

Induced Resistance in Tomato Plants Against Fusarium Wilt Invoked by Nonpathogenic *Fusarium*, Chitosan and Bion

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The potential of nonpathogenic *Fusarium oxysporum* strain Avr5, either alone or in combination with chitosan and Bion, for inducing defense reaction in tomato plants inoculated with *F. oxysporum* f. sp. *lycopersici*, was studied *in vitro* and glasshouse conditions. Application Bion at concentration of 5, 50, 100 and 500 µg/ml, and the highest concentration of chitosan reduced *in vitro* growth of the pathogen. Nonpathogenic *F. oxysporum* Avr5 reduced the disease severity of Fusarium wilt of tomato in split plants, significantly. Bion and chitosan applied on tomato seedlings at concentration 100 µg a.i./plant; 15, 10 and 5 days before inoculation of pathogen. All treatments significantly reduced disease severity of Fusarium wilt of tomato relative to the infected control. The biggest disease reduction and increasing tomato growth belong to combination of nonpathogenic Fusarium and Bion. Growth rate of shoot and root markedly inhibited in tomato plants in response to tomato Fusarium wilt as compared with healthy control. These results suggest that reduction in disease incidence and promotion in growth parameters in tomato plants inoculated with nonpathogenic *Fusarium* and sprayed with elicitors could be related to the synergistic and cooperative effect between them, which lead to the induction and regulation of disease resistance. Combination of elicitors and nonpathogenic *Fusarium* synergistically inhibit the growth of pathogen and provide the first experimental support to the hypothesis that such synergy can contribute to enhanced fungal resistance in tomato. This chemical could provide a new approach for suppression of tomato Fusarium wilt, but its practical use needs further investigation.

Keywords : Biocontrol, Elicitor, *Fusarium oxysporum*, Tomato

Fusarium wilt of tomato caused by pathogenic formae speciales of the soil-inhabiting fungus *Fusarium oxysporum* (Schlecht.) f. sp. *lycopersici* (Sacc.) Snyder et Hansen.

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cause severe losses of yield in tomato (*Solanum lycopersicum* Mill.). This pathogen occurs throughout most tomato-growing areas and devastates the crop (Mes et al., 1999). Control of pathogen is difficult to eradicate from soil because they produce resting spore (chlamydospores) that can survive for many years, and there are few means of eliminating them once a field has become infested. Use of bioactive products, commonly referred to as plant activators that induce systemic acquired resistance (SAR) in plant to limit pathogenesis of many plant pathogens (Sticher et al., 1997). SAR can be induced by biotic and abiotic elicitors, which are the initial biochemical signals recognized by the plant cell being infected by fungi or other pathogens (Davis et al., 1993). Several products have been used as inducers of resistance in many plants species against various pathogens, including chitosan (Eikemo et al., 2003; Tiuterev et al., 1996) and Acibenzolar-S-methyl (ASM) (Kamal et al., 2008; Oostendorp et al., 2001).

Chitosan has been shown to control post harvest infection of *Botrytis cinerea* and *Rhizopus* sp. in strawberry fruit (Reddy et al., 2000) and increase shelf life of sliced strawberry fruit (Yoo and Lee, 1999). It has been reported that both soil and foliar plant pathogens fungal, bacterial and viral may be controlled by chitosan application (Bautista et al., 2006).

ASM with commercial name Bion is a systemic compound used for the control of plant diseases that it has minimal fungicidal and bactericidal activity, but induces host plant resistance via a unique mode of action that mimics the natural SAR response found in most plant species (Wade, 2006). Studies have shown that, under field conditions, ASM protects against a broad spectrum of pathogen in several crops (Gorlach et al., 1996).

Integrated control of plant pathogens by combining selected biocontrol agents is currently one of the most talked about yet controversial avenues for minimizing damage caused by pathogens (Dandurand and Knudsen, 1993; Lemanceau and Alabouvette, 1991; Samia and El-Khallal, 2007). Although the potential benefits of such combinations have been demonstrated in some cases (Duffy and Weller, 1995), other attempts failed to provide significantly better disease control (Dandurand and Knudsen,

1993).

