Occurrence of Fusarium wilt on summer squash caused by *Fusarium oxysporum* in Assiut, Egypt

A. F. Mahmoud[*]

Plant Pathology Department, Faculty of Agriculture, Assiut University, 71526 Assiut, Egypt

Abstract

Fusarium wilt of summer squash was first noticed in April and May 2012 in Assiut-Egypt. Symptoms included wilting of the foliage, withering of older leaves and eventually died during growth. The causal pathogen was identified as *Fusarium oxysporum* Schlecht. Pathogenicity tests of the obtained isolates were demonstrated via artificial inoculation, and it satisfied Koch's postulates. Because summer squash is of great economic importance in Egypt, biological control of the disease was carried out by the biocontrol agent *Gliocladium catenulatum*. The effect of *G. catenulatum* against *F. oxysporum* was investigated *in vitro* and tested on summer squash plants under greenhouse conditions. *G. catenulatum* isolates were able to inhibit the growth of *F. oxysporum* in dual culture. In greenhouse, application of *G. catenulatum* was tested at three different times. All treatments were effective to reduce disease severity. Application of *G. catenulatum* preceding the inoculation with *F. oxysporum* reduced significantly the incidence of Fusarium wilt compared to plants inoculated with *F. oxysporum* alone. The disease suppression was occurred when *G. catenulatum* applied five days before infested soil by *F. oxysporum*. Obtained results indicate that *G. catenulatum* is very effective biocontrol agents offer potential benefit in Fusarium wilt of squash, and should be harnessed for further biocontrol applications. To our knowledge, this is the first report of *F. oxysporum* causing wilt of summer squash in Assiut, Egypt.

Key words: biological control, Fusarium wilt, *Fusarium oxysporum, Gliocladium catenulatum,* summer squash.

[*] **Corresponding author:** A. F. Mahmoud,
Tel.: +201273883747, Fax: +20882331384
E-mail: amer.mahmoud@agr.au.edu.eg

Introduction

Cucurbitaceae is one of the most favorable vegetable for human nutrition. Cucurbit crops constitute a major portion of all vegetables and are grown in different regions in Egypt. Summer squash (*Cucurbita pepo* L.) is one of the most important vegetable crops which can be readily produced at a low cost during the winter season of Egypt. Several fungi have been identified that infects squash worldwide. Among those, Fusarium wilt is an important infection limiting both longevity and productivity of the plants. It is known to occur worldwide and has been described as a plant pathogen causing wilt, yellowing, and death of plants (Farr & Rossman, 2015; Lee and Park, 2001). Mingshan et al., 2002 reported that *Fusarium oxysporum* f.sp. *niveum* is the causal organism of wilt disease of some cucurbitaceae plants. *Fusarium oxysporum* Schlechtend is a cosmopolitan soilborne plant pathogen that causes vascular wilt and cortical rot diseases of more than 100 agricultural crops of economic importance (Both, 1971). The Cucurbitaceae plant family is affected by several vascular wilt diseases caused by different forma specialis of the fungus *Fusarium oxysporum*, which are morphologically similar, but generally host-specific (Egel & Martyn, 2007). The pathogen persists indefinitely in infested soil (Martyn, 1996). Optimal conditions for development of Fusarium wilt occurs when soil temperatures are between 25-27°C and there is low soil moisture (Kleczewski & Egel, 2011). If temperatures exceed 33°C, *Fusarium* will not develop (Sitterly, 1972). Control of wilt diseases depends mainly on fungicides which are expensive, can cause environmental pollution and may cause the selection of pathogen resistance (Lumsden & Locke, 1989; Minton, 1986). Antagonistic microorganisms are able to suppress growth and development of phytopathogenic fungi (Phillipy, 1988). *Gliocladium catenulatum* is an effective biocontrol against several root and foliar greenhouse pathogens. The biocontrol agent forms dense networks of hyphae on plant roots, grows internally in root epidermal cells and produces hydrolytic enzymes, all of which lead to a reduction in pathogen propagules (Chatterton & Punja, 2010). It was reported to suppress Fusarium root and stem rot caused by *Fusarium oxysporum* f. sp. *radicis-cucumerinum* on cucumber plants grown hydroponically in rockwool medium. Densities of the biocontrol agent appeared to increase in the presence of the pathogen (Chatterton et al., 2008). Also, it has been shown to be effective in reducing pre- and post-emergence damping-off caused by *P. ultimum* in pansy and snapdragon and post-emergence damping-off caused by *R. solani* in salvia (McQuilken et al., 2001). In addition, *G. catenulatum* was successfully controlled Fusarium root and stem rot on cucumber plants, resulting in disease levels that were not significantly different from fungicide-treated plants (Rose et al., 2003). Antibiotic production by biocontrol fungi has most commonly been reported for isolates of *Trichoderma* and *Gliocladium* (Whipps, 2001). An endophytic *Gliocladium sp*. has also been found to produce volatile organic compounds (VOC) that have strong antimicrobial

