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Full Length Research Paper

Biological control of *fusarium* wilt of tomato by antagonist fungi and cyanobacteria

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Biological control of *Fusarium oxysporum* f. sp*. lycopersici* (FOL) causing wilt disease of tomato was studied *in vitro* as well as under pot conditions. Dual culture technique showed that *Aspergillus niger, Penicillium citrinum*, *Penicillium* sp*.* and *Trichoderma harzianum* inhibited the radial colony growth of the test pathogen. Methanol extract of *Nostoc linckia* and *Phormidium autumnale* showed moderate and minor zone of inhibition. Maximum seed germination was observed in seeds treated with *N. linckia* (93%), whereas, *T. harzianum, P. autumnale, P. citrinum* showed 80% seed germination, while *A. niger* treated seeds showed 50% germination. Under pot conditions, the plant heights, fresh and dry weight of plants were found to be increased significantly ($p \leq 0.05$) in all treatments except in *P. autumnale* amended soil. Similar results were observed in chlorophyll (a+b) content of treated plants. Maximum control of wilt disease was observed with *T. harzianum* (44.4%) treated plants as compared to FOL inoculated plants. Whereas, effectiveness of the other antagonists were recorded in the following order: *A. niger* (35.6%), *N. linckia* (33.3%), *P. citrinum* (24.4%), and *P. autumnale* (0.9 %).

Key words: Biological control, *fusarium* wilt, tomato, antagonist fungi, cyanobacteria.

INTRODUCTION

Fusarium oxysporum f. sp*. lycopersici* (FOL) is a known pathogen of tomato plant which is an economically important crop (Suárez-Estrella et al., 2007). Tomato yield is significantly reduced by *F. oxysporum* f. sp. *lycopersici* because it can destroy roots of tomatoes at growth stages. Numerous strategies have been proposed to control this fungal pathogen (Biondi et al., 2004; Ahmed, 2011). Currently, the most effective method in preventing tomato from fusarium wilt is mixing of tomato seeds with chemical fungicides. However, the use of chemical fungicides can be harmful to other living organisms besides reduction of soil microorganisms (Lewis et al., 1996). Therefore, public concern is focused on alternative methods of pest control, which can play a role in integrated pest management systems to reduce our dependence on chemical pesticides (Sutton, 1996).

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As with other vascular plant diseases, sanitation measures are difficult to apply (Brayford, 1992). Hence, strategies aiming at replacement of chemical pesticides by hazardous free biological agents can be a reasonably good choice.

Potential agents for biocontrol activity are rhizosphere competent fungi and bacteria, which in addition to their antagonistic activity are capable of inducing growth responses by either controlling minor pathogens or by producing growth stimulating factors.

Numerous studies have demonstrated reduced incidence of diseases in different crops after supplementing the soils with fungal or bacterial antagonists (Mukhopadhyay, 1987; Smith et al., 1990; Bashar and Rai, 1994; Singh et al., 2002; Akrami et al., 2011; Ahmed, 2011). Cyanobacteria have also been studied for the control of plant pathogenic fungi, particularly soil borne disease (Hewedy et al., 2000; Tassara et al., 2008; Kim and Kim, 2008). Recent developments in commercialization of biological control products have accelerated the approach of fungal antagonists (Fravel

et al., 2003).

This study was carried out to assess the efficacy of certain biocontrol agents for the management of wilt disease of tomato.

MATERIALS AND METHODS

Isolation and maintenance of pathogen and antagonist microorganism

F. oxysporum f.sp. *lycopersici* was isolated from naturally infected tomato plants grown from a commercial scale in greenhouse.

Dominant rhizosphere fungi of tomato were isolated from plants severely affected by wilt disease caused by *F. oxysporum* f. sp. *lycopersici*. These dominant rhizosphere fungi were isolated by soil plate method as described by Dhingra and Sinclair (1995) using potato dextrose agar (PDA) medium. *Trichoderma* spp. was isolated on selective media of Elad and Chet (1983). All species were maintained on PDA slants and were stored at 4°C till use.

