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Biological control of *Fusarium oxysporum* f.sp. *radicis-cucumerinum*, the casual agent of root and stem rot of *Cucumis sativus* by non-pathogenic *Fusarium oxysporum*

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The aim of this study was to evaluate the ability of non-pathogenic *F. oxysporum* isolates from suppressive soil in Sri Lanka to control Fusarium root and stem rot of *Cucumis sativus* L. (cucumber) under greenhouse conditions. Three non-pathogenic *F. oxysporum* isolates were included in the study. *Fusarium* isolates were first checked *in vitro* for pathogenic and antagonistic potential. In greenhouse evaluations, the non-pathogenic *F. oxysporum* isolates were introduced to root system just before the seedlings were replanted in pathogen infested soil. The plants were evaluated for disease development after three weeks. Results of the greenhouse pot experiments revealed that, two non-pathogenic *F. oxysporum* isolates significantly reduced Fusarium root and stem rot incidence. However, combining the non-pathogenic fungal isolates did not enhance the plant protection. These data provide evidence that the non-pathogenic isolates have potential to protect cucumber plants from Fusarium-rot. The most appropriate mechanism of action behind the disease suppression in this system would be suppression of saprophytic growth of the pathogen and depriving a niche rather than direct antagonism.

Key words: Cucumber, biological control, non-pathogenic Fusarium, *F. oxysporum*

1. Introduction

Fusarium root and stem rot is regarded as one of the most devastating diseases in cucurbits affecting cultivations in many countries around the world (Pavlou & Vakalounakis, 2005). Since late 19's, root and stem rot caused by *Fusarium oxysporum* f. sp. *cucumerinum* has become a threat to cucumber cultivations particularly in southern parts of Sri Lanka. *Cucumis sativus* L. (cucumber) is one of the most important vegetable crops in Sri Lanka and local varieties are susceptible to Fusarium diseases, causing significant crop loss annually. The fungus survives as chlamydospores, which come into contact with roots, will germinate to infect the root systems. After infection, the pathogen will colonize the vascular system leading to wilting, and eventually, plant mortality occurs. Once the pathogen is established, it survives in the fields for several years thereby affecting future production of the crop. The pathogen causes initially stem and root rot lesions leading to wilting by colonizing the plant vascular system and eventually resulting in seedling or mature plant mortality.

Chemical control methods which are effective against Fusarium root and stem rot of cucurbits are limited. Therefore, control strategies focus mostly on preventing the pathogen from being introduced into disease-free areas, and the development of disease resistant varieties (Lemanceau and Alabouvette, 1993). Biological control offers potential alternatives to combat many soil-borne pathogens (Spadaro and Gullino, 2005). Several reports have previously demonstrated the successful use of biological control agents against Fusarium diseases of various crops. Non-pathogenic strain of *F. oxysporum* Fo47 controls Fusarium wilt of carnation (Lemanceau et al., 1992), tomato and melon (Alabouvette, et al., 1993), Asperagus (Blok et al., 1997), and Fusarium wilt of flax (Duijff et al., 1999). Several biocontrol bacteria, including *Pseudomonas* spp., *Serratia marcescens*, *Bacillus* sp., *Streptomyces* sp. have been used to control Fusarium wilt diseases (Scher and Baker, 1982). Moreover, use of *Trichoderma* spp. on banana (Thangavelu et al., 2003), arbuscular mycorrhiza (AM) on banana (Jaizme-vega et al., 1998) and soil amendment of Lettuce on cucumber (Pavlou and Vakalounakis, 2005) have also been reported. Several workers have used combinations of microorganisms in biological control experiments (Lemanceau & Alabouvette, 1993). However, according to our knowledge, any reports in relation to use of non-pathogenic *F. oxysporum* isolates for disease suppression of Fusarium root and stem rot are not yet reported in Sri Lanka. Therefore, the main objective of this research was to test the performance of non-pathogenic *F. oxysporum* to suppress Fusarium root and stem rot of cucumber under greenhouse conditions in Sri Lanka.

