faced by imminent problems: first, there are reports of an increasing number of fungicide - resistant strains of the soil borne pathogens (Jones, 1985) and second, a number of commonly used fungicides such as benomyl are under review in many countries due to health risk concern (Anonymous, 1988).

Thus, there is a growing need to develop alternative approaches for control of soil borne diseases: one approach that is being actively pursued involves the use of bioactive substances (Benhamou *et al.*, 1994). Among the most promising bioactive oligosaccharides is chitosan, a mostly deacetylated derivative of chitin occurring in the cell wall of several fungi, which is readily extracted from the chitin of crustacean shell wastes (Hadwiger *et al.*, 1988). Chitosan oligomers have attracted attention because of their unique biological properties including their inhibitory effect on the growth of various pathogenic fungi and their ability to be potent elicitors of plant defense reactions (Leuba and Stossel, 1986; Hirano and Nagao, 1989; El-Ghaouth *et al.*, 1992; Benhamou *et al.*, 1998 and Ragab *et al.*, 2001).

The biological control of pathogen populations by microbial antagonists is another goal among the proposed avenues for minimizing damage caused by pathogens. Side by side several bioagents are usually found along with the pathogens, which show an antagonistic reaction. For instance, most species belonging to the genus *Trichoderma* are able to antagonize many plant pathogenic fungi and sometimes give equal control effects with those of certain fungicides (Lynch, 1987; Sivan and Chet, 1989; Ibrahim *et al.*, 2001; Rasmy, 2002 and El-Abbasi *et al.*, 2003).Another fact of biological control concerns is the possibility of stimulating the natural plant disease resistance process by biotic or abiotic agents (El-Ghaouth *et al.*, 1994 and El-Gamal, 2003).

The objective of this research is to determine and elucidate, *in vitro* and under greenhouse conditions, the inhibitory effect of chitosan and three species of *Trichoderma* on Fusarium crown and root rot (FCRR) disease incidence on tomato, caused by *F. oxysporum* f.sp. *radicis-lycopersici*, as well as on some growth parameters of tomato plants .

Materials and Methods

Source of cultures:

Five fungal cultures, identified as *Fusarium oxysporum* f.sp. *radicis – lycopersici* (FORL) according to their morphological characters (Nelson *et al.*, 1982) and the host range experiments (Rowe, 1980 and Menzies *et al.*, 1990), were collected from diseased samples showing typical symptoms of Fusarium crown and root rot disease in winter plantation of tomato in Jeddah district, Saudi Arabia. Also, three isolates of *Trichoderma* spp., *i.e. T. harzianum, T. viride* and