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Antagonistic Activity of *Pseudomonas Fluorescens* Against *Fusarium Oxysporum* f. sp. *Nievum* Isolated from Soil Samples in Palestine

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Abstract

Watermelon is an important summer crop in Palestine, for several decades filling the needs of local market and some Arab countries. The yield of watermelon decreased dramatically in recent years due to severe infections with the soil borne fungal pathogen *Fusarium oxysporum f. sp. niveum* (FON). Soil fumigation with methyl bromide was commonly applied by Palestinian farmers until it was recently legally banned. Different control mechanisms were not feasible to overcome problems caused by the disease resulting in decreased watermelon cultivation in Palestine for the past 30 years. In this work, we have experimentally shown that *Pseudomonas fluorescens* was efficient in controlling FON infection and allowing normal seedling growth of both the root and shoot systems. Field experiments are necessary to further confirm the efficacy of biocontrol application.

Keywords: Biological Control, Watermelon, Fungi, Bacteria

1. Introduction

Watermelon (*Citrullus lanatus* L.) is an economically important vegetable crop in many parts of the world. In Palestine, it is one of the most important summer fruit crops. According to FAOSTAT (2014), the watermelon cultivated area was estimated to be 356 ha during the period from 2001-2008 with an average yield of 13,777 tons. In the following years, the watermelon area decreased by more than 65% and the total annual production was reduced by more than 75% (FAOSTAT, 2014). A number of reasons caused the dramatic decline in watermelon cultivation, of which Fusarium Wilts caused by formae speciales of *Fusarium oxysporum* that are generally considered host specific (Martyn, 2012; Ren et al., 2015; Meyer et al., 2016). *Fusarium oxysporum* f. sp. *niveum* (FON) is one of the most severe diseases in watermelon and a major limiting factor for watermelon production in the world (Martyn & McLanghlin, 1983; Peng et al., 2013). Due to high infestation rates of the disease in different regions in Palestine, farmers have abandoned growing watermelon. The use of fungicides is not always feasible due to economic and ecological reasons.

Currently, no effective fungicides or chemical disinfectants are available because FON can generate thick-walled chlamydospores that are highly resistant to soil fumigation (Besri, 2008; Peng et al., 2013). FON can survive in soil as saprophyte for many years (Notz, Maurhofer, Dubach, Haas, & Defago, 2002). Therefore, watermelons can only be replanted in locations with infected soil, after FON has been eradicated by preplan treatments with soil fumigants. However, the most effective soil fumigant, methyl bromide, has been phased out, and FON can transform itself into thick-walled chlamydospores, highly resistant to chemical fumigation (Lin, Chen, Liou, Huang & Chang, 2009), leaving hardly any means to control the soilborne FON safely, economically and effectively (Ren et al., 2015).

With increasing public awareness of the environmental implications of the extensive use of fungicides in agricultural practices, alternative strategies for the control of plant disease are being sought (Weller, 1988; Ellis et al., 1999). Biological control using antagonistic microorganisms alone, or as supplements to minimize the use

of chemical pesticides in a system of integrated plant disease management, has become more important in recent years (Hwang, 1993; Mao, Lewis, Hebber, & Lumsden, 1997).

Soilborne, non-pathogenic fast growing bacteria that are capable of antagonizing fungal phytopathogens might represent first choice biocontrol method. They show great promise with respect to protecting plant roots from fungal-induced diseases (O'Sullivan & Ogara, 1992; Walsh, Morrissey, & O'Gara, 2001). These bacteria are known by several generic names, including biological control agents (BCAs), plant growth promoting rhizobacteria (PGPR) and biopesticides (Walsh et al., 2001). The aim of this study was to test the activity of *P. fluorescens* isolate against the watermelon fungal pathogen *F. oxysporum* f. sp. *nievum* (FON).