Cyanobacteria samples were obtained from Phycology Laboratory, Botany Department, Faculty of Science, King Saud University, Saudi Arabia. These species were isolated from soils of different areas of Saudi Arabia according to standard isolation procedure (Rippka et al., 1979; Vaara et al., 1979). Each isolate was sub cultured in BG11 nutrition media for their cultivation and allowed to flourish at 20 to 30°C under constant light for 2 to 4 weeks.

Characterization of pathogen and antagonist agents

Based on microscopic studies, the pathogen was identified as *Fusarium oxysporum* on the basis of presence, shape and size of macro- and micro-conidia (Leslie and Summerel, 2006).

On the basis of culture characteristics and microscopic observations, the dominant rhizosphere fungi of tomato were, *Aspergillus flavus, A. niger, A. terreus, Alternaria alternata, Cladosporium cladosporioides, Penicillium citrinum*, *P. chrysogenum, Penicillium sp.* and *Trichoderma harzianum*.

In addition to botanical approach (light microscope morphology), cyanobacteria *Phormidium autumnale and Nostoc linckia* were identified by scanning electron microscopy.

In vitro screening of antagonist fungi against *F. oxysporum* f. sp. *lycopersici*

Antagonistic behavior of fungi was evaluated against *F. oxysporum* f. sp. *lycopersici in vitro* by dual culture techniques. 5 mm mycelial disc of FOL and antagonist fungi namely *A. niger, P. citrinum*, *Penicillium* sp. and *T. harzianum* were cut with the help of reverse side of sterilized tips of micropipette from the edge of 3 days old culture. One disc of each of all antagonists were placed on solidified PDA medium at one side of plates and one of FOL at opposite to antagonist. Plates were incubated at 28 ± 2°C. The radial growth of test pathogens in treated and control plates were recorded after 5 days of incubation and the per cent inhibition of mycelial growth of the pathogens was calculated by using following formula;

$$I = (C-T/C) \times 100$$

Where, I is the percent inhibition; C is the colony diameter in control plate and T is the colony diameter in treated plate.

Determination of cyanobacterial chlorophyll content and antifungal activity

Cell growth of *P. autumnale and N. linckia* was estimated by measuring cell dry weight and chlorophyll a content of 1 ml fresh cell pellets. Cells were harvested by centrifugation, washed twice with distilled water. After measuring chlorophyll content (Lichtenthaler and Buschmann, 2001) samples were dried to constant weight at 85°C for 12 h.

Cyanobacterial methanol extract was prepared by the method of Kim and Kim (2008). The antifungal activity was evaluated by measuring diameter of the inhibition zone formed around the well. FOL (1.0×10^6 spores/ml) were spread on PDA with the help of sterilized cotton swab. The extracts were placed in wells made on the pathogen inoculated agar plates. Wells containing only methanol served as the control. Plates were incubated for 3 days at 28 ± 2°C, and inhibition zones of mycelial growth around the wells were measured.

Determination of percent seed germination

Steam-sterilized sand was inoculated with the FOL spores (1.0×10^3 spores/g of soil mixture) before the seed were sown. Surface sterilized (0.1% sodium hypochlorite) tomato seeds (n = 5) were sown in each pot. Inoculum of *A. niger, P. citrinum*, *Penicillium* sp. and *T. harzianum* were prepared in the form of a conidial suspension (10^6 spores / ml) as described by Sivan et al. (1984). However, for cyanobacteria inoculation, 5 ml of fresh inoculum of *P. autumnale and N. linckia* were used. Tomato seedlings were raised in a glass house containing one of the antagonist microorganisms. Seedlings without any inoculation served as the control. Plants were watered regularly. After 14 days of sowing, percent seed germination was determined.

Determination of tomato plant growth and inhibition of *F. oxysporium* f. sp. *lycopersici* under pot conditions

Bioefficacy of the *A. niger, P. citrinum*, *T. harzianum, P. autumnale and N. linckia* was determined against phytopathogenic fungus under pot conditions.

