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EFFICACY OF INTEGRATING PLANT GROWTH-PROMOTING RHIZOBACTERIA AND THE FUNGICIDE THIOPHANATE-METHYL FOR CONTROLLING WATERMELON DAMPING-OFF AND WILT DISEASES CAUSED BY *Fusarium oxysporum* f.sp. *niveum*

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Abstract: Twenty bacterial isola-tes from soil rhizosphere of water-melon plants were *in vitro* screened for their ability to inhibit the mycelial growth of *Fusarium oxysporum* f.sp. *niveum* (Fon), the causal pathogen of watermelon damping-off and wilt diseases. Among the tested bacterial isolates, three isolates were found to inhibit the mycelial growth of the pathogen. The potential of three plant growth promoting rhizobacteria, *Bacillus cereus*, *Pseudomonas fluorescens* and *Pseudomonas putida* as well as Topsin-M alone or in combination was tested for controlling Fon in the greenhouse and field conditions. All tested treatments significantly reduced disease severity as compared to the non-treated infected control. Under greenhouse conditions, the fungicide, thiophanate-methyl (Topsin-M) caused the highest reduction in pre-emergence damping-off and wilt diseases (44.4 and 72.9%, respectively) followed by using *Pseudomonas fluorescens* combined with Topsin-M (37 and 71.8%, respectively). Under field conditions, the highest reduction percentage of disease (67.7.0%) was obtained after application of Topsin-M alone and *Pseudomonas fluorescens* + Topsin-M followed by using of *P. fluorescens* (59.7%) and *Bacillus cereus* + Topsin-M (51.6%).

Key words: Biocontrol, *Fusarium oxysporum* f.sp. *niveum*, *Bacillus cereus*, *Pseudomonas putida*, *Pseudomonas fluorescens*, Watermelon, Rhizobacteria, Topsin-M

Introduction

Watermelon (*Citrullus lanatus* (Trunb.) Matsum and Nakai) is widely consumed vegetable crop No. ten around the world. It is attacked by several viral, bacterial

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and fungal diseases (Abou-Jawdah *et al.* 2000 and Burdman *et al.* 2005). The most important fungal pathogens attacking watermelon, is *Fusarium oxysporum* f.sp. *niveum* that causes pre-emergence damping-off and wilt diseases in mature plants. Several reports recorded that such diseases are the limiting factors of watermelon production (Martyn and Bruton, 1989).

The causal pathogen had received a great attention in many countries. It attacks the host plant at different stages of the growth starting at the seedling to mature stages. It may attack the host earlier to cause pre-emergence damping-off. Control of this disease is difficult, several measures have been suggested to control the disease such as the use of resistant cultivars, sanitization practices and chemical control. Chemical control strategies have been used as seed dressing treatments (Fravel *et al.*, 2005) or using methyl bromide as fumigant (Cebolla *et al.*, 2000). Control of wilt disease depends mainly on fungicides, which are applied in furrow or as seed treatments (DeVay *et al.*, 1988). Since, fungicides are expensive, can cause environmental pollution and may cause the selection of pathogen resistance. Also, the effectiveness of fungicides may be reduced if they are absorbed, inactivated or decomposed by other soil managements (Lumsden and Locke, 1989 and Diehl and Fehrmann, 1999).

The problems associated with the use of agrichemicals have promoted investigation for agents of biological control of plant diseases as an alternative for pathogens managements (Fravel *et al.*, 2005 and Blanco *et al.*, 2007). Plant growth promoting rhizobacteria (PGPR) are naturally occurring as soil microorganisms that colonize roots and stimulate plant growth. Such bacteria have been applied to a wide range of agricultural species for the purposes of growth enhancement, including increased seed emergence, plant weight, and disease control. Yield increases between 10% and 20% with PGPR applications have been reported for several agricultural crops (Kloepper, *et al.*, 1991).