One specific aspect of integrated biological control that has not received much attention concerns the possibility of stimulating the natural plant disease resistance process by combining the effects of biotic inducers. In recent years, the process of plant immunization or induced resistance to disease has been the focus of considerable interest and has been abundantly documented (Uknes et al., 1992).

The purpose of the present study was to assess the potential benefit of activators (Bion and Chitosan) alone or in combination with bioagent nonpathogenic *F. oxysporum* strain Avr5 to suppression of Fusarium wilt disease of tomato caused by *F. oxysporum* f. sp. *lycopersici* and investigated their possible modes of action through changes in growth to induced resistance in infected tomato plants.

Materials and Methods

Microorganism and culture conditions. *F. oxysporum* f. sp. *lycopersici* race 1 (race 1, 2, 3) was isolated (7 isolates) from infected tomato seedlings in tomato fields of Kurdistan province in Iran. All the tested isolates infected tomato plants, but with different degrees of disease severity. Isolate of FOL5 elected for experiment.

Nonpathogenic *F. oxysporum* (Avr5) was provided by F. S. Djalilov (Phytopathology Dep. of Moscow Timiryazev Agricultural Academy). The fungal culture was stored in sterile sand tubes at 4°C. Isolates of pathogen and antagonistic used in this study were grown on potato-dextrose agar (PDA) in darkness at 22-25°C for 2 weeks. Spores from 14-day-old cultures were removed gently from the surface of each plate culture by adding sterile distilled water. Suspension were filtered through five layers of sterile gauze (cotton) and suspended in sterile distilled water (SDW), and the density of the macro and microconidia was determined with a hemocytometer and adjusted by dilution to the desired concentration for soil inoculation. Spores were more than 90% microconidia.

Elicitors. ASM (Bion WG 50; Novartis Ltd., Basel, Switzerland) and a chitosan (Sigma, USA, deacetylation degree: ~85%) were tested as putative activators of resistance to *F. oxysporum* f. sp. *lycopersici* in tomato. Chitosan and Bion were dissolved in distilled water and both elicitors were applied with 0.01% Triton X-100 as a dispersant. In addition to a nontreated control, water and 0.01% Triton X-100 in combination, were used as controls *in vitro* and greenhouse.

Effect of elicitors on *F. oxysporum* f. sp. *lycopersici* *in vitro*. Effect both activators (chitosan and Bion) were tested

in vitro on growth of pathogen. Both compounds were used in the concentrations 5, 50, 100 and 500 µg active ingredient per ml in PDA. Stock solutions were 10 or 5 mg/ml. Both elicitors were applied with 0.1% Triton X-100 as a dispersant. Media were prepared in 100 ml flasks containing 95 ml of media, and stock solutions of either ASM or chitosan were added to obtain the desired concentrations. Distilled water was added to each flask to bring the total volume to 100 ml. Five millilitres of distilled water was added to the control flasks. Chitosan was added to the media before being autoclaved, whereas ASM was added aseptically after the media had been autoclaved. In each of two trials, five Petri dishes, each containing 20 ml of media, were made for each concentration of ASM and chitosan. An agar disc (5 mm in diameter) with mycelium of *F. oxysporum* f. sp. *lycopersici* was placed in the center of each Petri dish. Then, Petri dishes were randomized and incubated at 24-26°C in incubator. After 120 hr, percentage inhibition of radial growth (PIRG) was determined as an estimate of the growth inhibition of pathogen by the elicitors. Data of mycelial growth rates were submitted to variance analysis. Duncan's multiple Range test was applied to compare means by using the program SPSS.