For pot trial, method described by Perveen et al. (2007) with slight modifications was used. Pots of 9 cm diameter were surface sterilized with 1% sodium hypochlorite and were filled with 100 g of autoclaved peat moss soil and sand mixed in a ratio of 5:1. Single seedling of tomato plant grown in sterilized soil was transplanted in each pot.

Four plates of FOL culture grown on PDA was scarped and was mixed in sterilized distilled water to get the final cfu of 1.0×10^6 spores/ml. FOL (10 ml) was added around experimental plants after removing soil. The pure inocula [3% (v/v)] of *A. niger, P. citrinum*, *Penicillium* sp., *T. harzianum, P. autumnale and N. linckia* were mixed with pathogen infested soils. The pots containing the soil pathogen inoculum mixture without the antagonists served as the control. Pots were arranged in glass house on a rack and watered with sterile water as per requirement. Plants were observed daily and symptoms and growth were recorded for one month. Plants were uprooted after one month to record the height, fresh weight and disease index (Perveen et al., 2007). Chlorophyll content of the third leaf from the apex was estimated according to the method of Lichtenthaler and Buschmann (2001).

Statistical analysis

All experiments were performed in triplicate. Data were analyzed by least significant difference (L.S.D.) test at probability of 0.05 to

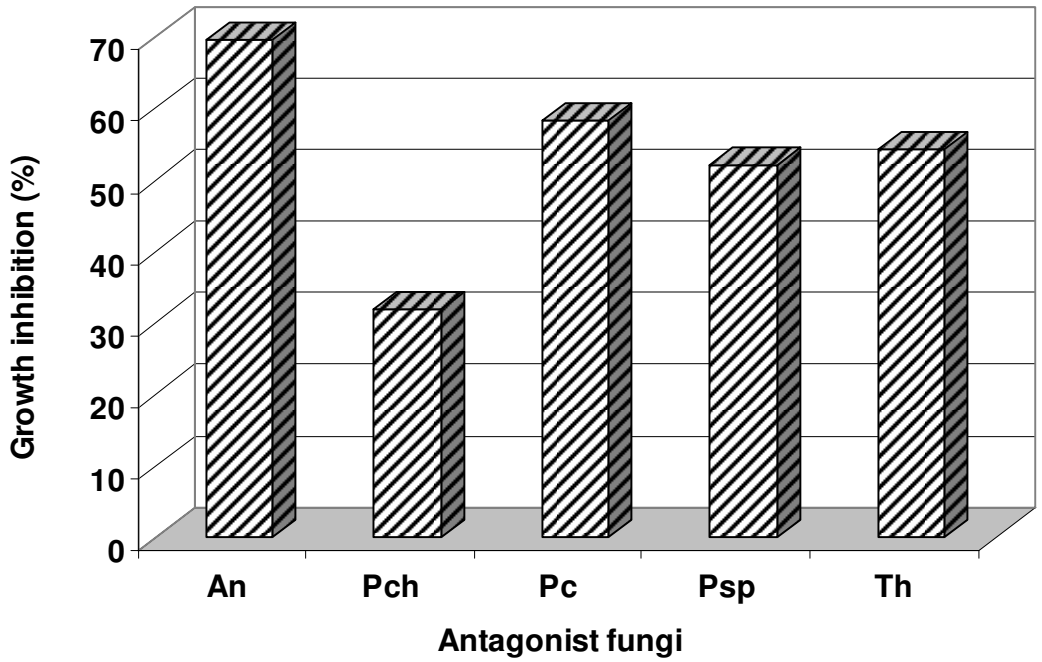

Figure 1. Growth inhibition of *F. oxysporum* f. sp. *lycopersici* by antagonist fungi under *in vitro* condition.
An = *A. niger,* Pch = *P. chrysogenum*, Pc = *P. citrinum, Psp = Penicillium* sp.*,* Th = *T. harzianum.*

Table 1. Chlorophyll content, dry weight and antifungal activity of cyanobacteria[a].