2. Materials and Methods

2.1. Isolation of *F. oxysporum* isolates from diseased and healthy plants

Pathogenic *F. oxysporum* isolates were isolated from the rhizosphere of *C. sativus* roots of diseased plants and non-pathogenic isolates were isolated from Fusarium root and stem rot suppressive fields located in Southern part (around Angunukolapalasse) of Sri Lanka. In order to isolate the causal agent of stem and root lesions of wilted *C. sativus* plants, thin sections of stem lesions at the soil line were taken and surface sterilized with 1% NaOCl for one minute, and then washed twice in sterilized distilled water (SDW). Small sections from the plant tissue were placed on antibiotic amended PCNB (in Booth, 1977) plates and incubated at 28℃. Agar plugs containing actively growing mycelium were transferred to potato dextrose agar (PDA) plates. Then the single spore cultures were obtained from these isolates. After following Koch's postulates, only pathogenic *F. oxysporum* isolates were selected and preserved under mineral oil. In order to isolate non-pathogenic *F. oxysporum* isolates, segments of the root system with adhering rhizosphere soil from healthy *Cucumis* plants were used and fungi were isolated on PCNB medium. To avoid the chance of isolation of pathogenic *F. oxysporum* isolates of other crops, infectivity assay was performed on other selected crops grown nearby cucumber fields.

2.2. Plant materials and growth conditions

Seeds of pathogen-free local variety *C. sativus* (Cucumber) were obtained from the Seed Certification Centre, Department of Agriculture, Sri Lanka. Plants were grown in plastic pots with 15 cm diameter containing 1.5 kg of non-sterile soil (1:1:1 w/w sand;clay;compost), and 50 mL of 25% Hoagland's solution was supplied 5 days after sowing and the second application was given 3 days after replanting in pots. Ten day old plants were used for all pot experiments. All the pot experiments were conducted in the glasshouse in the Department of Botany, University of Ruhuna. Plants were watered daily just to satisfy the field capacity but not in excess to drain off freely.

2.3. Pathogenicity tests

The pathogenicity of four *F. oxysporum* isolates, obtained from diseased and healthy *C. sativus* plants were tested. Commercial potato dextrose agar (PDA) was used to cultivate *F. oxysporum* isolates. First they were grown on half strength PDA plates for seven days at 28°Cunder cool-white and near-ultraviolet fluorescent light. Agar plugs containing actively growing mycelial tips were transferred to 500 mL Erlenmayer flasks containing 250 mL PDA liquid broth and the flasks were incubated in an orbital shaker at 28°Cfor 5 days. Then the culture was passed through cheesecloth to separate the mycelium from the spores and the final concentration of the spore suspension was adjusted to 10^8 spores/mL. Ten day old cucumber plants were inoculated by dipping gently washed root system in the spore suspension for 5 min. Inoculated seedlings were replanted in pots and observed for disease symptoms. Three seedlings per pot were planted and four pots were used for each isolate. Pathogen was reisolated from randomly selected diseased plants, the Koch's postulates were followed in order to identify the pathogenic *F. oxysporum* isolate(s).

2.4. In vitro plate assay for evaluation of antagonistic activity

In order to test non-antagonistic fungal activity against pathogenic isolate, agar plugs containing both categories of fungal isolates were placed on the PDA plates about 5 cm apart from each other and assessed for about 10 days whether any inhibition zone was appeared or not. Three replicate plates were used for each fungal isolate.

2.5. Preparation of nonpathogenic fungal inoculum for glasshouse pot experiments

The *F. oxysporum* isolates, which were identified as non-pathogenic isolates, from the *C. sativus* rhizosphere, were grown in liquid cultures as mentioned above. The conidial suspension was filtered through cheesecloth and diluted to a final concentration of 10^8 conidia mL^{-1}. Ten days old cucumber plants were inoculated by dipping the root system in the spore suspension for 10 min prior to transplant in pots containing pathogenic *F. oxysporum* infested soil.

