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Effect of Bacterial Isolates Obtained from Animal Manure Compost Extracts on the Development of *Fusarium oxysporum* f. sp. *radicis-lycopersici**

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Abstract: Dual culture of some bacterial isolates obtained from animal manure compost extracts with *F. oxysporum* f. sp. *radicis-lycopersici*, the causal agent of the Fusarium Crown and Root Rot of tomato, significantly inhibited the *in vitro* development of the pathogen comparatively to the untreated control. Among 14 isolates tested, 8 inhibited by 38 to 47% the mycelial growth of *F. oxysporum* f. sp. *radicis-lycopersici*. The transplantation of tomato seedlings (cv. Riogrande) in peat, previously treated by bacterial suspensions and inoculated with a conidial suspension of the pathogen (10^7 spores mL⁻¹), significantly reduced the Fusarium Crown and Root Rot severity compared to the untreated control. The most effective isolates were identified by means of the API system, as *Chryseomonas luteola*, *Serratia liquifaciens* and *Aeromonas hydrophila*.

Key words: *Lycopersicon esculentum*, biocontrol, compost, inhibition, root rot severity

INTRODUCTION

Fusarium oxysporum f. sp. *radicis-lycopersici* causes the Fusarium Crown and Root Rot in tomato and induces serious yield losses (Rekah *et al.*, 1999). Due to its soil borne origin, complete suppression of this pathogen from soil is difficult and fungicides use is limited by the risk of development of fungicide-resistant strains (Hibar *et al.*, 2006). Thus, more efficient control alternatives are required (Sivan and Chet, 1993).

Under Tunisian conditions and for Fusarium wilts biocontrol, Hibar *et al.* (2005) and Ayed *et al.* (2006) showed the antagonistic activity of fungi isolated from suppressive soils, against *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *tuberosi*. Daami-Remadi *et al.* (2006) reported the inhibitory effect of *Bacillus* sp. against *Fusarium solani*, *F. graminearum*, *F. oxysporum* f. sp. *tuberosi* and *F. sambucinum*, the causal agents of potato dry rot in Tunisia. Elsewhere, Idris *et al.* (2007) showed that some rhizobacteria isolated from the rhizosphere were efficient in controlling *F. oxysporum* Schlectend of sorghum.

For the biological control of *Fusarium* sp., composts and their extracts are also shown to be of potential value (Weltzien, 1992; McQuilken *et al.*, 1994; Hoitink *et al.*, 1997; Cotxarrera *et al.*, 2002; Pharand *et al.*, 2002) as compost microflora plays a major role in the suppression of plant pathogens. The pasteurization of compost destroyed its active microorganisms and consequently nullified their antagonistic effect (Hoitink *et al.*, 1991, 1997; Zhang *et al.*, 1998; Bess, 2000; Quarles, 2001; Ingham, 2002; Camozzi, 2003). Cotxarrera *et al.* (2002) showed that bacterial populations were frequent in compost and were implicated in the suppression of the Fusarium wilt of tomato. Some strains of *Bacillus subtilis* (Phae *et al.*, 1990), isolated from compost, showed suppressive effect

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against several phytopathogenic fungi such as *Verticillium dahliae* and *Fusarium oxysporum* f. sp. *lycopersici*. Stindt (1990) found that 33 bacterial antagonists, belonging to the genera *Pseudomonas*, *Bacillus* and *Enterobacter* isolated from cattle manure compost extracts, inhibited the conidial germination of *Botrytis cinerea*. Similarly, Ketterer (1990) isolated from extracts of composted horse manure, two bacterial strains with antagonistic properties on detached potato leaves against *Phytophthora infestans*. Antagonistic interactions with phytopathogenic fungi and mechanism of biological control were based on antibiosis, parasitism, induced resistance and competition for space and limited resources (Hoitink *et al.*, 1997).

Preliminary dual culture of some animal manure compost extracts with *Fusarium oxysporum* f. sp. *radicis-lycopersici*, showed inhibition of this pathogen (Kerkeni *et al.*, 2007). The aim of this study was to evaluate the individually effect of some bacterial isolates, obtained from the most suppressive compost extracts, against *F. oxysporum* f. sp. *radicis-lycopersici* and their ability to decrease the severity of the Fusarium Crown and Root Rot of tomato.

MATERIALS AND METHODS

Pathogen

F. oxysporum f. sp. *radicis-lycopersici* used in this study was isolated on 2006 from tomato plants showing typical symptoms of Fusarium Crown and Root Rot. The pathogen was cultured on PDA at 25°C for one week and stored at 4°C for long term preservation.