Plant material and growth conditions. Seeds of tomato (*Solanum lycopersicum* Mill.) c.v Belyi naliv-241 susceptible to races 1 and 2 of *F. oxysporum* f. sp. *lycopersici* was used in this study. Tomato's seeds at first were surface-disinfected in 75% ethanol for 1 min and immersing for 3 min in sterile double-distilled water prior to sowing. Then seed sown in sterilized loamy sandy soil and seeded in 64-cell plug trays (plug size 3.4 by 3.4 by 5 cm). Trays maintained in a glasshouse at 28-30°C, 60-70% relative humidity, 16 hr light, and 8 hr darkness and watered as required.

Assays for disease suppression and induced resistance by non-pathogenic *Fusarium* (Avr5). Twenty five days after growth of tomato, the seedlings were split in two parts with a sterile scalpel from the hypocotyls down to the root system. Each half of the root systems was planted in separate flask containing 300 ml- fold dilution of commercial nutrient stock solution. This so-called split root system, performed in order to physically separate the antagonist and the pathogen, has been previously described by Fuchs et al (1997). The non-split seedlings were planted in another flask containing 300 ml- fold dilution of commercial nutrient stock solution. Two days later, one side of each split plant was watered with conidial suspension of non-pathogenic *Fusarium* (10⁶ spores/ml). Three days after the inoculation of the biocontrol strains, the non-treated side of the split plants and the non-split plants were

watered with conidial suspension of pathogen (10^6 spores/ml). Thirty five days after inoculation disease severity was scored.

In order to exclude any direct contact between the antagonists and the pathogen in the split plants, the absence of nonpathogenic *Fusarium* on the root side infested with the pathogen, and in the stem was checked during microbial analyses performed at the end of the experiment. In order to this work, the presence of nonpathogenic *Fusarium* on the rhizoplane was evaluated after plating 100 μ l of the rhizoplane suspensions on Special Nutrient Agar (SNA; Nirenberg, 1976). Three Petri dishes were plated per suspension-dilution. The stems were surface-disinfected (flamed after dipping into a 95% ethanol solution) and cut into sections at 2, 5 and 10 cm above the split of the stem. Section were placed on SNA and incubated in 27°C, for 72 hr. The colonies of *F. oxysporum* strain Avr5 were discriminated from pathogen ones on the basis of their different morphology.

Effect of elicitors on tomato *Fusarium* wilt in greenhouse conditions. Three ml conidial suspensions of approximately 10^6 spores/ml of non-pathogenic *F. oxysporum* (Avr5) were added to each plug cell in time of planting seeds. Bion and chitosan applied on tomato seedlings at concentration 100 μ g a.i./plant; 15, 10 and 5 days before pathogen inoculation. After 25 days, plugs containing the tomato plants were transplanted into 1.2-liter pots containing sterile soil infested with the pathogen at a rate of 10^6 CFU/g soil. Control plants were treated with sterile water.

The pots were divided into eight groups (each group consist of 6 pots and two seedlings per pot) and treated as the follow:

- Plants of the 1st group were left without any treatments, distilled water combined with 0.01% Triton X-100 (non-infected control).
- Plants of the 2nd group were treatments only with non-pathogenic *Fusarium* (Avr5).
- Plants of the 3rd group were infected with pathogen at a rate of 10^6 CFU/g soil (infected control).
- Plants of the 4th group were inoculated with non-pathogenic *Fusarium* and infected with pathogen as described above.
- Plants of the 5th and 6th groups, at first sprayed 3 times as described above with Bion and chitosan in plug trays, respectively before transplanting to pots infested by pathogen.
- Plants of 7th and 8th groups were inoculated with non-pathogenic *Fusarium* at the time of planting seed as described above, sprayed 3 times with Bion or chitosan and infested by pathogen.

Disease assessment. Disease index (disease severity) was rated by using the following scale (Bora and et al., 2004): 0, no symptoms; 1, <25% of leaves with symptoms; 2, 26-50% of leaves with symptoms; 3, 51-75% of leaves with symptoms; 4, 76-100% of leaves with symptoms.