Integrating biological with chemical means has become an acceptable strategy for many pest managements. The benefits of this approach include reducing the number of chemical sprays and improving plant growth and quality. Also, there is motivation on the part of the grower to use biologicals to minimize the cost of fungicide applications, lessen the chance of developing resistance in the pest populations, and avoid potential environmental hazards (Elmer and McGovern 2004). The aim of this study was to evaluate the integrated application of PGPR with the fungicide, Topsin-M for

controlling damping-off and wilt diseases of watermelon.

Materials and Methods

Isolation and identification of the causal pathogen

The causal pathogen was isolated from naturally infected watermelon plants, which were showing wilt symptoms which were collected from different localities of Assiut Governorate in 2006 growing season. Identification of the pathogens was carried out by using the morphological characteristics of mycelia and conidia as described by Booth (1977). Hyphal tip technique and single spore isolation were used to obtain pure cultures of the developed fungus. Pure cultures of isolates were grown on Potato Dextrose Agar (PDA) and stored for further studies in refrigerator at 5°C on PDA slants.

Pathogenicity tests:-

Nine isolates of the Fusarium fungus were examined for their pathogenicity whether they cause pre-emergence damping-off and wilt diseases on watermelon cv. Giza 1. Inocula were prepared by growing the isolates in 250 ml conical flasks, each containing 100 ml of Czapek's liquid medium and were inoculated separately with 6 mm agar disc obtained from 7 days old culture of *Fusarium oxysporum* f.sp. *niveum*. The flasks were incubated at 25°C for 15 days. Mycelial growth of the fungus was decanted, washed with distilled water, suspended in of distilled water and blended for 5 minutes using a warring Blender. For soil infestation, 100 ml fungal suspensions (10^6 CFU/ml) were added to 30 cm diameter pots filled with sandy loamy soil, 7 days before planting. Soil and pots sterilization was carried out by 5 % formalin solution for 15 minutes. Soil was covered with poly-ethylene sheet for 7 days to retain the gas and left to dry for two weeks until all traces of formaldehyde disappeared.

Seed disinfestations was carried out by dipping the seed in 1% sodium hypochlorite solution for 3 minutes, then rinsed for several times with sterilized water. Pots containing non-infested soil were used as control. Nine seeds were sown in each pot. Four replicates were used for each isolate. The pots were kept under careful observation in greenhouse conditions of the Plant Pathology Dept., Fac. of Agric., Assiut Univ. Egypt, and examined for incidence of the Pre-emergence damping-off disease. Percentage disease occurrence was based on the number of nonemerged seeds in relation to the number of sown seeds. In case of wilt disease, sixty days after sowing, the wilt percentage was recorded by appling the CIAT scale, 1-9 as reported by Van Schoonhoven and Pastor-Corrales (1987), where 1 -no visible symptoms and 9 dead plants or severely infected.

with 100% foliage showing wilting, chlorosis and/or premature defoliation. The experiment was repeated twice.

Antagonistic bacteria:-

Twenty antagonistic bacterial isolates were isolated from rhizosphere of watermelon plants grown in an open field in Assiut, Egypt. The identification of these bacteria was confirmed according to their morphological, physiological and biochemical characters as stated in Bergey's Manual of Systematic Bacteriology (Schaad, 1980, Krieg and Holt, 1984). After screening of 30 bacterial isolates in different culture tests, several isolates were characterised as strains of *Bacillus cereus, Pseudomonas putida* and *Pseudomonas fluorescens*. Isolates were tested for their inhibitory effect against the wilt pathogen then the most potent isolate from each was used for *in vivo* experiments.

Greenhouse Experiments:-

The trial was carried out in the Greenhouse of Plant Pathology Dept. Faculty of Agriculture, Assiut Univ. Plants were grown in 30 x 20 cm pots in a soil mix containing sand, (2 kg/pots) and kept at the ambient conditions of the greenhouse at 30+5°C and watered when necessary. The bacterial inocula were prepared by growing the tested species in Tryptic Soy Broth for 24 h at 26°C, cells were harvested aseptically and diluted to be read at an optical density of 660 nm. Application of the biocontrol bacteria was conducted by soil drench with 30 ml bacterial suspension/pot (10^8CFU/ml) 48 hrs after soil infestation with the pathogen.The fungicide Topsin-M ,(thiophanate-methyl)70% (1.2-bis (3-methoxy carbonayl)-2-thiouredio benzene) TPM, (1 g/L) was used; watermelon seeds were soaked in fungicidal suspen-sions for 24 hours. Control seed were treated with sterile distilled water for the same periods.