Cyanobacteria	Chlorophyll a (mg)	Dry weight (mg)	Antifungal activity (methanol extract)
N. linckia	2.42	36	++ (moderate)
P. autumnale	7.73	103	+ (minor)

[a]Each value is an average of three replicates.

identify significant effect of a treatment. Duncan Multiple Range Test was used to evaluate the significant differences between treatments ($P \leq 0.05$). ANOVA analysis was done with the SPSS statistics software.

RESULTS

All tested antagonist fungi inhibited the colony growth of *F. oxysporum* f. sp. *lycopersici* at varying degree (Figure 1). The maximum inhibition (70%) was recorded with *A. niger.* However, *P. citrinum, T. harzianum* and *Penicillium* sp. showed considerable inhibition of 58, 54 and 58% respectively against the tested pathogen. *P. chrysogenum* showed 32% inhibition of pathogen.

It was found that 1.0 ml pellets of *P. autumnale* and *N. linckia* contains 2.42 and 7.73 mg chlorophyll a respectively. The methanol extract of *N. linckia* showed moderate (++) control of FOL. While minor (+) inhibition was shown by methanol extract of *P. autumnale (*Table 1).

On the basis of *in vitro* screening results, the selected antagonists were further tested for their effect on seed germination. Maximum seed germination (93%) was observed in seeds treated with *N. linckia.* Furthermore, *T. harzianum, P. autumnale* and *P. citrinum*, showed 80% seed germination, whereas *A. niger* showed 50% seed germination (Figure 2).

From Table 2, it is evident that plants' height and their fresh and dry weight have increased in all treated plants except *P. autumnale* amended soil as compared to the FOL inoculated control. All treatment showed significant ($p \leq 0.05$) increase in plant height as compared to FOL inoculated control. Maximum plant height was observed in plants treated with with *A. niger* and *N. linckia* (33.7 cm) while plants treated with *T. harzianum and P. citrinum* had 31.6 cm height. Minimum height of 29.3 cm was observed in *P. autumnale* treated plants. The plant fresh weight was found to be significantly ($p \leq 0.05$) increased in all except *P. autumnale* treated plants as compared to FOL inoculated control. Maximum plant fresh weight was shown by *N. linckia* (2.07 g) treated plants, followed by *T. harzianum* (1.81 g), *A. niger* (1.78 g) and *P. citrinum* (1.68 g). Dry weights of plants