Plant harvest and analysis. Twelve plants from each treatment were harvested after 35 days inoculation with pathogen. Leaves and roots were separated from plants and were used for different analysis and disease severity was rated at each harvest. Three pots of each treatment kept in greenhouse for three months to see the diseases improvement.

Determination of fresh weight. Seedlings from all treatments were removed, washed with distilled water, blotted with tissue paper, and fresh weight was determined.

Statistical analyses. The experiments were arranged in completely randomized design by 12 replication for each treatment (every treatment consist of 6 pots and two seedlings per pot). All data at first analyzed by least significant difference (LSD) testing. Duncan's multiple Range test was applied to compare means. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS). The experiment was replicated twice.

Results

All the concentrations of ASM reduced radial growth of

Table 1. Percentage of growth inhibition of *F. oxysporum* f. sp. *lycopersici* (at 24-26°C) on PDA media containing four different concentrations of ASM and chitosan. The average colony radius was calculated from fungal growth on five Petri dishes per treatment after 120 hr of growth

Treatment, concentration (μ g a.i./ml)	acibenzolar-S-methyl (ASM)	Chitosan
	Mean of radial growth (mm)	% growth inhibition
Control (Fol5)	87.0 ^c	–
5	81.4 ^d	6.4
50	68.0 ^c	21.8
100	53.2 ^b	38.8
500	29.0 ^a	66.6
LSD at 5 %	1.2	0.5

Data are means of 5 replicates.

Means in the column followed by different letters indicate significant differences among treatments at 0.05 according to Duncan's multiple range test.

Table 2. Effect of nonpathogenic *Fusarium oxysporum* Avr5 on development of tomato Fusarium wilt symptoms in non-split and split plants method

Treatment	Non-split plants		Split plants	
	Disease ^x severity	% reduction	Disease severity	% reduction
Control water	0.0 ^a	0	0.0 ^a	0
Control Fol5	3.6 ^b	–	3.4 ^b	–
Control Avr5	0.0 ^a	0	0.0 ^a	0
Avr5 ^a + Fol5 ^b	1.3 ^c	63.0	0.8 ^c	76.5
LSD at 5%	1.8		1.3	

Data are means of 5 replicates.

X, Disease severity (%wilt): 0, no symptoms; 1, <25% of leaves with symptoms; 2, 26-50% of leaves with symptoms; 3, 51-75% of leaves with symptoms; 4, 76-100% of leaves with symptoms

Means in the column followed by different letters indicate significant differences among treatments at 0.05 according to Duncan's multiple range test.

^anonpathogenic *Fusarium*; b, *F. oxysporum* f. sp. *lycopersici*

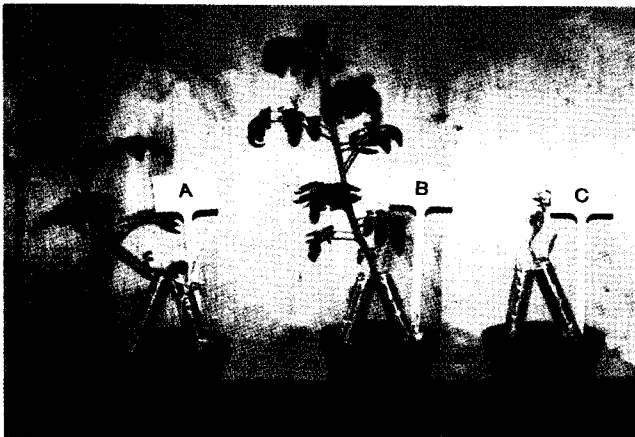


Fig. 1. Effect of nonpathogenic *Fusarium oxysporum* Avr5 on development of tomato Fusarium wilt symptoms in split plants method in greenhouse conditions. A, Control water; B, Fol5+Avr5; C, Control pathogen (Fol5)

pathogen *in vitro* significantly after 120 hr, whereas, chitosan reduced radial growth of pathogen, only in 500 µg a.i./ml. The highest concentration of ASM (500 µg a.i./ml) significantly reduced the growth rate (66.6%) of the colony of pathogen as compared control (Table 1).