Isolation of Compost Bacteria

A mature compost (>12 months), composed by 40% cattle manure, 40% sheep manure and 20% vegetable wastes and produced on 2006 at the composting-unit of the Technical Centre of Organic Agriculture of Chott Mariem-Tunisia, was used for compost extract preparation and bacteria isolation.

Compost bacteria were separately isolated on Glutamate-Mannitol (MG) medium based on yeast agar (Oxoid) (0.5 g L⁻¹), Glutamic acid (2 g L⁻¹), Mannitol (5 g L⁻¹), KH₂PO₄.3H₂O (0.5 g L⁻¹), NaCl (0.2 g L⁻¹), MgSO₄.7H₂O (0.2 g L⁻¹) and agar (Oxoid No. 3) (20 g L⁻¹). A serial dilution of compost extract up to 10⁻³ was carried out and then 10 µL aliquots of this dilution were spread onto MG medium plates. After 48 h of incubation at 27°C, bacterial colonies formed in the seeded media were individually resuspended into MG medium. The same procedure was repeated until having a purified bacterial culture. A total of fourteen bacterial isolates with different morphological characteristics were selected (CB1B1, CB1C, CB2A, CB2B, CB3A1, CB3B, CB3A2, CB3C, CB3D, CB4C, CB4D, CB5D, CB5B2 and CB7A1). They were sustained on King's B medium (King *et al.*, 1954) at 27°C. Their identification was realized by means of the API system (Idris *et al.*, 2007).

In vitro Bioassay of the Antagonistic Activity of the Compost Fungi

The antifungal activity of each bacterial isolate against *Fusarium oxysporum* f. sp. *radicis-lycopersici* was tested via the dual culture technique, as described by Fuchs (1993). The method consists of placing an active mycelial disc (6 mm in diameter) of the pathogen at the center of a 9 cm Petri plate containing freshly prepared King's B medium (King *et al.*, 1954). Compost bacteria were applied on the King's B plate as shown in Fig. 1. For untreated plates, an agar disc of *F. oxysporum* f. sp. *radicis-lycopersici* was placed at the center of the Petri dish but sterile distilled water was used instead of bacterial suspension. All plates were then incubated at 25°C and evaluated for pathogen growth inhibition after 5 days of incubation. Three replicates were used per elementary treatment.

To determine the inhibition rate of this pathogen by each of the tested compost bacteria, the radial fungal growth of *F. oxysporum* f. sp. *radicis-lycopersici* was noted by measuring the colony diameters for the control and treated plates (average of the two perpendicular diameters). The inhibition rate was calculated according to the formula used by Hibar *et al.* (2005) where:

Inhibition rate (%) = $(1 - (\text{Average diameter of the treated} / \text{Average diameter of the control})) \times 100$

In vivo Bioassay of the Antagonistic Activity of the Compost Bacteria

Plant Material

Lycopersicon esculentum Mill. «Priscas», cv. Riogrande was chosen for its susceptibility to *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Hibar, 2002).

Bacterial Inoculum Preparation

Colonies of 48 h old bacterial cultures on King's B medium were used for inoculation of 25 mL of a liquid M1 (i.e., (L⁻¹) 0.98 g K₂HPO₄·3H₂O, 0.4 g MgSO₄·7H₂O, 0.4 g CaCO₃, 3 g yeast agar and 10 g sucrose). The suspensions were incubated at 27°C for 24 h under continuous agitation at 120 rpm. After incubation period, an aliquot of 200 µL of each suspension was spread onto Petri plates containing King's B medium and incubated again for 24 h at 27°C. Cells from over-night cultures (27°C) were washed three times in sterile distilled water. Twenty five milliliter of bacterial suspensions were used for the treatment of tomato roots (Fuchs, 1993). Control plants were similarly treated but the bacterial suspension was replaced by sterile distilled water.

Preparation of the Pathogen

F. oxysporum f. sp. *radicis-lycopersici* mycelium taken from the colony edge was transferred to 150 mL of Potato Dextrose Broth (PDB) and incubated at 25°C for 5 days under continuous agitation at 120 rpm. After incubation period, the liquid culture was filtered and the conidial suspension was adjusted to 10⁷ spores mL⁻¹ by means of a Malassez cystometer (Hibar *et al.*, 2006).