The experiment was repeated twice under greenhouse condition in 2007 and 2008 growing seasons. Five replicates were used for each treatment. Each replicate consisted of 9 plants. Surviving seedlings and wilted plants were counted 15 and 60 days after sowing.

Field Experiments:-

The experiment was conducted at the Experimental Farm of Faculty of Agriculture, Assiut University, Assiut, Egypt in 2007 and 2008 growing seasons. Watermelon Giza 1 cultivar and isolate 6 of *Fusarium oxysporum* f.sp. *niveum* were used in this study. Field plots (3.x3.5 m) comprising 3 rows and 10 holes/row were used. Four plots were used as replicates for each treatment as well as for untreated control. Treatments were arranged in complete randomly design. Each row was infested with 550 ml of the inoculums of the pathogens one week before planting; each

hole received 55 ml of the fungal suspension. Application of antagonists and fungicide was carried out as mentioned in the greenhouse experiments. From each row 5 plants were used (15 plant/replicate). Healthy seedlings and wilted plants were counted 15 and 60 days after sowing and the percent of pre-emergence and wilted plants were calculated as mention before. All experiments were repeated twice

Statistical analysis

Analyses of variance (ANOVA) were carried out using MSTAT C program and the significance of differences among the treatments were determined according to the Duncan's multiple range test (DMRT) at $P \leq 0.05$. (Gomez and Gomez, 1984).

Results

Pathogenicity tests:

Nine isolates of *Fusarium oxysporum* were tested for their pathogenicity to watermelon plant cultivar Giza 1. Results in Table (1) showed that all tested isolates were pathogenic to watermelon plants. The percent of pre-emergence dead plants ranged between 12-22 %. Also, all isolates attacked watermelon plants and caused wilting symptoms up to 85%. However; isolates varied in their virulence, isolates No. 5 and 6 were more virulent than other isolates. According to these data isolate No.6 was selected for further experiments.

Table(1): Pathogenicity tests of the nine isolate of *Fusarium oxysporum* f.sp. *niveum* on watermelon plants under greenhouse conditions.

Fungal isolates	Pre-emergence damping-off	% wilt
1	18 c	82 a
2	13 ef	62 c
3	12 ef	75 b
4	16 cd	52 d
5	20 a	85 a
6	22 a	85 a
7	15 de	65 c
8	16 cd	55 d
9	18 c	85 a

Different letters indicate significant differences among treatments within the same column according to the Duncan's multiple range test (DMRT) at $P<0.05$

Greenhouse Experiments:-

Table(2): Effect of PGPR and Topsin-M (fungicides) alone or in combination in controlling artificially infected watermelon in greenhouse experiments.

Treatments	Pre-emergence damping-off		wilt	
	% Disease	% reduction	% Disease	% reduction
Control (healthy)	0 e	-	2 h	-
Control (infected)	27 a	-	85 a	-
Bacullius cereus (Bc)	20 b	25.9	70 b	17.6
Pseudomonas fluorescens (Pf)	18 bc	33.3	48 e	43.5
P. putida (Pp)	22 b	18.5	66 c	22.4
Topsin-M (T)	15 de	44.4	23 g	72.9
BC + T	19 bc	29.6	65 c	23.5
PF + T	17 cd	37.0	41 f	71.8
PP + T	20 b	25.9	60 d	29.4

Different letters indicate significant differences among treatments within the same column according to the Duncan's multiple range test (DMRT) at $P \leq 0.05$

Results in Table (2) indicated that application of *Bacillus cereus, Pseudomonas putida* and *Pseudomonas fluorescens* alone showed significant reduction in both pre-emergence damping-off and wilt diseases as compared to infected control. No significant differences were observed between their reductions effect on pre-emergence damping-off disease. Other treatments significantly reduced the percentage of wilt disease relative to the infected control. The fungicide, Topsin-M caused the highest reduction in pre-emergence damping-off and wilt diseases followed by using *Pseudomonas fluorescens* (Pf). On the other hand, a satisfactory percentage of disease reduction was obtained when both *Pseudomonas fluorescens* and Topsin-M were used in combination.