Results of the effect of the biocontrol agents (Avr5) on kinetics of disease severity in non-split and in split plants are shown in Table 2. In the split plants Avr5 reduced diseases severity more than with when that antagonist and pathogen were applied together (Table 2 and Fig. 1). In the split plants, nonpathogenic *Fusarium* could not be isolated from the side infested by the pathogen and from the tomato stem tissue. These results indicate that antagonists and pathogen remained spatially separated all through the experiment. In split plants, the absence of any direct contact

Table 3. Effect of nonpathogenic *Fusarium* and elicitors (Bion and chitosan) alone or in combination on disease severity (%) and percentage of reduction of vessel's necrosis of tomato plants infected with *F. o. f. sp. lycopersici* (FOL5) under greenhouse conditions after 35 days

Treatment	Disease severity ^x	% reduction of wilting	% reduction of nec ^y
Control water	0.0 ^a	0.0	0.0 ^a
Control Avr5	0.0 ^a	0.0	0.0 ^a
Control Fol5	3.5 ^f	–	3.0 ^g
Fol5+Avr5	1.2 ^c	65.7	87.6 ^c
Fol5+Bion	1.5 ^d	54.3	27.5 ^e
Fol5+Chitosan	2.3 ^e	37.1	32.0 ^f
Fol5+Avr5+Bion	0.8 ^b	77.1	94.0 ^b
Fol5+Avr5+chitosan	1.2 ^c	65.7	70.5 ^d
LSD at 5%	0.2 ⁰		0.8 ⁰

Data are means of 12 replicates.

X, Disease severity: 0, no symptoms; 1, <25% of leaves with symptoms; 2, 26-50% of leaves with symptoms; 3, 51-75% of leaves with symptoms; 4, 76-100% of leaves with symptoms

Means in the column followed by different letters indicate significant differences among treatments at 0.05 according to Duncan's multiple range Test.

Y, % reduction of vessel's necrosis of tomato plants infected with *F. oxysporum* f. sp. *lycopersici* compared with high of tomato plant

between the pathogen and the biocontrol strains prevented any microbial antagonism.

Application nonpathogenic *Fusarium* (Avr5), Bion and chitosan greatly reduced disease severity of tomato plants as compared with infected control (Table 3). The best result of protection of tomato plants against *F. oxysporum* f. sp. *lycopersici* was conferred by nonpathogenic *Fusarium* and Bion which reduced %wilt 65.7 and 54.3, respectively in comparison with control pathogen (Table 3). Also, chitosan and combination between chitosan and Avr5 significantly reduced Fusarium wilt, 37 to 65.7% (Table 3).

Results also showed that combination between Avr5 and Bion is more effective in reducing disease severity than them when applied alone (Fig. 2).

Change in growth parameters of tomato plant

The influence of all treatment on fresh weight of roots and shoots of tomato plants was determined 5 weeks after inoculation by pathogen. Fresh weights of shoots and roots of infested plants with pathogen were significantly lower than with treatment of control water (Table 4).

Treatments of Avr5 plus Bion and Avr5 plus chitosan significantly increased fresh weight of tomato shoots (g/plant) compared to control pathogen, respectively 117 and 101%. Treatments Avr5 and Bion alone increased fresh weights of shoots by 54.7% and 40.3%, respectively. Inoculation plants with Avr5 plus Bion or chitosan increased