Bioassay

Compost bacteria inhibiting by more than 40% the *F. oxysporum* f. sp. *radicis-lycopersici* mycelial growth in the *in vitro* assay were tested under greenhouse conditions. One month old tomato plants, cv. Riogrande, were transferred from alveolar flats; their roots washed and soaked for 15 min in each of the bacterial suspension already prepared. Plants were then transplanted into 10 cm diameter plastic pots containing an autoclaved peat (15 min at 120°C).

Tomato plants already treated individually with compost bacteria were directly inoculated with *F. oxysporum* f. sp. *radicis-lycopersici* by irrigation with 10 mL of conidial suspension (10⁷ spores mL⁻¹). Plants inoculated with the pathogen and irrigated by sterile distilled water were used as control.

Bioassay was conducted under greenhouse conditions at 25°C and under 12 h photoperiod (Pharand *et al.*, 2002). The plants were watered as needed. Ten replicate pots of each treatment were randomly placed. No fertilizer was added to plants. The experiment was conducted twice. Disease severity was determined 30 days after transplantation (Woo *et al.*, 1996), based on a symptom severity scale;

Where:

- 0 = Asymptomatic plants,
- 1 = Weakly infected plants (<50% of leaves chlorotic or wilted),
- 2 = High infected plants (>50% of leaves wilted but plants not dead),
- 3 = Dead plants.

At the end of the bioassay, the height, the mean shoot and root fresh weights of plants per elementary treatment were determined.

Identification of Bacterial Isolates

Based on the *in vitro* results, the most promising bacterial isolates were selected and identified to the species level by means of the API identification system assisted by Analytic Profile Index (API) plus computer software (bioMérieux® SA, Marcy-l'Etoile/France). Gram negative rod isolates with fermentative reaction were identified by The API® 20 E test strip while those with oxidative reaction were identified by the API® 20 NE test strip.

Experimental Design and Statistical Analysis

Data were arranged as a completely randomized design. Ten replicate pots per elementary treatment were used and the whole bioassay was repeated twice. Data were analyzed using SPSS statistical program version 11.0 and subjected to analysis of variance (ANOVA). Means were compared according to the Duncan's test.

RESULTS

In vitro Inhibition of *F. oxysporum* f. sp. *radicis-lycopersici* Growth by Compost Bacteria

The results in Table 1 showed that all tested bacterial isolates, significantly reduced the mycelial growth of *Fusarium oxysporum* f. sp. *radicis-lycopersici*, after incubation at 25°C for 5 days. All tested isolates were effective in reducing the mycelial growth by 3 to 47% compared to the untreated control; the most effective ones were CB3B, CB2A, CB7A1 (Fig. 1), CB4D, CB3A2, CB3C, CB3D and CB5B2, where pathogen growth was limited by more than 38%. The isolates CB5D, CB1C, CB3A1 and CB1B1 showed lower efficiency (<10%).

In vivo Inhibition of *F. oxysporum* f. sp. *radicis-lycopersici* Growth by Compost Bacteria Disease Severity

The ability of four selected compost bacteria to reduce the *F. oxysporum* f. sp. *radicis-lycopersici* development on tomato was assessed one month post inoculation. Symptoms induced by *F. oxysporum* f. sp. *radicis-lycopersici* were less severe in plants grown in substrates treated with tested bacteria, in comparison to the untreated control. The isolates CB3D and CB5B2 significantly inhibited by about 50% the tomato crown and root rot development, the disease severity was reduced to 1.1 and 1.4%, respectively. Control plants not treated with bacteria but inoculated only with the pathogen rendered 2.6% (Fig. 2).

Table 1: Inhibition rate of *Fusarium oxysporum* f. sp. *radicis-lycopersici* radial growth in presence of the compost bacteria (King's B, after six days of incubation at 25°C)

Bacterial isolates	Inhibition (%) of <i>Fusarium oxysporum</i> f. sp. <i>radicis-lycopersici</i>
CB3B	47 ^a
CB2A	40 ^a
CB7A1	44 ^a
CB5D	5 ^d
CB2B	24 ^b
CB1C	9 ^{cd}
CB4C	20 ^{bc}
CB4D	39 ^a
CB3A2	39 ^a
CB3C	38 ^a
CB3D	42 ^a
CB3A1	3 ^d
CB1B1	8 ^{cd}
CB5B2	43 ^a
Control	0 ^d

Different letter(s) within columns represent values that are significantly different at $p = 0.05$ based on ANOVA and Duncan's test. Each value represents the mean of 3 values