2. Methods

2.1 Isolation of FON Plant Pathogens

Soil and plant samples naturally infested with FON were used for the isolation of pathogenic strains of the fungus. Isolation from soil samples was conducted using the soil dilution plate technique modified from Nishimura (2007). One gram of soil was suspended in 99 ml of 0.05% water agar. Serial dilutions (10^{-3}, 10^{-4} and 10^{-5}) were prepared and 100 µl from each solution were spread on selective medium for FON isolation; selective medium was prepared as described by Leslie and Summerell (2006).

For isolation of the pathogen from plant materials and debris, samples were washed thoroughly under running tap water, blotted to remove excess water, cut into 0.5 cm pieces that were placed on the selective medium.

2.2 Selection of Biological Control Agents (BCAs) to FON

Bacteria were isolated from healthy and infected watermelon plants as well as from infested and non-infested soil samples. Bacterial extracts were suspended in 0.085% NaCl, diluted serially and 100 µl were spread on Nutrient Agar media supplemented with 50 µg ml^{-1} cyclohexamide.

Four-day old cultures of FON grown on 7-mm diameter disks were placed centrally on potato dextrose agar (PDA) plates were used as targets for bacterial mediated antagonism. Bacterial suspensions (~ 5 x 10^9 cfu ml^{-1}) in 20 µl of 24 hrs old broth cultures were placed on 0.5 cm sterile paper disks. The assembled test cultures were incubated for 48 hrs at 22 ℃ and inhibition of mycelial growth was noted. Bacteria that inhibit fungal growth were selected for further greenhouse studies. Identification of *P. fluorescens* was done as mentioned in Barghouthi (2010).

2.3 Pathogenicity of Bacteria to Host Plant (Wtermelon)

In order to test whether the selected bacteria were pathogenic to host plants, 2-3 true leaf stage plants (grown in peat moss vermiculite mix 2:1, v/v) were treated by root drench with different concentrations of bacterial suspensions (10^7-10^9 cfu ml^{-1}). Control was done by treatment of seedlings with sterile water. Seedlings were incubated in climate rooms of 12 hrs day length and day-night temperature of 26 ℃ and 16 ℃. Plant height, fresh and dry weights of root and shoot, and damage were assessed and recorded after the plant reached a stage of development of 4-5 true leaves (Montealegre et al., 2003).

The pathogenicity of bacterium was tested on watermelon seeds as well. Seeds were immersed in bacterial suspensions (10^7-10^9 cfu ml^{-1} in 2% methylcellulose, pH 7.0) for 60 seconds (Abuamsha, Salman, & Ehlers, 2011). Seeds were sown in peat moss vermiculite mix (2:1, v/v) at the above mentioned conditions. Measurements of time of emergence, heights and fresh and dry weights of shoots and roots were recorded when the seedling reached the 2-3 true leaf stage (Montealegre et al., 2003).

2.4 Molecular Identification of Isolated Fusarium Spp

Isolated fungal strains were identified using PCR according to the method of Z. Zhang, J. Zhang, Wang, and Zheng (2005). Specific primers for FON Fn-1 (5'-TACCACTTGTTGCCTCGGC-3') and Fn-2 (5'-TTGAGGAACGCGAATTAAC-3') were used to produce a 327 bp PCR product. PCR was performed in 25 µl reaction mixtures containing 1 µl genomic DNA, 0.5 µM primers, a 0.5-µl mixture that contained 50 mM of each dNTP, 2.5 µl 10 PCR buffer, 2 mM Mg^{2+}, 2.5 µl 1% BSA, 0.25 µl Tw-20, and 1.25 U of Taq DNA polymerase. Amplification was performed using PCR System DNA thermal cycler programmed for one cycle at 94 ℃ for 5 min, followed by 35 cycles at 94 ℃ for 30 s, 54 ℃ for 30 s, and 72 ℃ for 30 s. A final 7-min extension at 72 ℃ was conducted. PCR products were separated on 1.5% agarose (in TAE buffer) for 60 min at 70V and visualized after staining with ethidium bromide (0.2 µg) under UV transilluminator.