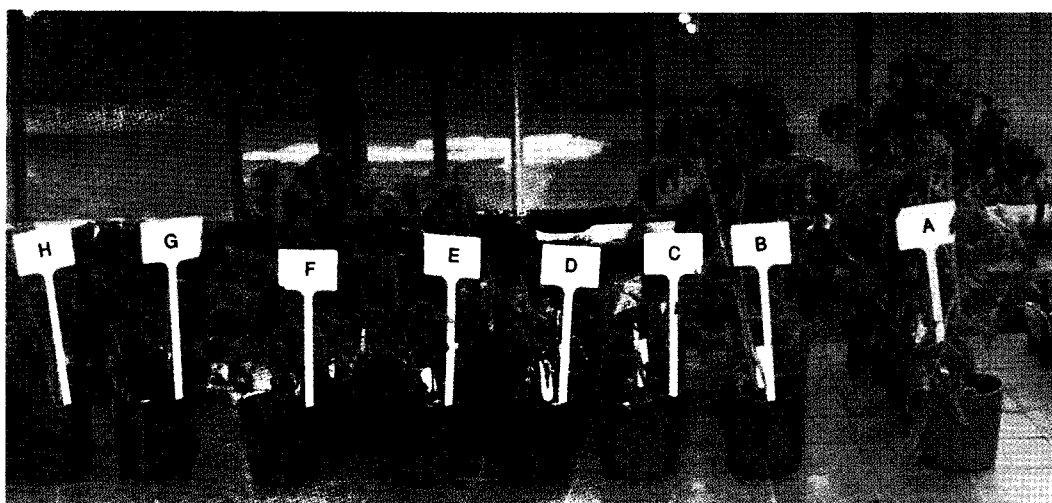


Fig. 2. Effect of nonpathogenic *Fusarium*, chitosan and Bion on control of Fusarium wilt of tomato under greenhouse condition. **A**, Control water; **B**, Control Avr5; **C**, Control pathogen (Fol5); **D**, Fol5+Chitosan; **E**, Fol5+Avr5+chitosan; **F**, Fol5+Avr5+Bion; **G**, Fol5+Avr5; **H**, Fol5+Bion

Table 4. Effect of nonpathogenic *Fusarium* and elicitors (Bion and chitosan) alone or in combination on changes in shoot and root growth of tomato plants infected with *F. oxysporum* f. sp. *lycopersici* (Fol) under greenhouse conditions 35 days after inoculation

Treatment	MSL (cm)	MRL (cm)	FWS (g/plant)	FWR (g/plant)
Control water	44.6 ^g	10.2 ^f	65.0 ^g	7.9 ^e
Control Avr5	44.9 ^h	10.0 ^c	65.7 ^h	7.9 ^e
Control Fol5	13.5 ^a	4.8 ^a	27.8 ^a	3.7 ^a
Fol5+Avr5	32.5 ^d	7.9 ^b	43.0 ^d	6.2 ^c
Fol5+Bion	27.6 ^c	7.5 ^b	39.0 ^c	5.8 ^{bc}
Fol5+Chitosan	19.4 ^b	7.5 ^b	35.5 ^b	5.7 ^b
Fol5+Avr5+Bion	38.0 ^f	9.3 ^d	60.5 ^f	7.6 ^e
Fol5+Avr5+chitosan	34.0 ^e	8.7 ^c	56.0 ^e	6.8 ^d
LSD at 5%	0.6	0.5	0.8	0.6

Data are means of 12 replicates.

MSL, mean shoot length; MRL, mean root length; FWS, fresh weight of shoots; FWR, fresh weight roots

Fresh weights are expressed as g⁻¹ plant.

Means in the column followed by different letters indicate significant differences among treatments at 0.05 according to Duncan's multiple range test.

shoot and root growth of tomato plants more, than with plants, only inoculated with Bion and chitosan (Table 4). Results showed that application Avr5 plus Bion were more pronounced in increasing shoot and root growth, while Avr5 plus chitosan had a lower effect (Table 4).

Also, treatment of nonpathogenic *Fusarium* plus Bion increased mean of shoot length and mean root length of tomato plants more than control pathogen and the other treatments (Table 4).

In remained treatments in greenhouse for three month no

diseases improvement and disease symptoms were observed.