activity against soilborne fungi (Strobel, 2006). The volatile organic compound annulene was produced in the greatest amount by *Gliocladium sp*. (Stinson et al., 2003). *G. catenulatum* (*C. rosea* f. *catenulata*) have shown mycoparasitic activity against several plant pathogenic fungi *in vitro* (McQuilken et al., 2001). Microscopic observations showed that the biocontrol agent destroyed hyphal cells of *S. sclerotiorum* and *Fusarium spp*. through direct contact, resulting in collapse and disintegration of the host cells without visible penetration (Huang, 1978). Hyphae of *G. catenulatum* were observed to coil loosely around hyphae of *P. ultimum* and *R. solani*, causing partial destruction (McQuilken et al., 2001). Enzymatic hydrolysis is believed to be involved in the penetration and dissolution of pathogen cell walls by *G. catenulatum* (Lahdenpera & Korteniemi, 2005). The aim of this work was to identify the causal pathogen of summer squash wilt disease. The biological control of such disease was also studied.

Materials and methods

Isolation and purification of the causal pathogen: Samples of summer squash vegetation showing typical Fusarium wilt symptoms were collected from several fields in Assiut governorate- Egypt in April and May 2012. The infected tissues were surface disinfected with 2% sodium hypochlorite solution for 2 min, rinsed twice in sterile distilled water, dried between sterile filter paper. Then infected tissues were cultured on Potato Dextrose Agar medium (PDA) amended with streptomycin sulphate (120mg L^{-1}) and incubated for three days at 25°C in the dark. Pure cultures of the developing fungi were obtained using single hyphal-tip isolation technique (Dhingra and Sinclair, 1995). The pure cultures were kept at 5°C in the refrigerator for further studies. Hyphae were examined under scanning electron microscope (SEM) to determine the morphological characteristics of the fungus (using Jeol JSM 5400 LV- scanning microscope, Japan), at the electron microscopy unit, Assiut University- Egypt.

Pathogenicity tests: Pathogenicity tests were carried out at greenhouse of Plant Pathology Department, Faculty of Agriculture, Assiut University. Pathogenic capabilities of eight isolates of *Fusarium oxysporum* were tested on Eskandrani squash cultivar. Inocula of the tested isolates were prepared by growing in sterilized conical flasks (500 ml) containing barley medium (100g barley supplemented with 2g glucose + 1g yeast extract + 100 ml distilled water) and incubated at 25±1°C for 15 days. Sterilized pots (35 cm in diameter) were filled with sterilized sandy-loam soil which mixed thoroughly with equal amounts of *F. oxysporum* inoculum at the ratio of 1% soil weight, mixed well and thoroughly irrigated. Soil infestation was carried out three days before sowing seeds. Each pot was planted with ten squash, surface disinfected, seeds (cv. Eskandrani). Pots containing 1% non-infested barley medium were used as control. Four pots were used as replicates. The prevailing temperatures under greenhouse conditions during pathogenicity tests were 20±2°C (minimum) and 26±2°C (maximum). The plants were irrigated when necessary and daily observed for infection.