Field Experiments:-

Table(3): Effect of PGPR and Topsin-M (fungicides) alone or in combination in controlling artificially infected watermelon in a field experiments.

Treatments	Pre-emergence damping-off		% wilt	
	% Disease	% reduction	% Disease	% reduction
Control (healthy)	0 d	-	5 h	-
Control (infected)	19 a	-	62 a	-
Bacullius cereus (Bc)	10 b	47.4	57 b	8.1
Pseudomonas fluorescens (Pf)	7 b	63.2	25 f	59.7
P. putida (Pp)	9 b	52.4	44 c	29.0
Topsin-M (T)	5 c	73.7	20 g	67.7
BC + T	8 b	58	30 e	51.6
PF + T	6 c	68.4	20 g	67.7
PP + T	7 b	63.2	38 d	38.7

Different letters indicate significant differences among treatments within the same column according to the Duncan's multiple range test (DMRT) at $P \leq 0.05$.

Data in Table (3) show that the highest reduction in pre-emergence damping-off was obtained by the application of T followed by Pf+T then Pf and PP+T. The application with the tested bacteria gave a significantly considerable degree of protection against the pathogen under field conditions. The highest percentage reduction in percent aged wilt (67.7.0%) disease was obtained after the application of T and Pf+T followed by using of BC (59.7) and BC +T (51.6%).

Effect of plant growth promoting rhizobacteria (PGPR) on water melon yield under field conditions

Table(4): Effect of PGPR and the fungicide Topsin-M alone or in combination on yield in watermelon under the field conditions.

Treatments	Yield Ton/faddan	% Increased
Control (healthy)	10 de	43
Control (infected)	7 f	-
Bacullius cereus (Bc)	11 d	57
Pseudomonas fluorescens (Pf)	13 bc	85.7
P. putida (Pp)	10 de	43
Topsin-M (T)	10 de	43
BC + T	14 b	100
PF + T	15 a	114
PP + T	14 b	100

Different letters indicate significant differences among treatments within the same column according to the Duncan's multiple range test (DMRT) at $P \leq 0.05$.

Data presented in Table (4) showed that yield of watermelon plants treated with *P. putida* and planted in the soil infested with Fon were nearly equal to the control. While, the yield of watermelon plants inoculated with *P. fluorescens* was significantly increased compared to the infected control. Overall, obtained yield after treatment with *P. fluorescens* was higher than those observed after treatment with other bacterial antagonists. Also, combination of bioagents with the fungicide significantly increased the yield in comparison to the other treatments.

Discussion

Biological control of soil-borne plant pathogens has been suggested as an alternative to the hazardous and expensive chemical fungicides for controlling plant disease (Vidhyasekaran *et al.*, 1997). The potential of root-colonizing bacteria to protect watermelon from damping-off and wilt diseases caused by *Fusarium oxysprium* f.sp. *niveum* (Fon), has recently been demonstrated in laboratory and greenhouse experiments (Suarez-Esterlla *et al.*, 2007). In the present study, three PGPR bacterial isolates were found to produce detectable inhibition zones against the pathogen (data not show). The production of clear inhibition zones in dual culture screens is due to the production of certain antagonistic metabolites which may could have several mechanisms for biological control of some plant pathogens (Swadling and Jeffries, 1998).