Discussion

Concentration of 500 µg a.i./ml of Bion and chitosan had more effect on pathogen *in vitro*. This concentration reduced the growth rate of *Phytophthora fragariae* var. *fragariae* in strawberry plant (Eikemo et al., 2003). Bion significantly reduced growth of *Microdochium nivale*, *Rhizoctonia solani* and *P. fragariae* var. *fragariae* *in vitro* at concentration ≥100 µg a.i./ml (Eikemo et al., 2003; Hofgaard et al., 2005).

In split plants, the disease suppression was shown more effectively than in non-split plants. Therefore, our results describe the ability of nonpathogenic *F. oxysporum* (Avr5) to induce systemic resistance against Fusarium wilt in tomato. The spatial separation between nonpathogenic *Fusarium* strains used to induce resistance and the challenging pathogen in the split root system led to the conclusion that the reduction of the disease incidence by the inducing microorganisms was plant mediated (Hoffland et al., 1996). Such a reduction confirms the ability of biocontrol strain and of pathogenic *F. oxysporum* belonging to another forma speciales than the pathogen to induce resistance has been shown several times in tomato (Duijff et al., 1998; Fuchs et al., 1997).

Results of effect of elicitors and nonpathogenic *Fusarium* on disease in greenhouse indicated that, all treatments significantly reduced disease severity of tomato Fusarium wilt relative to the infected control. These treatments improve plant health through reducing wilt symptoms, vascular invasion and sporulation of pathogen. These results are in harmony with other researches (Hofgaard et al., 2005). Bion and its analogues have suppressed Fusarium diseases

on some crop (Gorlach et al., 1996) and it was effective in controlling tomato Corky root and no phytotoxic symptoms were observed by any material in the field (Bubici et al., 2006). Treatment Bion alone or in combination with *Pseudomonas fluorescens* reduced bacterial spot incidence in tomato and significantly increased tomato yield (Anith et al., 2004; Kamal et al., 2008). Reports have indicated that chitosan has the capacity to induce resistance to *F. oxysporum* in susceptible tomato plants when applied as a root dressing, foliar spray, and seed dressing by restricting pathogen growth to the outer root tissues and eliciting a number of defence reactions, including structural barriers (Benhamou et al., 1998).

Application of nonpathogenic *F. oxysporum* strain Avr5, Bion, or chitosan and combination of Avr5 with elicitors significant reduction disease severity and increase in growth parameters of tomato plant. Chitosan concentrations directly affected the number of root lesions on tomato seedlings after *F. oxysporum* f. sp. *lycopersici* inoculation (Benhamou et al., 1994). These experiments demonstrated that use of defense activators can enhance resistance to Fusarium wilt in tomato.

Results revealed that a synergistic action was found between nonpathogenic *F. oxysporum* strain Avr5 with Bion and chitosan on the growth promotion and protection of tomato against *F. oxysporum* f. sp. *lycopersici*. Combination of elicitors and nonpathogenic *Fusarium* synergistically inhibit the growth of fungi and provide the first experimental support to the hypothesis that such synergy can contribute to enhance fungal resistance in tomato plant. Also, a synergistic effect of *P. fluorescence* and Bion inoculation on shoot and root growth were found in tomato against bacterial spot disease (Kamal et al., 2008).

Application of bioagent nonpathogenic *F. oxysporum* Avr5 and elicitors enhanced shoot and root growth of tomato, especially when applied together. Results of treatment of control pathogen showed that infection by *F. oxysporum* f. sp. *lycopersici* markedly decreased fresh weights in roots of tomato plants. This phenomenon might be related to the toxin produced by the fungi, which affected K⁺ uptake and stomata function leading to uncontrolled transpiration and excessive loss of water leading to wilted plants (Aducci et al., 1997).

In conclusion, this chemical could provide a new approach of control of tomato Fusarium wilt, but its practical use needs further investigation. Furthermore, plant activator may also induce SAR effective against other tomato diseases (Inbar et al., 1998; Louws et al., 2001) not included in our experiments. Application these materials to diseases management and their practical use needs further investigation.

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