FORMULATIONS OF *BACILLUS* SPP. AND *PSEUDOMONAS FLUORESCENS* FOR BIOCONTROL OF CANTALOUPE ROOT ROT CAUSED BY *FUSARIUM SOLANI*

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Abstract: The aim of this study was to evaluate the different carrier formulations of antagonistic bacteria on incidence of root rot disease of cantaloupe. Twenty-seven isolates of bacteria isolated from rizosphere cantaloupe plants (collected from different localities of the Assiut Governorate, Egypt) were tested *in vitro* against the growth of *Fusarium solani.* The tested isolates exhibited varied percentages of mycelial inhibition of *F. solani.* The highly antagonistic bacteria isolates were identified as *Bacillus subtilis*, *Bacillus cereus,* and *Pseudomonas fluorescens*. The effect of talc based powder and wood flour as various carrier formulations of antagonistic bacteria were tested on incidence of cantaloupe root rot disease in greenhouse and field experiments. All tested carrier formulations of antagonistic bacteria significantly decreased the disease index percentage ($p > 0.05$) of root rot disease compared with the control, in greenhouse or in field experiments. Application of the wood flour formulation to the infested soil at the time of planting, gave the lowest disease (21.75%) index percentage compared to an application fifteen days before planting (26.83%). The reverse effect occurred in the case of the talc based powder formulation application. In field experiments, during the two growing seasons of 2009 and 2010, wood flour formulation gave the same effect in the reduction of the disease index when added before planting or at the time of planting to soil infested with the pathogen. However, application of the talc formulation at the time of planting showed the least disease index compared to when it was applied fifteen days before planting. In general, wood flour formulation significantly decreased the disease index when compared with the talc formulation. In all the formulations, a number of viable colonies of bioagents were decreased gradually by prolonging the storage time at 4°C. Storage time was prolonged up to five months. But in the case of *B. subtilis* on talc and *B. cereus* on wood flour formulations, storage time needed to be prolonged up to seven months.

Key words: *B. cereus, B. subtilis*, cantaloupe, *F. solani, P. fluorescens,* root rot

INTRODUCTION

Root-rot disease caused by soil-born fungi is the most important disease of many crops. Several fungi were recorded as causal pathogens of root-rot and wilt disease such as *Rhizoctonia solani* and *Fusarium solani* (Abouzeid *et al*. 1990; Abouzeid *et al*. 1997). One of the fungi which was most isolated from discolored vascular tissue or root rot of melon (*Cucumis melo*) was *Fusarium solani* (Aegerter *et al*. 2000). *F. solani* can cause severe economic losses to field and greenhouse grown cucumber (Kaulizakis 1997; Roberts *et al*. 2005). Control of the disease depends mainly on fungicides, which are applied in furrows or as seed treatments (DeVay *et al*. 1988). Fungicides are expensive, though, and can cause environmental pollution. The use of fungicides may also cause the selection of pathogen resistance. In addition, the effectiveness of fungicides may be reduced if they are absorbed, inactivated or decomposed by other soil managements (Lumsden and Locke 1989; Diehl and Fehrmann 1999). Therefore, many trials for using biocontrol to overcome this problem have been carried out (Sallam *et al*. 2009). The application of biological controls using antagonistic micro-organisms has proved to be successful for controlling various plant diseases in many countries (Sivan and Chet 1986). Good results have been obtained with gram-positive *Bacillus* spp. and gram-negative *Pseudomonas* spp. in the control of several plant pathogens, including *Fusarium* spp. (Haas and Defago 2005). Seed treatment with *Bacillus* spp. actively controlled three fungal root diseases (Kim *et al.* 1997). Also, *Pseudomonas cepacia* or *Pseudomonas fluorescens* applied to pea seeds acted as a biological control agent against Pythium damping-off and Aphanomyces root rot and was able to reduce disease incidence (De Chial *et al.* 2003; Debode *et al.* 2007).

The development of formulations and delivery systems for biocontrol by using antagonistic microorganisms to suppress the incidence of diseases caused by soil born pathogens is a great importance (Çiğdem and Merih 2005). There are several approaches described that optimize the formulation of a biocontrol agent, like the ap-

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plication of appropriate carrier materials (Vidhyasekaran *et al*. 1997; Krishramuthy and Grarananickam 1998; Ali *et al*. 2001) and formulation additives (Schmidt *et al*. 2001). Ideal formulation additives should improve the biocontrol efficacy of the antagonist but should not support the growth of the pathogen or cause any damage to the host plant (Wiyono *et al*. 2008).