2.5 In Vitro Inhibition of Fungal Growth

The antagonistic activity of bacteria against FON was determined using the dual culture technique (Salman,

2010). Each bacterial strain was streaked on the center line of a PDA plate and incubated for 24 hrs at 28 ℃. Then two disks of FON grown for 4 days on PDA medium were placed about 3 cm apart from the bacterial streak. Cultures were then incubated at 22 ℃ for 48 hrs. Control experiments used sterile distilled water instead of bacteria. The distance between the bacterial streaks was recorded and the effect of the bacteria was determined by measuring the inhibition zone of mycelial growth.

2.6 Greenhouse Pot Experiments

Watermelon (*Citrullus lanatus* (Thunb)) was used as the target test plant species. Seed treatment with the bacteria was done as mentioned above. For infesting soil with *F. oxysporum* sp. *niveum*, pots (9 cm diameter; 450 cm^3) were 66% filled with peat moss vermiculite mix (2:1, v/v). Small agar pieces (1/16 of an agar plate; 3 (7 mm diameter) discs from plates grown with FON (4-days old) were placed on the soil surface of each pot. Subsequently, the inoculum of each pot was then covered with a top layer of soil. The pots with infested soil and control-non-infested soil were watered and incubated for 3-days under greenhouse conditions as mentioned above, before sowing five watermelon seeds per pot (Vogt & Buchenauer, 1997). Disease development was recorded by examining a cross section of water melon stems after three weeks of sowing the seeds. During experimental period, all plants were fertilized weekly with commercial N:P:K (8:8:6) fertilizer.

2.7 Statistical Analysis

All experiments were done in triplicates and repeated three times. Statistical analysis was done using XlStat (Adinosoft). Significant differences were computed using ANOVA after Tukeys HSD test at $P< 0.05$.

3. Results

Six FON isolates (F161, F162, F163, F164, F241, and F243) were obtained from infected watermelon plants. All six isolates were shown to be FON based on PCR using *F. oxysporum* specific primers that produced a 327bp PCR amplicon (Fig. 1).