2.6. Evaluation of Fusarium stem and root rot suppression in glasshouse trials

A pathogenic isolate of *F. oxysporum* f. sp. *cucumerinum* was grown on PDA as previously mentioned. In order to prepare soil inoculum, agar plugs containing actively growing mycelium tips were used to inoculate slightly pressed sterilized corn seeds mixed with pigeonpea (*Cadjanus cadjan*) seed powder in 500 mL Erlenmeyer flasks. This mixture was incubated at room temperature for 10 days. Tests were carried out to establish the relationship between inoculum density and disease severity. Disease severity (DS) was assessed as number of plants affected as described in a visual disease scale 0 - 2, where 0 = no symptoms, 1 = vascular disease symptoms in the stem, root and stem rot, wilt or without wilt, 2 = dead or almost dead (Pavlou and Vakalounakis, 2005). In these experiments 20 g of infested corn seeds were added to 500 g of soil. The inoculated soil was mixed and dispended in 15 cm diameter plastic pots. Ten day old cucumber plants were uprooted with care, and adhering soil clumps were removed by gently shaking, and dipped the root system in a conidia suspension (10^8 conidia/mL) of nonpathogenic *F. oxysporum* for 10 min prior to replant in pots. Three plants were accommodated in one pot, and four replicates were included for each treatment, and the entire experiment was repeated twice. The experimental design was a Randomized Complete Block Design (RCBD). The number of diseased plants were recorded. Cucumber plants were classified as healthy when disease symptoms were absent. Disease incidence (DI) (%) was estimated as the percentage of infected cucumber plants.

2.7. Statistical analysis

The data were analyzed by ANOVA using SPSS version 10.0 statistical software (SPSS, SAS Institute USA) to evaluate differences in disease incidence and disease severity. Before analyses were carried out, data on percentage of disease incidence (DI) were arcsine-transformed. Differences between treatments were determined by Fisher's LSD test at 5% significance level.

3. Results

3.1. Characterization of *F. oxysporum* isolates

On the basis of morphological characters, four isolates originating from cucumber plants grown in Southern part of Sri Lanka were identified as *F. oxysporum* (Booth, 1977). As only a single cultivar of cucumber is grown in this area, race determination of these isolates was not done. In pathogenicity tests it was found that only one isolate was pathogenic. This isolate was designated as *F. oxysporum* f. sp. *cucumerinum* SLS04. This isolate was not pathogenic on melon, pumpkin, and other tested cucurbitaceae crops grown in the same area.

Taking into account the symptoms observed in cucumber plants, it was confirmed that the one of the isolates was *F. oxysporum* f. sp. *cucumerinum*. Rest of the *F. oxysporum* isolates were not pathogenic either on *C. sativus* or other cucurbits, they were identified as nonpathogenic isolates (Table 1). All fungal isolates produced abundant oval shaped microconidia, abundant sickle-shaped macroconidia

Table 1 Description of *F. oxysporum*isolated from Fusarium root and stem rot suppressive soils in cucumber fields and infected cucumber plants.

F. oxysporum isolates	Colony characters	Pathogenicity
FOA1	Fast growth, White	Non-pathogenic
FOA2	Fast growth, Pale Yellow	Non-pathogenic
FOA3	Moderate growth, White	Non-pathogenic
SLS04	Fast growth, White	Pathogenic

Table 2 Effects of non-pathogenic Fusarium isolates on Fusarium root and stem rot disease severity of cucumber in greenhouse experiments.

Treatment[1]	Disease severity[2]	
	Trial I	Trial II
Uninoculated control	0 a[3]	0 a
FORC only	1.8 b	1.9 b
FORC+FOA1 only	0.9 c	0.8 c
FORC+FOA2	0.8 c	0.8 c
FORC+FOA3	1.8 b	1.7 b

[1] FORC = *F. oxysporum* f. sp. *cucumrinum*; FOA = non-pathogenic isolates of *F. oxysporum*;
[2] Disease severity was assessed with 0-2 visual scale, in which 0 = no symptoms, 1 = vascular discoloration in the stem, root and stem rot, wilt, 2 = dead or almost dead plants.
[3] Figures followed by the same letter within a column are not significantly different according to Fisher's LSD test ($P < 0.05$).

and chlamydospores. All isolates produced white aerial mycelium except FOA2. *F. oxysporum* FOA2 was slightly yellow to tan when aged. No inhibition zones were observed between the non-pathogenic and pathogenic isolates of *F. oxysporum* when tested in plate assay.

3.2. Evaluation of Fusarium wilt suppression in the greenhouse pot experiments

Among the three non-pathogenic isolates of *F. oxysporum* evaluated for potential biological control ability, only two isolates significantly ($P<0.05$) reduced severity of Fusarium root and stem rot (Table 2). In glasshouse trials, disease incidence (DI) % was also significantly low ($P<0.05$) in non-pathogenic *F. oxysporum* FOA1 and FOA2 inoculated plants. However, no further protection was observed in combined use of non-pathogenic fungal isolates (Figure 1).