Table 2: Fusarium crown and root rot severity on tomato plants observed after 30 days, in sterilized peat treated with compost bacteria in comparison to the untreated control (means of ten plants)

Treatments	Control	CB3B	CB3D	CB7A1	CB5B2
Disease severity	2.6 ^a	1.72 ^{bc}	1.1 ^c	2.1 ^a	1.4 ^c

Disease severity ranked from 0 (asymptomatic plants) to 3 (dead plants). Different letter(s) represent values that are significantly different at $p = 0.05$ based on ANOVA and Duncan's test

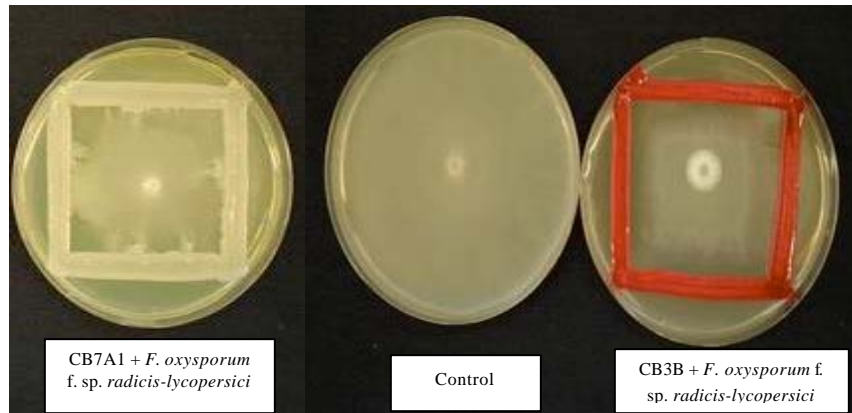


Fig. 1: Inhibition of growth of *Fusarium oxysporum* f. sp. *radicis-lycopersici* by some bacteria isolated from compost extracts in comparison to the untreated control

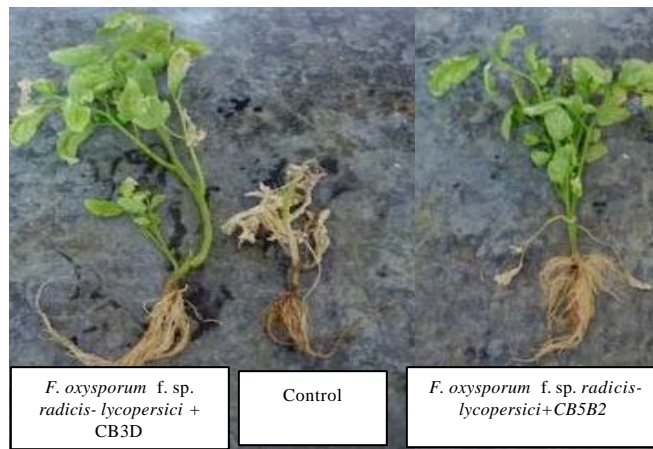


Fig. 2: Improvement of plant growth of one month old tomato plants (cv. Riogrande) with compost bacteria. Control: substrates with *Fusarium oxysporum* f. sp. *radicis-lycopersici*

Plant Height

Results in Table 2 showed that the amendment of substrates with compost bacteria significantly enhanced the plant height comparatively to the untreated control when isolates such as CB3D, CB3B and CB5B2 were used. In fact, tomato plants growing in those substrates were higher than 18 cm comparatively to 10.6 cm for the untreated control plants (Fig. 2).

Table 3: Effect of the treatment of substrates with compost bacteria on plant height, shoot and root fresh weights of one month old tomato plants (cv. Riogrande)

Treatments	Control	CB3B	CB3D	CB7A1	CB5B2
Plant height (cm)	10.60 ^b	19.70 ^a	21.00 ^a	16.00 ^{ab}	18.90 ^a
Shoot fresh weight (g)	3.80 ^c	10.09 ^{bc}	17.64 ^a	7.94 ^c	14.58 ^{ab}
Root fresh weight (g)	1.26 ^c	12.22 ^a	6.40 ^b	11.83 ^a	4.06 ^{bc}

Control: substrates with *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Each value represents the mean of 10 values. Different letter(s) represent values that are significantly different at $p = 0.05$ based on ANOVA and Duncan's test

Shoot and Root Fresh Weights

The inoculation of plants by *F. oxysporum* f. sp. *radicis-lycopersici* only significantly decreased the fresh weights of plants, compared to plants treated by the bacterial isolates and inoculated (Table 3, Fig. 2). In fact, the treatment by CB3D, CB3B and CB5B2 isolates enhanced the shoot fresh weight of plants by more than 60%, in comparison to the inoculated but untreated control.