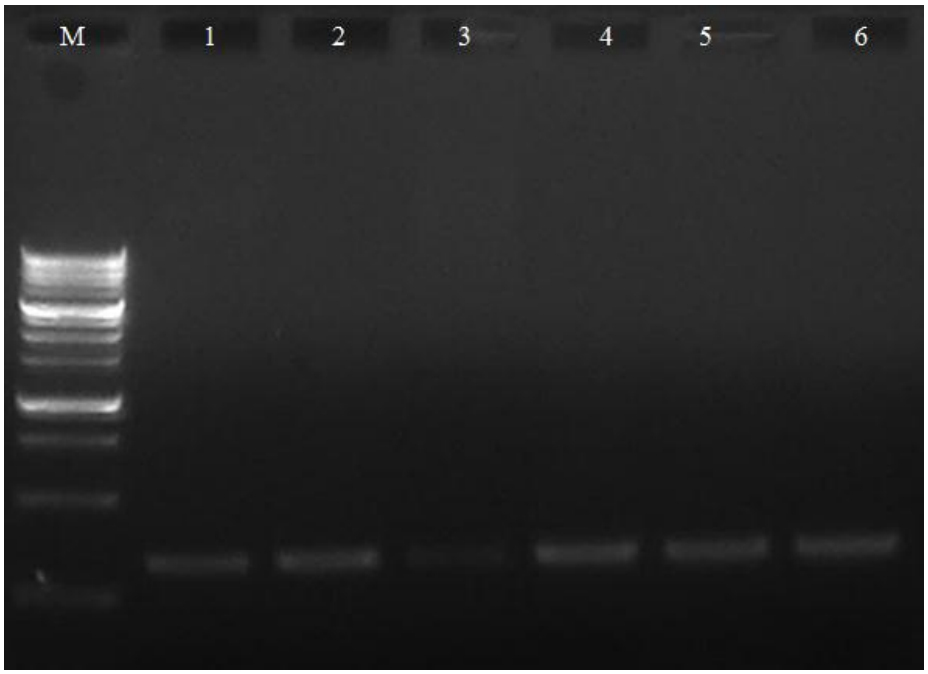

Figure 1. PCR products of 6 FON isolates F161 (lane 1), F162 (lane 2), F163 (lane 3), F164 (lane 4), F241 (lane 5) and F243 (lane 6) after amplification with FON specific primers Fn-1 and Fn-2. PCR products were separated on 1.5% agarose gel in 1X TAE buffer at 80 V for 1 h. Lane M, 100bp marker.

All fungal isolates were detected in reinfected watermelon seedlings. However, only isolate F161 showed browning symptoms in cross sections of the seedlings (Fig. 2). The isolate was used for further studies to evaluate the efficacy of bacteria against the pathogen. Inhibition zones on PDA against FON isolates were obvious in the presence of *P. fluorescens* (Fig. 3). A large inhibition zone (8 mm) in the presence of the bacteria was significantly higher ($P < 0.05$) against FON isolate F161 (Table 1). The lowest inhibition zone was recorded against FON isolate F162.

Table 1. In vitro antagonistic effects of *P. fluorescens* isolates against six FON isolates on PDA medium.

FON isolate	Inhibition distance (mm)	Average diameter of FON (mm)
F161	8 [a]	9.7 [d]
F163	5.4 [b]	11.5 [c]
F241	5.4 [b]	13 [ab]
F243	4.1b [c]	11.6 [bc]
F164	3.6 [c]	13.1 [ab]
F162	2.9 [c]	12.4 [bc]
FON	0 [d]	14.2 [a]

Data with different letters in the same column are significantly different after ANOVA at $P < 0.05$ using Tukeys HSD test.

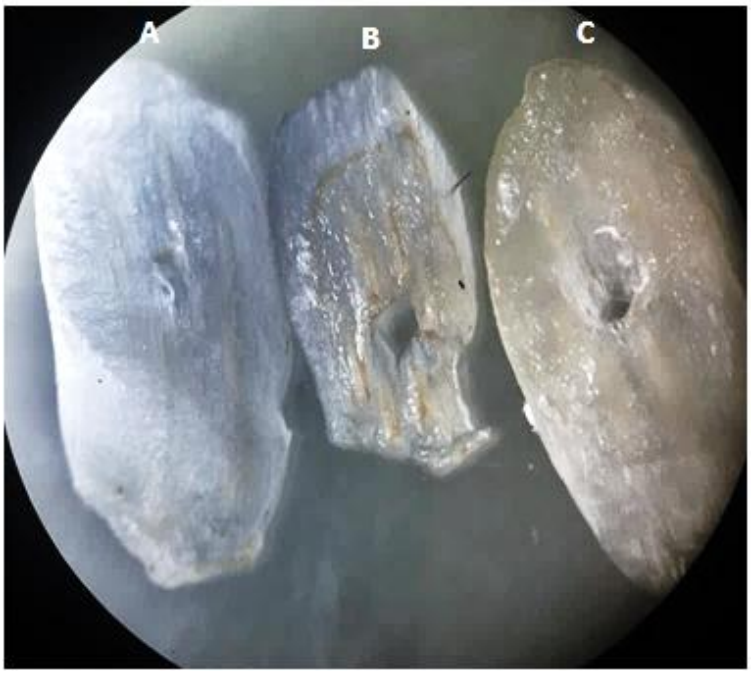

Figure 2. Stem Cross section showing vascular browning of watermelon stem (A) Control; (B) Infected with the FON isolate F161 and treated with of *P. fluorescens* and (C) Infected with FON isolate F161. Browning of tissue was due to fungal growth; indicating tissue infection.