Disease severity assessment: Disease severity was estimated after 8 weeks post-sowing, as a wilting percentage, using the rating scale in which infected plants were classified. Plants with typical Fusarium wilt symptoms were assessed according to the type of symptoms that was observed using a numerical grades ranging from 0 to 5 as follows:

(0)= No symptoms; (1)= 1-<20 % of plant leaves are yellow, and of discoloration of the vascular system; (2)= 20-<40 % of plant leaves are yellow, and of discoloration of the vascular system; (3)= 40-<60 % of plant leaves are yellow, and of discoloration of the vascular system; (4)= 60-<80 % of plant leaves are yellow, and of discoloration of the vascular system; (5)= 80-≤100 % of plant leaves are yellow, and of discoloration of the vascular system. The number of dead and healthy plants was also recorded.

Disease severity (%) =

$$\frac{\sum\left[(N\times0)+(N\times1)+......(N\times5)\right]}{5T}\times100$$

Where: (N) = the number of plants corresponding to the numerical grade, 0, 1, 2, 3, 4 and 5. (5T) = the total number of plants (T) multiplied by maximum numerical grade (5).

Isolation of bioagent: *Gliocladium catenulatum* was isolated from the soil and rhizosphere of healthy squash plants. Isolation was carried out by serial dilution technique on Potato Dextrose Agar (PDA) medium amended with streptomycin sulphate (120mg L-1). PDA culture plates were incubated at 25±2ºC. Obtained isolates, which belong to *Gliocladium* spp. were purified by single

spore technique according to Brown, 1924 and kept at 5°C for further studies.

Efficacy of *Gliocladium catenulatum* against *Fusarium oxysporum in vitro*: Antagonistic capability of seven isolates of *G. catenulatum* was tested against *F. oxysporum* (no. 3) *in vitro*. Dual culture technique was followed; mycelial disks 5 mm in diameter were cut from the edges of actively growing colonies of *F. oxysporum* and *G. catenulatum* isolates, and were placed opposite each other, 1 cm from the edge of 9 cm Petri dishes containing PDA medium, amended with streptomycin sulphate (120mg L^{-1}). Petri dishes inoculated with *F. oxysporum* alone served as control. Each pair was replicated four times and incubated for five days at 25±2°C in darkness, then colony diameter of *F. oxysporum* was measured using ruler. The inhibition percent in mycelial growth of *F. oxysporum* was calculated using the formula as follow:

$$\text{Inhibition (\%)} = \frac{A - B}{A} \times 100$$

Where: A = Fungal growth of control; B = Fungal growth of treatment.

Efficacy of *G. catenulatum* on controlling *Fusarium* wilt of squash under greenhouse conditions: *G. catenulatum* isolate (no. 6) that produced the highest antagonistic effect against *F. oxysporum in vitro* was selected to investigate its ability to reduce the incidence of Fusarium wilt in squash plants under greenhouse conditions. Inoculums of *F. oxysporum* (no. 3) and *G. catenulatum* were prepared using Potato Dextrose Broth (PDB) amended with streptomycin sulphate (120mg L^{-1})