Weller and Thomashow (1993) reported that some rizobacteria have the capability of producing antibiotics *in vitro* and *in vivo* that may not only be toxic to other micro-organisms in soil but also have a negative effect on the plant growth. Which confirm the present study that there was no indication of direct toxic effect of the bacteria on our experimental plant. Plant growth-promoting rhizobacteria may be relatively host specific, cultivar specific, or non specific in root-colonizing ability in plant growth promotion (Schroth and Becker 1990). In the present investigation, different levels of protection against *Fusarium oxysprium* f.sp. *niveum* were observed and could be attributed to a host specific reaction of the bacterial strain, related to their abilities to colonise rhizosphere or to their mode of action. Application of mixtures of antagonistic micro-organisms, preferably with different modes of action, has been proposed as a strategy to increase the efficacy and improve the consistency of disease control (Schisler *et al.* 1997). In the field trial carried out in the present study, watermelon infected with *Fusarium oxysprium* f.sp. *niveum* and treated with *Pseudomonas fluorescens* improved their growth compared to control plants.

Infection of watermelon plants with *Fusarium oxysprium* f.sp. *niveum* and treated with PGPR was significantly increased yield as compared to infected plants. Increase in yield following bacterial treatment has been reported by several authors in different plant species (Kloepper, *et al.*, 1989). Several modes of action were suggested to control some plant diseases by using certain strains of rhizobacteria (Dwivedi and Johri, 2003 and Ran *et al.*, 2005). Pseudomonads exert a protective effect on the roots through antagonism towards some fungal plant pathogens by producing metabolites e.g lytic enzymes, auxins, indole-3-acetic acid, gibberellins, siderophores and antibiotics (Haas and Keel, 2003 and Ran *et al.*, 2005). PGRP may also, play a significant role in plant nutrition either by their ability to release mineral nutrients from the soil or by their interactions with mycorrhizas and rhizobia (Ran *et al.*, 2005).

The present study demonstrated that, in general, when biologicals were integrated with fungicides, the level of disease suppression was better than that achieved by bioagents alone. This conclusion agrees with several other investigations which reported that PGPR have a potential as part of a strategy that includes sanitation and fungicides (Elmer and McGovern 2004 and Someya *et al.*, 2000).

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فعالية التكامل بين البكتريا المشجعة للنمو ومبيد الثيوفانيت مثيل لمقاومة أمراض موت البادرات والذبول فى البطيخ المتسبب عن الفطر *Fusarium oxysporum f.sp. niveum*

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تم دراسة تأثير عشرين عزلة من البكتريا المعزولة من تربة نباتات البطيخ لدراسة قدرتها على تثبيط النمو الميسليومى للفطر *Fusarium oxysporum f.sp. niveum* المسبب لمرض ذبول البطيخ . وقد وجد ان ثلاث عزلات من هذه البكتريا هى الاكثر تثبيط للنمو الميسليومى للفطر المسبب للمرض وهى *Bacillus cereus*, *Pseudomonas fluorescens*, *Pseudomonas putida* . تم دراسة تاثير هذه العزلات الثلاث منفردة او مع مبيد التوبسين م على تثبيط المرض فى الصوبة وفى الحقل. فى تجارب الصوبة أدت كل المعاملات الى خفض معنوى فى شدة المرض مقارنة بالنباتات السليمة. كان مبيد التوبسين م هو الاكثر فعالية فى تثبيط المرض سواء فى الحد من موت ما قبل الانبات او الذبول (٤٤,٤ و ٧٢,٩ ٪ على التوالي) يليه فى ذلك المعاملة بالبكتريا *Pseudomonas fluorescens* مع المبيد حيث كان الخفض بمقدار (٣٧ و ٧١,٨ ٪ على التوالي) وتحت ظروف الحقل تبين ان اكبر انخفاض فى شدة المرض حدث عند المعاملة بالمبيد مع البكتريا *Pseudomonas fluorescens* (٦٧,٧ %) يليها فى التثبيط البكتريا *Bacillus cereus* (59.7%) ثم فى النهاية المعاملة بالبكتريا *Bacillus cereus* مع المبيد (٥١,٦%)