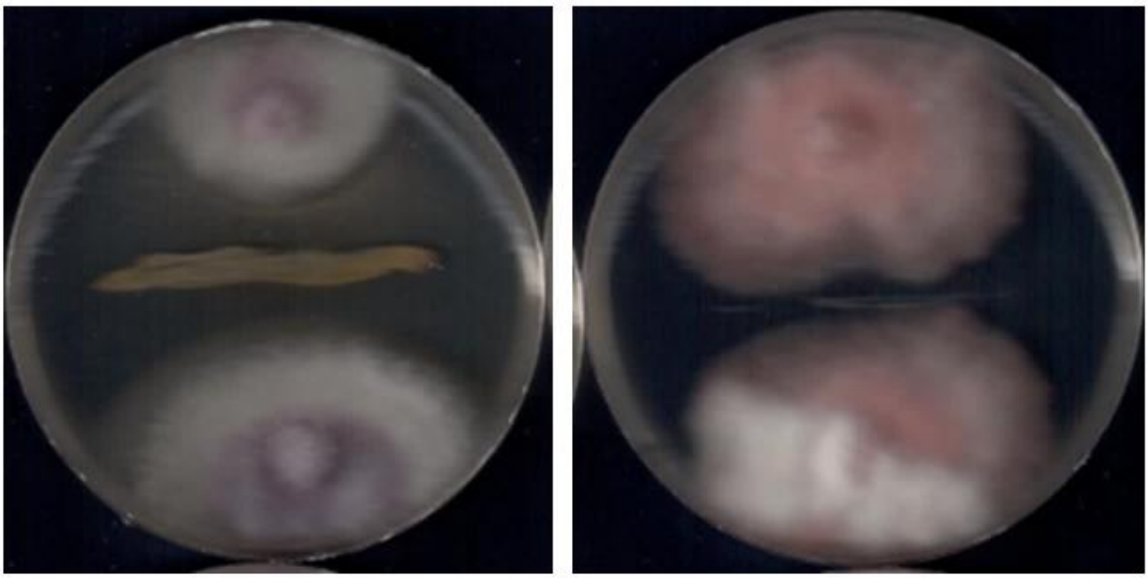

Figure 3. Inhibition caused by *P. fluorescens* against FON isolate 161 (left) on PDA medium.

Under pot experiments, watermelon seedling grown in the presence of the bacteria showed higher growth rates compared to control seedlings or seedlings infected with the pathogen (Fig. 4). Growth promotion effect of the bacteria was measured and the results proved the bacteria could enhance both fresh and dry weights of the plant (Table 2). Shoot fresh and dry weights of watermelon seedlings treated with the bacteria (5.112±0.159 and 1.03±0.105, respectively) were significantly higher ($P < 0.05$) than that in the control seedlings (3.40±0.448 and 0.52 ±0.116, respectively). Seedlings Infected with FON showed significantly lower ($P < 0.05$) fresh shoot and root dry weights (1.59±0.209 and 0.31±0.068, respectively). The same observations were recorded for dry shoot and root weights (Table 2).

Figure 4. Seedlings of watermelon three weeks after sowing, (A) infected with FON isolate F161 (B) infected with isolate F161 and treated with *P. fluorescens* (C) treated only with *P. fluorescens* and (D) negative control, not treated. Notice that B, C, and D are well developed relative to A which is infected but not treated.

Table 2. Fresh and dry weight of water melon seedling three weeks after sowing growth promotion effect of *P. fluorescens* on watermelon.

