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Characterization and selection of *Bacillus* sp. strains, effective biocontrol agents against *Fusarium oxysporum* f. sp. *radicis-lycopersici*, the causal agent of Fusarium crown and root rot in tomato

Neila SAIDI[1]*, Soulwene KOUKI[1], Fadhel M'HIRI[2], Mohamed R. HAJLAOUI[3], Meriam MAHROUK[1], Hadda OUZARI[1], Naceur JEDIDI[1], Abdennaceur HASSEN[1]

[1]Centre de Recherches et des Technologies des Eaux, Laboratoire Traitement et Recyclage, B.P. 95-2050, Hammam lif, Tunis; [2]Centre International de Technologie de l'Environnement, Boulevard du leader Yasser Arafet, 1080 Tunis; [3]Laboratoire de Phytopathologie, Institut National de la Recherche Agronomique de Tunisie, Avenue Hédi El karray, Ariana, Tunis, Tunisia

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Abstract - The antagonistic activities of 20 *Bacillus* isolates were tested with dual culture and greenhouse conditions against *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) race 0, the causal agent of Fusarium crown and root rot of tomato. Under dual culture, 10 isolates inhibited mycelial growth > 38% and the most effective inhibited fungal growth > 50%. The 20 *Bacillus* isolates were tested for production of volatiles, cyanide, antibiotics, and phosphorus solubilisation; 15 isolates produced volatiles that inhibited growth of pathogens, 9 isolates produced cyanide, 10 produced antibiotics, and five solubilised phosphorus. Greenhouse experiments with the same 20 isolates revealed the effectiveness of 12 strains, which increased the percentage of healthy plants in the tested cultivar from 66 to 96%. The best disease control was achieved by isolates B11, B5, B17, and B18. However, B11 and B17 were the only isolates that produced cyanide, antibiotics, solubilised phosphate and showed 44% inhibition of fungal growth. The selected strains could be considered in plant growth promotion and biological disease control.

Key words: biocontrol, Fusarium root rot, tomato, *Bacillus* sp., seed bacterization.

INTRODUCTION

Fusarium crown and root rot of tomato, which is caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL), was discovered in Japan (Yamamoto *et al.*, 1974), and subsequently identified in many other regions, including North America, Europe, and Israel. In the United Kingdom, the disease was identified in 1988 (Hartman and Fletcher, 1991) and was subsequently confirmed in several nurseries (Green *et al.*, 2003). An increase in early injury to the roots and collar of tomato plants caused by FORL was also observed in Tunisia (Hajlaoui *et al*., 2001).

Fusarium crown and root rot of tomato is an important soil-borne disease, with the potential to limit productivity in greenhouses and open fields. Yield losses were ranged from 20-60% (Jarvis *et al.*, 1983). Chemical fungicides are typically used to manage the disease; however, repeated applications may be responsible for the appearance of fungicide-resistant strains of the bacteria (Benhamou and Belanger, 1998; Sierotzki and Ulrich, 2003).

* Corresponding Author. Phone: 216-71-788-436, 99-641-367; Fax: 216-71-410-740, E-mail: neila_saidi@yahoo.fr

Biological control of Fusarium crown and root rot of tomato, in the form of natural microbial populations in soils, has been recognized for over 70 years (Fravel *et al.,* 2003). Biological control in these 'supportive' soils primarily results from antagonistic and competitive abilities of natural microbial communities and is generally recognized to be among the best and most successful examples of biological controls (Cook and Baker, 1983). The potential of applying domestic compost to control crown and root rot of greenhouse-grown tomato caused by FORL was investigated by Pharand *et al*. (2001) and Reuveni *et al*. (2002). Indeed, several organisms antagonistic to soil-borne root pathogens have been isolated from composts (Suarez-Estrella *et al.,* 2007).

Bacillus spp. are attractive candidates for biological control agents because they produce active antagonistic metabolites, are abundant in soils, and readily form endospores that survive under adverse environmental conditions (Handelsman *et al.,* 1990; Silo-Suh *et al.,* 1994). *Bacillus* spp. are well known antibiotic producers with antagonistic activity against fungal and some bacterial pathogens (Krebs *et al.,* 1998; Yu *et al.*, 2002) and these traits appear to contribute to establishment and persistence of the antagonist in the plant (Krebs *et al.,* 1998). *Bacillus*

spp. generate spores that are easily formed, and have high viability compared to vegetative cells (Bochow *et al.,* 1995).