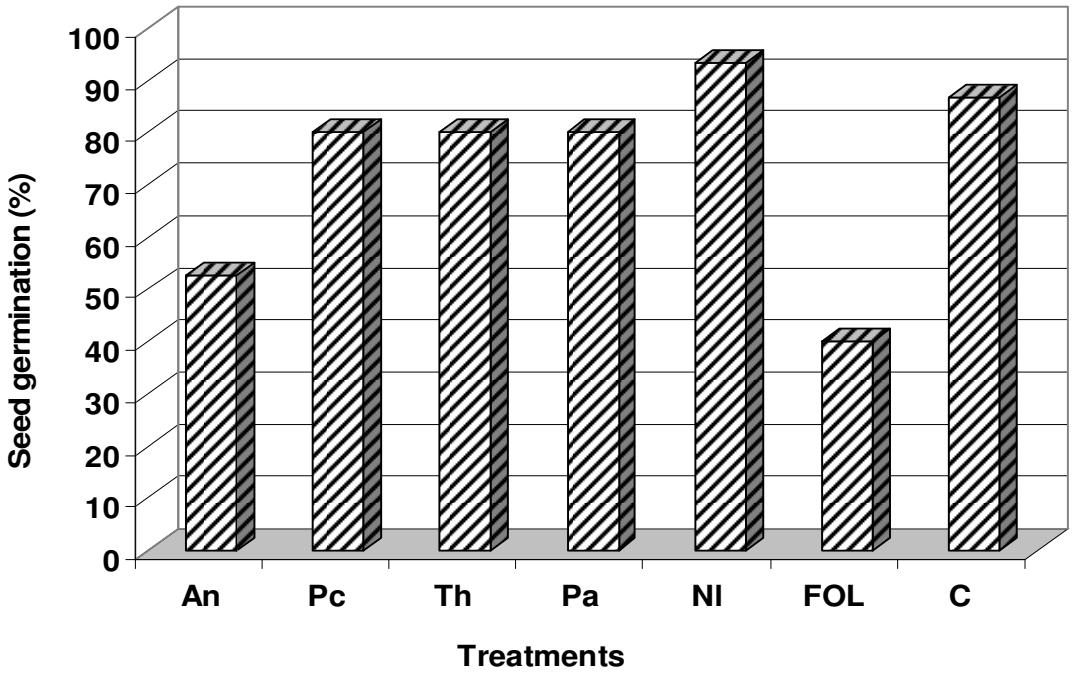

Figure 2. Effect of antagonists on seed germination. C = Uninoculated control, FOL = *F. oxysporum* f. sp. *lycopersici* only, An = *A. niger,* Pc = *P. citrinum,* Th = *T. harzianum,* Pa = *P. autumnale,* Nl = *N. linckia.*

Table 2. Influence of screened antagonists on the growth of tomato plant and wilt disease incidence under pot conditions[a].

Treatment	Plant height (cm)	Plant fresh weight (g)	Plant dry weight (g)	Chl a+b (µg/mg)	Disease incidence (%)
C	35.3^{x}	2.94^{y}	1.81^{v}	10.11^{x}	0.00
FOL	23.7^{y}	1.28^{z}	0.53^{w}	1.91^{y}	75.00^{z}
An + FOL	33.7^{x}	1.78^{x}	0.57^{x}	5.75^{x}	48.33^{x}
Pc+ FOL	31.6^{x}	1.68^{x}	0.59^{x}	5.38^{x}	56.67^{y}
Th+ FOL	31.6^{x}	1.81^{x}	0.71^{y}	8.00^{x}	41.67^{x}
Pa+ FOL	29.3^{x}	1.22^{z}	0.49^{w}	4.93^{y}	74.33^{z}
Nl+ FOL	33.7^{x}	2.07^{x}	0.87^{z}	7.71^{x}	50.00^{x}

[a]Each value is an average of three replicates. Data followed by different letters in the column are significantly different (P≤ 0.05) according to Duncan's multiple range test. C = Uninoculated control, FOL= *F. oxysporum* f. sp. *lycopersici,* An = *A. niger,* Pc = *P. citrinum,* Th = *T. harzianum,* Pa = *P. autumnale,* Nl = *N. linckia,* Chl = Chlorophyll.

treated with *N. linckia* and *T. harzianum* was increased significantly ($p \leq 0.05$) as compared to FOL inoculated control. However, rest of the treatment showed non significant increase in plant dry weight. Similarly, chlorophyll a+b has increased significantly ($p \leq 0.05$) in all treated plants as compared to FOL inoculated control. Maximum value was observed in *T. harzianum* treated plants (8.00 µg/mg) followed by *N. linckia* (7.71 µg/mg), *A. niger* (5.75 µg/mg), *P. citrinum* (5.38 µg/mg) and *P. autumnale* (4.93 µg/mg).

Under pot conditions, maximum control of the wilt disease was observed with *T. harzianum* (44.4%) as compared to FOL inoculated plant. Effectiveness of the other antagonists was recorded in the following order: *A. niger* (35.6%), *N. linckia* (33.3%), *P. citrinum* (24.4%). *P. autumnale* showed negligible wilt disease control (0.9%).