and incubated for 20 days at 25±2°C in the dark. Inoculums were harvested by passing the liquid culture through double layer cheesecloth, and adjusted to $5x10^5$ conidia mL^{-1}. Sterilized pots (35 cm in diameter) were filled with sterilized sandy-loam soil and arranged in complete randomized design. Drench of 50 ml suspension ($5x10^5$ spore mL^{-1}) of *G. catenulatum* was applied to each pot. Whereas, drench of 100 ml suspension ($5x10^5$ conidia ml^{-1}) of *F. oxysporum* was applied to each pot. Applications of *G. catenulatum* were carried out via three methods: (1) inoculation with *G. catenulatum* at 1, 2, 3, 4 and 5 days before inoculation with *F. oxysporum;* (2) inoculation with *G. catenulatum* at the same time of inoculation with *F. oxysporum;* (3) inoculation with *G. catenulatum* at 1, 2, 3, 4 and 5 days after inoculation with *F. oxysporum.* In all cases, inoculums of *G. catenulatum* or *F. oxysporum* were mixed thoroughly with the soil, then watered and left to insure establishment and distribution of the inoculums in soil. Ten sterilized squash seeds (cv. Eskandrani) were sown into each pot one day after soil infestation with *G. catenulatum* or *F. oxysporum.* Positive controls were inoculated similarly with *F. oxysporum* only, while negative controls were treated with distilled water. There were four replicates pots per treatment. The experiment was repeated twice. The prevailing temperatures during the experiment were 20±2°C (minimum) and 22±2°C (maximum). The plants were irrigated when necessary and daily observed for infection. Disease severity was estimated after 8 weeks post-sowing, as described previously in pathogenicity tests. The percentage of reduction in the incidence of Fusarium wilt disease was calculated as follow:

$$\text{Reduction (\%)} = \frac{A - B}{A} \times 100$$

Where: A= disease incidence of positive control; B= disease incidence of treatment.

Results

Characterization of disease symptoms: The first symptom usually noticed in the field is wilting of the leaves. Wilting is followed by a yellowing of the leaves and finally necrosis, necrotic lesions may be present in older parts of the plant that are near the soil line or just below the crown of the plant. The wilting generally starts with the older leaves and progresses to the younger foliage (Figure 1A). Initial symptoms often occur as the plant is beginning to vine, and wilting may occur in only one runner leaving while the rest of the plant apparently unaffected (Figure 1B). Within several days, the entire plant may wilt and die (Figure 1C). Affected plants that do not die are often stunted and have considerably reduced yields; in older plants disease is most severe at the fruit set stage (Figure 1D). Under high inoculum pressure, seedlings may damp-off as they emerge from the soil. The primary diagnostic symptom of Fusarium wilt is a discoloration of the vascular system (xylem), which can be observed readily in longitudinal or cross section of roots or stems.
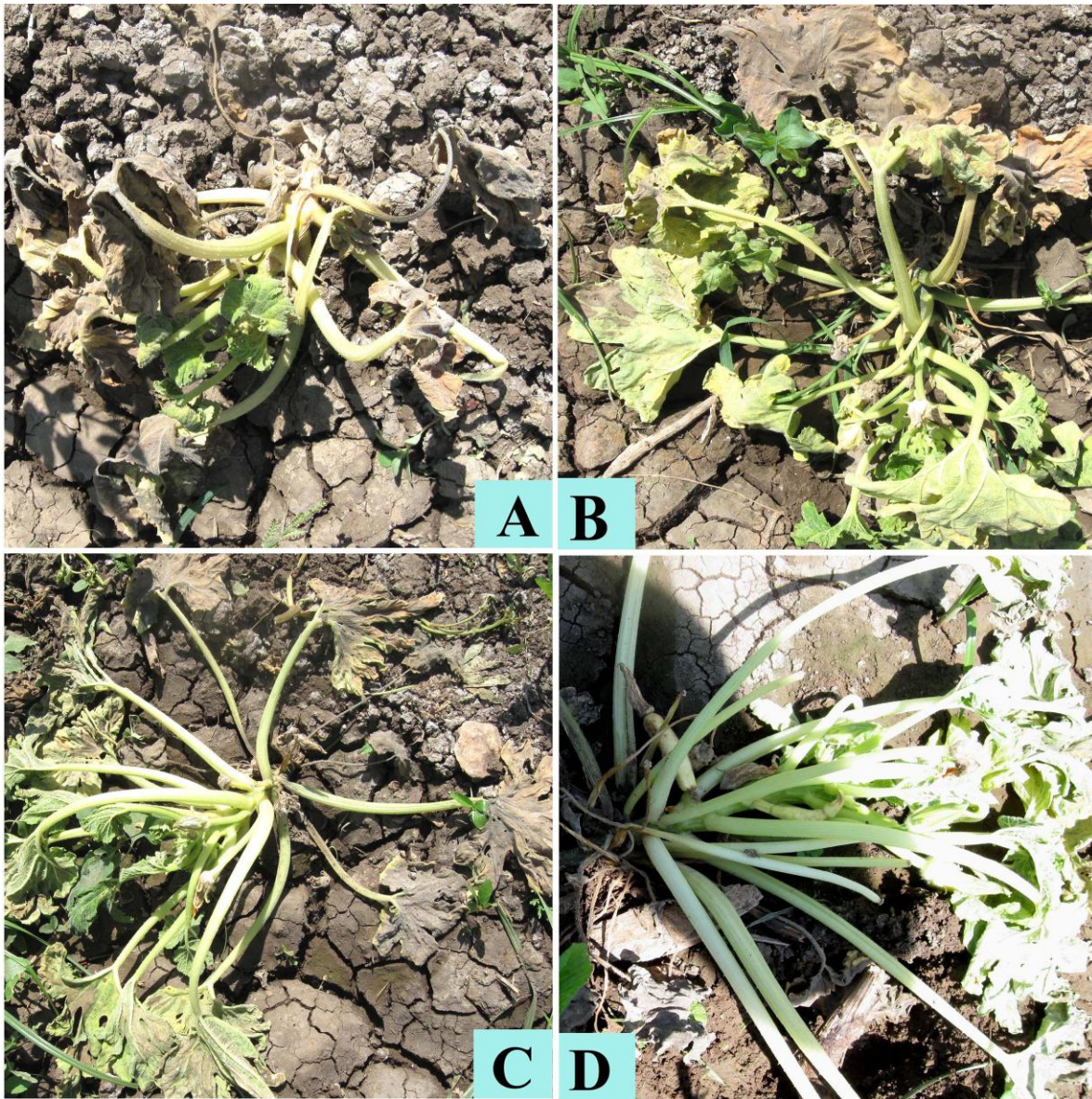
Figure 1: Fusarium wilt caused by *F. oxysporum* on summer squash. The wilting was starts with the older leaves and progresses to the younger foliage (A), wilting was occur in only one runner leaving while the rest of the plant apparently unaffected (B). Within several days, the entire plant was wilt and die (C), and plants that do not die were often stunted and have considerably reduced yields (D).