4. Discussion

F. oxysporum population of the study area was found to be dominated by non-pathogenic isolates and this was in agreement with other reports (Nel et al., 2006).

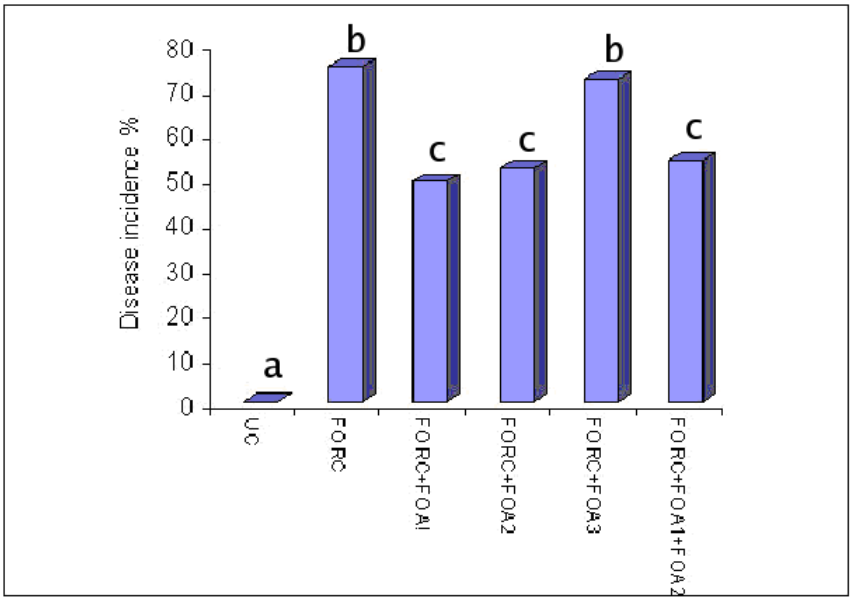

Figure 1 Disease incidence % showing with different treatments, bars with a common letter do not differ significantly at $P < 0.05$.

Previous studies on other Fusarium wilt diseases have found that the population of saprophytic *Fusarium oxysporum* is more diverse (Edel et al., 1995). It also reflects from this study that the four isolates identified as *F. oxysporum* showed some morphological and culture differences (Table 1). Molecular study with more isolates collected from throughout the Island will reveal the real population structure of the *F. oxysporum* in Sri Lanka.

Two non-pathogenic *F. oxysporum* isolates, which were morphologically different, significantly reduced the Fusarium root and stem rot incidence of cucumber in glasshouse trials. The number of non-pathogenic isolates, obtained in this study was rather low compared to the previous reports (Nel et al., 2006). This could be related to different edaphic factors and/or due to the crop species. However, Nel et al., (2006) have reported that only two isolates, out of 24 isolates of non-pathogenic *F. oxysporum* isolates were able to significantly reduce the Fusarium wilt of banana indicating that finding good biological control agents is a rear incident.

The results of the current study also provided evidence for the ability of selected non-pathogenic *F. oxysporum* to suppress Fusarium rot and stem rot. Previous reports showed that the combination of *F. oxysporum* Fo47 and *Pseudomonas putida* increased disease suppression by either antagonists alone (Park et al., 1988). Moreover, Rose and Parker (2003) reported that Fusarium root and stem rot of cucumber could be controlled by combination of biocontrol agents and chemical treatments and they have found that addition of crab/shrimp shells significantly enhanced seedling growth and decreased the disease severity indicating some other mechanism(s) was involved in that system. Therefore, it would be worthwhile to extend this research in combination with rhizobacteria as well.

At the same time possibility of induction of induced systemic resistance (ISR) by non-pathogenic fungal isolates can not be ruled out. Research is being focused to understand the mechanism of action of non-pathogenic *F. oxysporum* isolates to protect cucumber plants from the pathogen.

In conclusion, the current study provides evidence that suppression of Fusarium root and stem rot of cucumber caused by *F. oxysporum* f. sp. *cucumerinum* can be controlled by using selected non-pathogenic *F. oxysporum* isolates.

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