	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)
Control	3.40±0.448 [b]	0.20±0.047 [cd]	0.52±0.116 [bc]	0.03±0.006 [ab]
P. fluorescens	5.112±0.159 [a]	0.35±0.024 [ab]	1.03±0.105 [a]	0.05±0.012 [a]
F161	1.59±0.209 [c]	0.08±0.009 [d]	0.31±0.068 [c]	0.01±0.008 [b]
F161+*P. fluorescens*	4.28±1.0138 [ab]	0.25±0.041 [bc]	0.64 ±0.140 [b]	0.03±0.017 [ab]

Data with different letters in the same column are significantly different after ANOVA at $P < 0.05$ using Tukeys HSD test.

4. Discussion

In this study, it was found that some bacterial isolates were capable of preventing the growth of *F. oxysporum* f. sp. *niveum* under *in vitro* conditions and in pot experiments, respectively. Reports on using bacteria as biocontrol agents against plant pathogens and pests have increased in recent decades. In this work, screening bacterial isolates for anti- *F. oxysporum* activity; 167 bacterial isolates were screened as described in methods section for anti *F. oxysporum* activity. The biological control approach of plant pathogens and diseases has been studied for many years, and the introduction of beneficial microorganisms into soil or the rhizosphere has been proposed for the biological control of soilborne crop diseases (Joffe, 1986; Burge, 1988; Cook, 1993). Many studies have been conducted on the application of antagonistic microbes, such as Pseudomonas spp., for the control of Fusarium wilt (Tu & Chang, 1983; Duijff et al., 1999).

The ability of *P. fluorescens* to control the growth of *F. oxysporum* pathogenic isolates as represented by F161 was demonstrated *in vitro* assay and confirmed by pot experiments. The biocontrol activity of *P. fluorescens* observed in vitro experiments were reproduced in pot experiments.

P. fluorescens has shown great potential to controlling fungal infection. In addition, a significant ($P < 0.05$) enhancement of the growth of control seedlings treated with the bacterium relative to untreated control indicates that in addition to controlling infection, *P. fluorescens* somehow stimulated seedling growth either by provision or solubilization of nutrients and/or further protection of plants against an invisible plant pathogen.

Seedling wet or dry weight produced consistent results that were significantly different for each applied combination; poor growth of infected seedlings, enhanced growth of *F. oxysporum* infected or non-infected seedlings receiving *P. fluorescens* treatment.

Wet or dry weight of shoot and root systems revealed that significant difference existed between infected and bio-controlled seedlings. The *P. flueorescens* protected seedlings; fresh shoot weight was 4.28±1.0138 g which was higher than the F161 infected seedlings vs. 1.59±0.209 for the infected seedlings ($p < 0.05$; $n \geqslant 9$). Protection allowed >2.6 folds enhancement in seedling shoot weight and enhanced growth >1.6 folds. *P. fluorescens* enhanced growth of seedlings in the absence of experimental infections possibly through the provision of other plant promoting factors or extended protection against unseen microbes or factors. The results are illustrated in Figs. 2-4 and Table 2.

Non-chemical strategies for the control of crop diseases are of considerable interest due to environmental and health concerns about the adverse effects of synthetic pesticides (Reuveni, 1995). Therefore, it is likely that there will be greater reliance on the use of microorganisms as antagonists of plant pathogens in the future (Hall, 1995; De Cal, Sztejnberg, Sabuquillo, & Melgarejo, 2009). However, the spectrum of activity of microorganisms as biocontrol agents is usually narrower than that of chemical pesticides (Baker, 1991; Janisiewicz, 1996). In addition, the inconsistent performance of microorganisms in commercial agriculture has limited their use as agents for controlling plant pathogens (Backman, Wilson, & Murphy, 1997; De Cal et al. 2009).

Application of Pseudomonas or its byproducts to field crops (before, during, and/or after sawing) such as watermelon and other similar crops that are afflicted by Fusarium, may improve the productivity and yields of such crops. Successful field biocontrol application will contribute to better agricultural methods. Furthermore, this method is cost effective, easily applicable and may accumulate beneficiary results on the long run; i.e. each successive year should be an improvement over the preceding year. Moreover, environmentally, it is a better choice than chemically based control methods.

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