The objective of the present study was to evaluate some antagonistic bacterial agents against *F. solani in vitro* and *in vivo.* Preparation of different carrier formulations of antagonistic bacteria, its effect on root rot of cantaloupe in greenhouse and field conditions, and the effect of the storage period on biological activity of formulating antagonistic bacteria were also investigated.

MATERIALS AND METHODS

Preliminary test for antagonistic capability of certain isolated microorganisms against *F. solani in vitro*

Twenty-seven bacterial isolates were isolated from different localities of the Assiut governorate. Isolates were from rhizosphere of cantaloupe plants were isolated according to the method described by Dhingra and Sinclair (1995). They tested against the pathogenic isolate of *F. solani in vitro*. The pathogen was isolated from naturally infected roots of those diseased cantaloupe plants showing root rot symptoms. Identification was according to the morphological characteristics of mycelia and spores as described by Booth (1977) and Domsch *et al.* (1980). The tested isolates of bacteria were grown on Nutrient Sucrose Agar medium (NSA) (Peptone 5 g, beef extract 3 g, sucrose 5 g, agar 20 g, and distill water 1,000 ml) and incubated at 28°C for one day, and used as inocula. Petri plates (9 cm in diameter) containing Potato Dextrose Agar (PDA) medium were inoculated in the middle by disks (5 mm in diameter) of pathogenic fungi, then inoculated with the tested bacterium on two opposite sites of the pathogen inoculum. Three replicates were used of each treatment. Inoculated plates with pathogenic fungus only, were used as the control. After a 5 day incubation period at 25°C, the linear growth of the tested pathogen was recorded when the growth of the pathogen covered the plate surface in the control treatment. The percentages of mycelial growth inhibition were calculated according to following formula:

Percentage of mycelial growth inhibition = [A–B/A] x 100

where:
A – the length of the hyphal growth in the control,
B – the length of hyphal growth in the tested isolate.

The antagonistic bacterial isolates which gave a higher percentage of mycelia growth inhibition were identified according to their morphological, physiological culture and biochemical activities according to Palleroni (1984) and Sneath *et al*. (1986).

Efficiency of certain biocontrol agents on the incidence of cantaloupe root-rot diseases

Preparation of talc-based powder and wood flour formulations of bacteria

The highly antagonistic bacterial isolates, *B. subtilis* (isolate No. 19), *B. cereus* (isolate No. 24) and *P. fluorescens* (isolate No. 20) were selected for this study. Isolates of antagonistic bacteria were grown in 250 ml flasks. Each flask contained 100 ml of Nutrient Broth (NB) and Carboxy methyl cellulose (10 g). Sterilized talc (1 kg) was prepared for the talc powder formulation. Talc formulation or sterilized wood flour formulation was mixed in each flask. The pH was adjusted to 7 by adding calcium carbonate. Bacterial suspension ($2x10^8$ /cfu/ml) was added to the mixture and mixed well under sterile conditions. The materials (35% moisture content) were packed in polyethylene bags sealed and stored at 4°C until used as described by Jayaraj *et al*. (2006).

Effect of the application of antagonistic bacteria on incidence of cantaloupe root rot caused by *F. solani* under greenhouse and field conditions

Greenhouse experiments

This experiment was carried out in the 2009 growing season, in the greenhouse of Plant Pathology Dept., Faculty of Agriculture, Assiut University. Inocula of *F. solani* was growing on Barley Medium (150 g barley + 50 g clean sand + 4 g glucose + 0.2 g yeast extract + 200 ml water) in 500 ml flasks and incubated at 25±2°C for 15 days. Sterilized pots (25 cm in diameter) were filled with sterilized clay soil (pots and clay soil sterilized with a 5% formalin solution for 15 min.) and infested by isolate *F. solani* at a rate of 3% of clay soil (w/w), two weeks before planting. The formulated antagonistic bacteria were added to the infested soil at a rate of 1.5% of soil (w/w) in pots, two weeks before or at the time of planting. Each pot was sown with 4 sterilized seeds of cantaloupe cv. Paquito (seeds sterilized for 2 min. in 2% sodium hypochlorite solution, then rinsed several times in sterilized distilled water and dried between sterilized filter papers). Three pots were used for each treatment as replicates. Untreated pots with antagonists were used as the control. The disease index percentage of root rot was measured at the end of the experiment (after 45 days from planting) as reported by Paternotte (1987) and Rajput *et al.* (2008) as follows: 0 – No infection, 1 – 1–25% infection, 2 – 26–50% infection, 3 – 51–75% infection, 4 – 76–100% infection. The estimation of the disease index percentage was carried out as follows:

$$\text{Disease index} = \sum \frac{(nx1) + (nx2) + \ldots}{tn} \times 100$$

where:
tn – the total number of plants,
n – number of plants in each group of diseased plants (1, 2, 3 ...)

Field experiments

These experiments were carried out in the 2009 and 2010 growing seasons. Seeds of cantaloupe cv. Paquito were sown in plots (3x3.5 m) which had 2 rows (1.5 m).

Each row contained 2 hills, spaced 50 cm apart. Every hill was sown with 4 sterilized seeds and three replicates were used for each treatment. Inoculum of *F. solani* (approximately 35 g) was placed in each hill two weeks before planting and each formulation of antagonistic bacteria was added to infested soil (approximately 17 g/hill), 15 days before planting or at the time of planting. Plots containing inoculum of *F. solani* without the antagonistic formula were used as the control.

The disease index was measured at the end of the experiment (12 weeks after planting) as described before.

Effect of the storage period on biological activity of formulated antagonistic bacteria

The effect of a storage period at 4°C on the biological activity of formulating antagonistic bacteria was tested in the laboratory from 1–8 months. One gram of each different type of formulation was suspended in 200 ml of sterile distilled water every month. One hundred micro liters of the respective dilution of formulations was plated onto Nutrient Sucrose Agar medium (NSA) in Petri dishes and incubated at 28°C. There were 3 replicates per treatments. Bacterial colonies were counted after 24 h (Jayaraj *et al*. 2006).

Statistical analysis

All experiments were performed twice. Analyses of variance were carried out using the MSTAT-C, 1991 program version 2.10. Duncan's multiple range test was employed to test for significant differences between treatments at p = 0.05 (Gomez and Gomez 1984).

RESULTS AND DISCUSSION

The preliminary test for antagonistic capability of certain bacteria against growth of *F. solani in vitro*

Results presented in table 1 indicate that all tested antagonistic bacterial isolates showed a different inhibitory effect against the growth of the tested pathogens. Isolates No. 23, 20, and 21 showed a high percentage of inhibition growth against the growth of *F. solani,* followed by isolates No. 12, 13, 14, 15, 17, 18, and 19. Isolates No. 19, 20, and 24 were selected and identified as *B. subtilis*, *P. fluorescens* and *B. cereus,* respectively. These isolates were used in the following study. Such results are in agreement with those reported by Thomashow *et al*. (2002). *Bacillus* spp. and *Pseudomonas* spp. produce antibiotics which can suppress one or more pathogens. The inhibitory effect of antagonistic bacteria such as *B. subtilis* and *P. fluorescens* against growth reduction of phytopathogenic fungi may be due to the production of hydrolytic enzymes that can degrade cell walls, iron-chelating siderophores, and several cyclic lipodepsipeptides (LDP) Kim *et al*. 2008.

Effect of the application of antagonistic bacteria formulations on the incidence of cantaloupe root rot caused by *F. solani* under greenhouse conditions

Data in table 2 indicate that application of antagonistic bacteria formulations to pathogen infested soil significantly decreased root rot disease of cantaloupe compared with infected control. Talc based powder formulation showed a higher disease index percentage when applied fifteen days before planting than when it was applied at the time of planting. But, the application of the wood flour formulation at the time of planting significantly decreased the disease index percentage compared to when it was applied fifteen days before planting. The wood flour formulation significantly reduced the disease index (24.29%) compared to the talc formulation (33.56%). Formulation of *B. subtilis* showed the highest reduction in the percentage of the disease index (26.73%) followed by *P. fluorescens* (29.85%) and *B. cereus* (30.20%). Such results are in agreement with results of Nahed (2007) and Sarhan *et al*. (2001). Rhizobacterial strains of *Pseudomonas* and *Bacillus* spp. are used to reduce soil-born pathogens including *Fusarium* spp. (Weller and Cook 1983; Weller 1988; Postma *et al*. 2000; Pavlou *et al*. 2002). The application of biological control using antagonistic microorganisms proved to be successful for controlling various plant diseases in many countries and was the best control measure under greenhouse conditions.