Identification of the causal pathogen: Eight isolates were obtained from vascular tissue of the infected summer squash plants. In culture, colonies growing rapidly, 4.5-5 cm in 4 days, aerial mycelium white, becoming purple, with discrete orange sporodochia (Figure 2A). Conidiophores unbranched, the monophialides bearing microconidia are short (Figure 2B). Macroconidia are fusiform, slightly curved, pointed at the tip, mostly three septate, basal cells pedicellate, 23-54 x 3-5µm. Microconidia are abundant, never in chains, formed in false-heads on short monophialides on the hyphae and were hyaline, smooth, oval to ellipsoidal, aseptate, one-septate, or very rarely two-

septate, slightly constricted at the septa, 4-11 x 2.2-3.5µm (Figure 2C). Chlamydospores are terminal or intercalary, hyaline, smooth or rough-walled, usually produced singly or in pairs (Figure 2D). It is distinguished easily from *F. solani* by the shorter phialides in the aerial mycelium. Based on the morphological and culture characteristics, the fungus was identified as *Fusarium oxysporum* according to Snyder and Hansen, 1940; Both, 1971; Armstrong and Armstrong, 1978; Gerlach and Nirenberg, 1982; Nelson et al., 1983; Kleczewski and Egel, 2011.
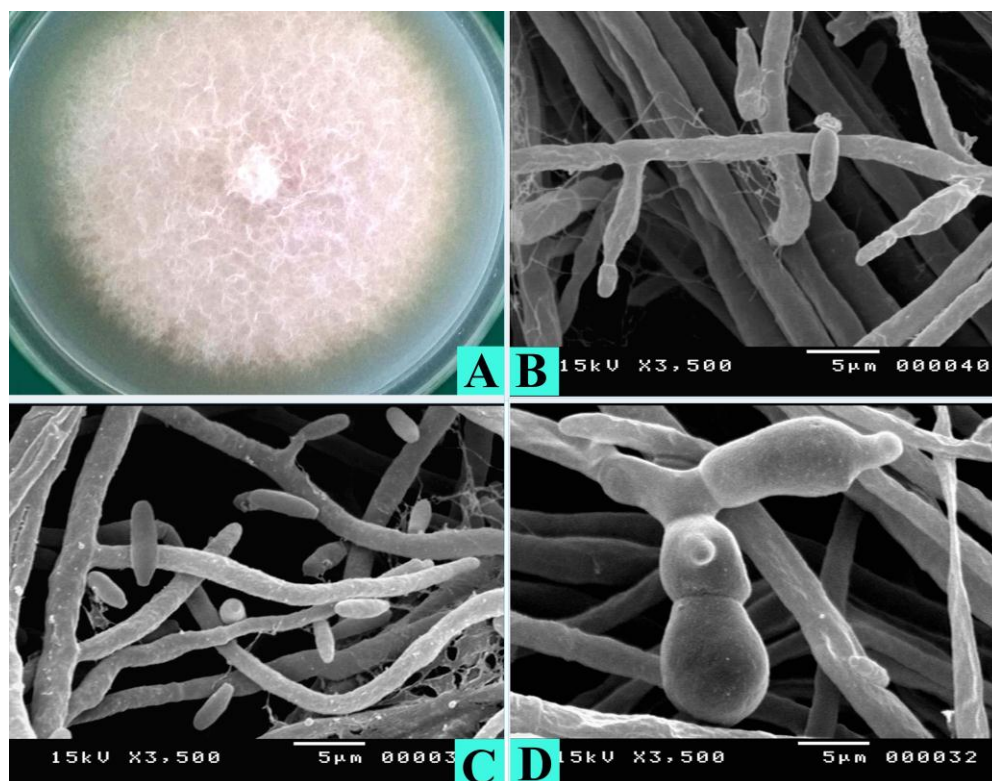

Figure 2: Colony of *F. oxysporum* on PDA for 6 days in darkness (A), scanning electron microscope (sem) image of short monophialides bearing microconidia (B), (sem) image of microconidia (C), and (sem) image of chlamydospores (D).

Pathogenicity tests: Data in Table (1) indicate that, all tested isolates are able to infect summer squash plants producing symptoms of Fusarium wilt disease. These symptoms were identical to those originally observed in the field. Isolates of *F. oxysporum* varied significantly in their virulence; isolate (no. 3) proved to be the most aggressive one and showed the highest percentage of disease severity followed by isolate (no. 7 and 2). Whereas, *F. oxysporum* isolate (no. 1 and 6) produce the lowest percentage of disease severity. No symptoms were observed in the control plants. Other *F. oxysporum* isolates were varied significantly in their pathogenic capability. According to these results isolate (no. 3) was selected for using in following study. To satisfy the requirements of Koch's postulates, *F. oxysporum* was reisolated from

inoculated squash plants from the greenhouse, and their identities confirmed as described before.

Table 1: Pathogenicity tests of *F. oxysporum* isolates on squash (cv. Eskandrani) under greenhouse conditions.

Isolates No	Disease severity (%)*
F. ox.1	61.25 E
F. ox.2	74.25 AB
F. ox.3	78.75 A
F. ox.4	67.00 CD
F. ox.5	69.50 BCD
F. ox.6	65.00 DE
F. ox.7	77.50 A
F. ox.8	69.75 BCD
Control	0.0 F

*Means within the same column followed by different letters are significantly different (P≤0.05) based on LSD.