Root fresh weights increased for all treatments with antagonistic bacteria, compared to the untreated control. No significant improvement in root weight was noted on plants treated by CB7A1 and CB3B isolates, which were shown to be less effective in reducing disease severity (Table 2). The increase in the root fresh weights by these both isolates was about 89% compared to the control.

Identification of Bacterial Isolates

Based on the results of the API tests, the four most effective bacterial isolates tested, were identified as *Chryseomonas luteola* (CB7A1), *Serratia liquifaciens* (CB3B) and *Aeromonas hydrophila* for CB5B2 and CB3D.

DISCUSSION

The present study showed that some bacterial isolates obtained from an animal manure compost extract showed an inhibitory effect against the causal agent of the Fusarium Crown and Root Rot of tomato. Eight bacterial isolates, among the 14 tested, inhibited the pathogen growth by more than 38%. These results supported the findings of Phae *et al.* (1990), Stindt (1990), Ketterer (1990) and Weltzien (1992), who reported that compost extracts contain microorganisms, including bacteria with antagonistic potential against several pathogens.

In a previous work conducted *in vitro*, Kerkeni *et al.* (2007) showed that compost extracts used for isolation of tested bacterial isolates, also inhibited the growth of this same isolate of *F. oxysporum* f. sp. *radicis-lycopersici* by 42.6%. This suggests that compost extracts contain biocontrol agents that are more efficient when used alone as is the case of isolates CB3B (47%) and CB7A1 (44%).

In the bioassay, some tested isolates such as CB3D and CB5B2 were shown to be the most effective in reducing the Fusarium Crown and Root Rot severity and also in enhancing the plant growth parameters (height and shoot fresh weights). This suggests that these bacterial isolates are also able to promote the plant growth by induced plant resistance to inoculation and probably by affecting directly the pathogen by several modes of action and/or variable types of antifungal metabolites produced (Williams and Asher, 1996). In fact, Daami-Remadi *et al.* (2006) and Chérif *et al.* (2002) showed that antagonistic bacteria act by producing enzymes that cause hyphal alterations such as generalized cytoplasm disorganisation, complete protoplasm loss and fungal cell wall disintegration. Singh *et al.* (1999) reported that chitinolytic enzymes were important in the biological control of soil borne pathogens because of their ability to degrade fungal cell walls, of which a major component is chitin. These enzymes were proved to be involved in the antagonistic activity; they act by breaking down and dissolving the polysaccharides, responsible for the rigidity of fungal cell walls (Howell, 2003).

The effectiveness of bacteria isolated from compost, as biocontrol agents, against plant diseases was previously reported by Weltzien (1992), Ketterer (1990) and Stindt (1990). Phae *et al.* (1990), showed an antagonistic effect of some bacteria originating from compost, against several pathogens including *F. oxysporum* f. sp. *lycopersici*. Kwok *et al.* (1987) identified different species of bacteria such as *Bacillus cereus*, *Pseudomonas* sp. and *Flavobacterium balustinum* suppressive of *Rhizoctonia solani*. El-Masry *et al.* (2002) also isolated from compost several bacterial microorganisms such as *Bacillus* sp. with inhibitory effect against pathogens such as *Pythium debaryanum*, *Fusarium oxysporum* f. sp. *lycopersici* and *Sclerotinia bataticola*.

The four isolates CB3D, CB3B, CB7A1 and CB5B2, inhibiting by more than 40% the *in vitro* pathogen growth and shown to be effective in reducing disease severity *in vivo*, were identified as *Chryseomonas luteola* for CB7A1, *Serratia liquefaciens* for CB3B and *Aeromonas hydrophila* for CB5B2 and CB3D. The genus *Serratia* was found to possess antifungal properties (Kurze and Bahl, 2001). In the same way, Sneh *et al.* (1985) demonstrated that *Serratia liquefaciens* inhibited the Fusarium wilt of carnation. Inbar and Chet (1991) reported that strains of *Aeromonas* were effective biological control agents against *Sclerotium rolfsii* and *Rhizoctonia solani*. Further studies are required for elucidating the several modes of action of these bacterial isolates, quantifying their effect on disease incidence rather than on disease severity for their eventual test under field conditions and in naturally infected soils.

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