Table 1. Percentage of mycelia growth inhibition of antagonistic bacteria against *F. solani in vitro*

Bacterial isolates	% mycelia growth inhibition *F. solani*
1	54.06 e
2	53.33 eg
3	41.83 fi
4	47.6 fgi
5	45.0 fgi
6	65.73 cdj
7	62.93 ce
8	56.3 ej
9	61.5 ce
10	56.3 ej
11	36.26 hi
12	69.83 cb
13	79.63 b
14	74.63 bd
15	77.77 b
16	67.60 cd
17	77.96 b
18	76.46 b
19	74.6 bd
20	88.3 ab
21	82.4 ab
22	65.53 cd
23	91.66 a
24	63.06 c
25	53.30 eg
26	68.53 cd
27	61.66 ce

Means in a column followed by the same letter do not significantly differ according to Duncan's multiple range test ($p < 0.05$)

Table 2. Effect of the application of the formulated antagonistic bacteria on incidence of cantaloupe root rot caused by *F. solani* under greenhouse conditions

Antagonistic bacteria	Disease index [%]				mean
	wood flour		talc		
	before*	with**	before*	with**	
Bacillus subtilis	38.87 d	6.933 a	25.00 b	50.00 f	30.20 c
Pseudomonas fluorescens	33.33 c	33.33 c	44.43 e	8.30 a	29.85 b
Bacillus cereus	8.30 a	25.00 b	6.933 a	66.7 g	26.73 a
Mean	26.83 b	21.75 a	25.46 b	41.67 c	
Mean	24.29 b		33.56 a		

The 100% infected control

Means in a column followed by the same letter do not significantly differ according to Duncan's multiple range test ($p < 0.05$)

*formulated antagonistic bacteria added to the soil two weeks before planting

**formulated antagonistic bacteria added to the soil at the same time as planting

Field experiments

The results in tables 3 and 4 indicate that applied formulations of antagonistic bacteria to infested soil with pathogen, during the 2009 and 2010 growing seasons, showed the highest reduction of the disease index percentage compared to the control. Applying the wood flour formulation to infested soil at the time of planting significantly reduces disease, compared to when it is applied fifteen days before planting. Application of the talc formulation showed the least disease index at the time of planting, compared to when it is applied fifteen days before planning. When compared to the talc formulation, the wood flour formulation significantly decreased the disease index. The formulation of *B. cereu*s and *P. fluorescens* showed a higher reduction disease index compared with the formulation of *B. subtilis*. These results were reported by several workers as the best antagonists against several soil and seed born plant pathogens (Sallam *et al*. 2008; Poddar *et al*. 2004). A number of strains of *Pseudomonas* spp. used as a seed treatment with cell suspensions have been found to be effective in controlling several soil-born diseases. Also, carriers could improve product stability, shelf life, and also protect the bacteria against environmental extremes in soil. Coley-Smith and Holt (1990) and Rose *et al*. (2004) reported that application of formulations (wood flour and talc based powder) of biocontrol

Table 3. Effect of the application of the formulated antagonistic fungi on incidence of cantaloupe root rot caused by *F. solani* under field conditions in the 2009 season

Antagonistic bacteria	Disease index [%]				mean
	wood flour		talc		
	before*	with**	before*	with**	
Bacillus subtilis	25.00 c	19.17 b	24.37 c	31.4 d	24.98 c
Pseudomonas fluorescens	37.13 e	16.67 b	25.93 c	9.033 a	22.19 b
Bacillus cereus	7.23 a	25.93 c	29.60 d	17.93 b	20.17 a
Mean	23.12 b	20.59 a	26.63 c	19.46 a	
Mean	21.86 a		23.04 b		

The 100% infected control

Means in a column followed by the same letter do not significantly differ according to Duncan's multiple range test ($p < 0.05$)