Identification of bioagent: Seven isolates were identified as *Gliocladium catenulatum* Gilman & Abbott (syn. *Clonostachys rosea* f. *catenulata* Gilman & Abbott), based on their morphological characteristics of mycelia and conidiophores on Czapek's agar medium according to Gilman and Abbott, 1927; Schroers et al., 1999; Schroers, 2001.

Efficacy of *Gliocladium catenulatum* against *Fusarium oxysporum in vitro*: *In vitro* assay was carried out by dual culture technique. Antagonism was measured as reduction in pathogen colony diameter. Antagonistic capabilities of seven isolates of *G. catenulatum* were tested against the highly pathogenic isolate of *F. oxysporum* (no. 3). Data presented in Table (2) indicated that, all tested isolates

of *G. catenulatum* were able to inhibit the growth of *F. oxysporum*. The tested antagonistic isolates were varied in their antagonistic effect; *G. catenulatum* isolate (no. 6) showed the highest antagonistic effect towards *F. oxysporum* on dual culture. Moreover, *G. catenulatum* isolate (no. 1) significantly reduced the growth of *F. oxysporum* in culture with the inhibition rate of 64.44%; followed by isolate (no. 5) with the inhibition rate of 63.33%. On the other hand, the other isolates of *G. catenulatum* gave a moderate antifungal effect on the mycelium growth of *F. oxysporum* with inhibition rate from 58.33 to 60.27%.

Efficacy of *G. catenulatum* on controlling Fusarium wilt of squash under greenhouse conditions: Based on effectiveness of *G. catenulatum* in dual culture, isolate (no. 6) was investigated for its ability to reduce the incidence of Fusarium wilt disease of squash plants under greenhouse conditions. Obtained results are summarized in Table (3), and indicated that, application of *G. catenulatum* preceding the inoculation with *F. oxysporum* reduced significantly the incidence of Fusarium wilt compared to plants inoculated with *F. oxysporum* alone. Highest suppression of Fusarium wilt was occurred when *G. catenulatum* applied five days before infection by *F. oxysporum*. Applications of *G. catenulatum,* (50 ml; 5×10^5 spore mL^{-1}; to each pot) reduced significantly the incidence of Fusarium wilt on squash plants in all treatments. Obtained results declared that, applications of *G. catenulatum* five days before *F. oxysporum* reduced the incidence of Fusarium wilt on squash plants at the rate

of 83.75%. On the other hand, suppression of Fusarium wilt was significantly occurred when *G. catenulatum* applied three and four days before the pathogen. Furthermore; *G. catenulatum* has been shown to reduce the incidence of Fusarium wilt when applied at the same time with *F. oxysporum*. Moreover, the reduction of Fusarium wilt was observed when even the bioagent applied after, one and two days, following infested soil with *F. oxysporum*. There was no significant effect of *G. catenulatum* when applied at, four and five days, after application of the pathogen. Over all, application of antagonistic fungi before infested soil with *F. oxysporum* gave the highest reduction of disease severity compared with the other times of application.

Table 2: Effect of *G. catenulatum* on colony diameter of *F. oxysporum* in dual culture.

Treatments	Colony diameter of *F. oxysporum* (cm)*	Inhibition of *F. oxysporum* growth (%)
F. ox.+ G. catenulatum-1	3.20 C	64.44
F. ox.+ G. catenulatum-2	3.57 B	60.27
F. ox.+ G. catenulatum-3	3.70 B	58.88
F. ox.+ G. catenulatum-4	3.75 B	58.33
F. ox.+ G. catenulatum-5	3.30 C	63.33
F. ox.+ G. catenulatum-6	2.80 D	68.88
F. ox.+ G. catenulatum-7	3.65 B	59.44
Control	9.0 A	0.0

*Means within the same column followed by different letters are significantly different (P≤0.05) based on LSD.