DISSCUSSION

Antagonistic activity of isolated fungi presented in Figure 1 showed varying degree of inhibition of *F. oxysporum* f. sp. *lycopersici* by *A. niger, P. chrysogenum, P. citrinum, T. harzianum* and *Penicillium* sp. The growth inhibition of

FOL by antagonist fungi is possibly attributed to the secretion of antibiotics by the fungi (Upadhyay and Rai, 1987) or other inhibitory substances produced by the antagonists such as geodin, terricin, terric acid, aspergillic acid, dermadin, etc. (Brian et al., 1945). The degree of effectiveness varies according to the nature, quality, and quantity of antibiotics/inhibitory substances secreted by the antagonists (Dennis and Webster, 1971a, b; Skidmore and Dickinson, 1976).

Antifungal activity of methanol extract of *P. autumnale* and *N. linckia* (Table 1) showed moderate (++) and minor (+) inhibition of FOL. Kim and Kim (2008) reported inhibition of *F. oxysporum* f. sp. *lycopersici* by extracts of *N. commune* FA-103. Similarly, several reports have shown that the extracts of *Nostoc* species significantly inhibited the growth of phytopathogenic fungi (Biondi et al., 2004; Zulpa et al., 2006; Tassara et al., 2008). Cyanobacteria produce biologically active compounds that have antifungal activity (Kulik, 1995; Schlegel et al., 1998) and antibiotic and toxic activity against plant pathogens (Bonjouklian et al., 1991; Kiviranta et al., 2006).

Result presented in Figure 2 indicates that in general all antagonist microorganisms tested has increased the percent seed germination. Barring *P. autumnale,* plant growth was increased by all antagonists and also was able to decrease the wilt disease incidence as compared to FOL inoculated control under pot conditions (Table 2). The results showed that the reduction of disease severity was associated with an increase of the vegetal growth including the plant height as well as the plant fresh and dry weights.

N. linckia reduced disease incidence by 33.3% however, *P. autumnale* was unable to control wilt. Several scientists reported that cyanobacteria produce exopolysaccharides that can function as energy source for fungi and also produce plant growth regulators, such as abscisic acid, ethylene, jasmonic acid, auxin, and cytokinin-like substances, the cytokinin isopentenyl adenine (Caire et al., 1993; Fish and Codd, 1994; Stratmann et al., 1994; Borowitzka 1995).

A. niger, P. citrinum, T. harzianum have almost similar effect on wilt severity and increase in plant growth (Table 2). Whipps and Mc Quilken (1993) reported that *A. niger, A. terreus, G. virens, P. citrinum, T. harzianum* and species of *Bacillus* control soil-borne diseases. Bashar and Rai (1994) observed that *A. flavus, A. niger,* and *T. viride* amended in soil suppressed the growth of *F. oxysporum* f. sp. *ciceri* and exhibited strong fungistatic activity against germination of conidia of test pathogen. Mondal et al. (2000) reported that two metabolites (2-carboxymethyl 3-n-hexyl maleic acid and 2-methylene-3-hexylbutanedioic acid) isolated from *A. niger* AN27, had increased germination, shoot length, root length and biomass of cauliflower seedlings.

It is well known that *Trichoderma* can parasitize fungal pathogens and produce antibiotics, besides the fungus have many positive effects on plants: increased growth and yield, increased nutrient uptake, increased fertilizer utilization efficiency, increased percentage and rate of seed germination and induced systemic resistance to plant diseases (Harman et al., 2004). Recently, Akrami et al. (2011) found that three isolates (*T. harzianum*, *T. asperellum*, *T. virens*) were effective against *Fusarium* rot of lentil.

Ahmed (2011) studied the growth promoting ability of *T. koningii* and a white sterile fungus (which did not fructify) on tomato plants grown in soil inoculated with wilt pathogen *F. oxysporum* f. sp. *lycopersici*. He reported that antagonistic rhizosphere PGPF suppressed the deleterious soil microbes by competing at the active sites, reduced the intensity of disease development and subsequently, stimulated the growth/yield of plants.

The present study indicates that *N. linkia, T. harzianum*, *A. niger* and *P. citrinum* can be explored further for the biological control of wilt disease of tomato which may help to obtain a higher yield and good health in agriculture.

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