*formulated antagonistic bacteria added to the soil two weeks before planting

**formulated antagonistic bacteria added to the soil at the same time as planting

Table 4. Effect of the application of the formulated antagonistic bacteria on incidence of cantaloupe root rot caused by *F. solani* under field conditions in the 2010 season

Antagonistic bacteria	Disease index [%]				mean
	wood flour		talc		
	before*	with**	before**	with**	
Bacillus subtilis	21.67 d	18.33 c	26.87 e	31.00 g	24.13 c
Pseudomonas fluorescens	36.77 h	18.80 c	25.53 e	10.00 b	23.11 b
Bacillus cereus	7.70 a	25.00 e	29.47 f	21.00 d	20.79 a
Mean	22.04 b	20.71 a	27.29 c	20.67 a	
Mean	21.38 a		24.64 b		

The 100% infected control

Means in a column followed by the same letter do not significantly differ according to Duncan's multiple range test ($p < 0.05$)

*formulated antagonistic bacteria added to the soil two weeks before planting

**formulated antagonistic bacteria added to the soil at the same time as planting

Table 5. Effect of the storage period on viability of bacterial formulation

Formulation	CFU/ml after storage period (months)								
	zero	one	two	three	four	five	six	seven	eight
B. subitlus + talc	$2x10^8$	$2.80x10^6$	$2.60x10^6$	$2x10^6$	$1.55x10^6$	$1.45x10^6$	$5x10^5$	$2.5x10^5$	0
B. subitlus + wood flour	$2x10^8$	$4x10^6$	$3.10x10^6$	$2.50x10^6$	$2.20x10^6$	$7.5x10^5$	0	0	0
P. fluorescens + talc	$2x10^8$	$2.65x10^6$	$2.45x10^6$	$2x10^6$	$1.70x10^6$	$5x10^5$	0	0	0
P. fluorescens + wood flour	$2x10^8$	$6.25x10^6$	$6x10^6$	$5x10^6$	$2.50x10^6$	$5x10^5$	0	0	0
B. cereus + talc	$2x10^8$	$3.50x10^6$	$2.50x10^6$	$1.40x10^6$	$1x10^6$	$9x10^5$	0	0	0
B. cereus + wood flour	$2x10^8$	$5.85x10^6$	$5.15x10^6$	$5x10^6$	$4.50x10^6$	$3.50x10^6$	$1.25x10^6$	$5x10^5$	0

Whereas CFU/ml means colony forming unit/ml

agents were more effective when added at the time of planting than when added two weeks before planting. Carrier formulations of *B. subtilis* and *P. putida* caused a significant high reduction of lettuce root rot disease (Weller and Cook 1983; Howie and Suslow 1991; Parke *et al*. 1991; Levy *et al*. 1992; Amer and Utkhede 2000). The biocontrol mechanism can be either induction of systemic resistance or antibiosis (Nandakumar *et al*. 2001). Use of *Bacillus* spp. resulted in rapid colonization of all tissues in tomato, including the vascular stele, and induced resistance against *F. oxysporum*. Similarly, *Pseudomonas* spp. also have the ability to suppress parasitic root pathogens via the production of biologically active substances. They also synthesize the enzyme that modulates hormone levels, limit the available iron via the production of siderophores, and kill pathogens by producing antibiotics

Effect of the storage period on the biological activity of formulated antagonistic fungi and bacteria

Data in table 5 indicate that in all the formulations, a number of viable colonies of bio-agents were gradually decreased by prolonging the storage time up to five months at 4°C. This was true except for *B. subtilis* on talc. and *B. cereus* on wood flour formulations, in which it took up to seven months to decrease bioagents. After four months of storage, formulations caused a high reduction in bacterial colonies. But, in the case of *B. subtilis* on talc and *B. cereus* on the wood flour formulation, colonies of bio-agents were decreased after five months. Such results are in agreement with the results of Vidhyasekaran and Muthamilan (1995). They assessed the efficacy of various carriers in sustaining the population of these strains during storage. They found that in talc-based and peat-based formulations, the bacteria even survived up to 240 days of storage although the population declined after 30 days, while in chickpea seeds treated with talc-based formulations, *P. fluorescens* survived on the seeds for at least 180 days.

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