Discussion

The results of the current study clearly demonstrate that *Fusarium oxysporum* Shlechtend is the causal pathogen of Fusarium wilt of summer squash in Assiut, Egypt. All the obtained isolates of *F. oxysporum* are pathogenic and cause severe wilt in squash plants in pathogenicity tests. *F. oxysporum* induced Fusarium wilt on summer squash has been reported worldwide, such as in Mexico, Poland, and Greece (Farr & Rossman, 2015). To our knowledge, this is the first report of *F. oxysporum* causing Fusarium wilt on summer squash in Assiut, Egypt. Because summer squash is of great economic importance in Egypt, disease management was tried, *in vitro* and under greenhouse conditions. The results of biological control study showed that *Gliocladium catenulatum* provided protection to squash plants against Fusarium wilt.

Table 3: Efficacy of *G. catenulatum* on controlling Fusarium wilt of squash under greenhouse conditions.

Treatments, and application times of *G. catenulatum*	Fusarium wilt severity (%)*	Reduction (%)
F. ox. + *G. catenulatum* (1 day, before *F. ox.*)	30.75 G	61.56
F. ox. + *G. catenulatum* (2 days, before *F. ox.*)	29.50 G	63.12
F. ox. + *G. catenulatum* (3 days, before *F. ox.*)	19.25 H	75.93
F. ox. + *G. catenulatum* (4 days, before *F. ox.*)	17.50 H	78.12
F. ox. + *G. catenulatum* (5 days, before *F. ox.*)	13.00 I	83.75
F. ox. + *G. catenulatum* (at the same time)	33.00 F	58.75
F. ox. + *G. catenulatum* (1 day, after *F. ox.*)	36.25 E	54.68
F. ox. + *G. catenulatum* (2 days, after *F. ox.*)	41.50 D	48.12
F. ox. + *G. catenulatum* (3 days, after *F. ox.*)	44.50 C	44.37
F. ox. + *G. catenulatum* (4 days, after *F. ox.*)	52.00 B	35.00
F. ox. + *G. catenulatum* (5 days, after *F. ox.*)	52.50 B	34.37
F. ox. alone (positive control)	80.00 A	0.0
Uninfected control	0.0 J	100

*Means within the same column followed by different letters are significantly different (P≤0.05) based on LSD.

In greenhouse, application of *G. catenulatum* preceding the inoculation with *F. oxysporum* reduced significantly the incidence of Fusarium wilt compared to plants inoculated with *F. oxysporum* alone. The disease suppression was occurred when *G. catenulatum* applied five days before soil infestation by *F. oxysporum.* In previous studies, pathogen challenge occurred within 24 hours to 3 days after *G. catenulatum* was applied (Rose et al., 2003; McQuilken et al., 2001). The mechanisms of action of *G. catenulatum* involved in disease suppression may be due to the ability of the bioagent to be rhizosphere competent and can endophytically colonize roots as well as stems of squash plants. Chatterton et al., 2008 found that colonization of *G. catenulatum* in the root zone reduced pathogen development and disease incidence. An endophytic *Gliocladium sp*. has also been found to produce volatile organic compounds (VOC) that have strong antimicrobial activity (Strobel, 2006). And that are inhibitory against soilborne fungi. The volatile organic compound annulene was produced in the greatest amount by *Gliocladium sp* (Stinson et al., 2003). Microscopic observations showed that the biocontrol agent destroyed hyphal cells of *Fusarium spp*. through direct contact, resulting in collapse and disintegration of the host cells without visible penetration (Huang, 1978). Hyphae of *G. catenulatum* were observed to coil loosely around hyphae of *P. ultimum* and *R. solani*, causing partial destruction (McQuilken et al., 2001). In conclusion, the results indicate that *G. catenulatum* will be most effective when applied early in the growing season, and it will be more advantageous in greenhouse crops.

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