In addition to their antibiotic properties, *Bacillus* spp. enhance their antagonistic effects against fungal pathogens by competition or exploitation, which includes predation and direct parasitism (Muninbazi and Bullerman, 1998; Walker *et al.,* 1998). Parasitism is expressed through degradation of the cell walls of pathogenic fungi and relies on the production of extracellular lytic enzymes. For example, several *Bacillus* species produce enzymes that degrade chitin, the insoluble linear polymer of β-1,4-N-acetylglucosamine, which is the second most abundant polysaccharide in nature and a major component of most fungal cell walls (Bottone and Peluso, 2003). Not surprisingly, many of these species are also potential biocontrol agents against fungi.

We tested *Bacillus* isolates for production of volatiles, cyanide, antibiotics, and phosphorus solubilisation. Volatiles produced by bacteria can influence both fungal mycelial growth rate and enzyme activity (Fiddaman and Rossall, 1993) and inhibit spore germination in a variety of fungal species (Mackie and Wheatley, 1999). Cyanide is a volatile inhibitor of microbial growth (Bakker and Schippers, 1987). As already mentioned, some bacterial antagonists produce antifungal antibiotics (Sadfi *et al*., 2002). Most of these antibiotics are cyclic peptides composed entirely of amino acids, but some may contain other components (Katz and Demain, 1977). Phosphate solubilisation consists of liberating soluble phosphorus from insoluble $Ca(PO_4)_2$. Some bacteria are able to dissolve the mineral phosphate and to make it available for plants (Troeh and Thompson, 1993). Solubilisation of phosphates by bacteria and fungi is of practical importance (Cheng and Yang, 2009). Application of the phosphate-solubilising microbes *Agrobacterium*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Aspergillus* and *Trichoderma* around the roots of plants, in soils, and in fertilizers has been shown to release soluble phosphorus, promote plant growth, and protect plants from pathogen infection (Rodríguez and Fraga, 1999; Rudresh *et al*., 2005; Ouahmane *et al*., 2007).

This study selected and characterized isolates of *Bacillus* spp. with antagonistic activity to Fusarium crown and root rot of tomato caused by FORL. Bacilli were isolated from biosolids and mature compost and assessed for their effects on fungal growth and development of disease in dual cultures and under greenhouse conditions.

MATERIALS AND METHODS

Origin of microorganisms. Isolates of *Bacillus* were obtained from mature municipal compost or biosolids. Municipal compost was obtained after 5 months of aerobic treatment at the Centre International de Technologie de l'Environnement near Tunis. Compost was analyzed at the end of the composting cycle, yielding: pH, 8.1; total N (1.3%), C (17.8%), C/N (13.7), neutral detergent fibre of hemi-cellulose and cellulose + lignin (22.0%), acid detergent fibre of cellulose + lignin (20.0%), and neutral detergent fibre minus acid detergent fibre (= hemi-cellulose, 2.0%). Stabilized biosolids at Charguia, Tunisia were analyzed after aerobic treatment of urban wastewater, yielding: water content (30.0%), organic matter (65.0%), C/N (12.5).

A 5-g sample of municipal compost or biosolids was thoroughly mixed in 45 ml sterile water in sterile flasks. Spores from aerobic, Gram-positive bacteria were isolated after heating the suspensions at 80 °C for 10 min. Individual cultures were isolated by serially diluting the suspension and plating a portion of the diluent on Trypticase Soy agar (TSA, Difco Laboratories, Detroit, MI). Pure cultures were obtained by repeatedly restreaking isolated colonies on new TSA plates. Selected *Bacillus* strains were examined for Gram reaction, oxygen requirement, and catalase activity (Sneath, 1986). Endospore formation was tested on Nutrient Agar medium (NA, Oxoid, Basingstoke, Hampshire, UK) amended with 0.003% (wt/v) manganese sulphate (Logan and Berkely, 1984).

All isolates were purified and tested for ability to suppress the tomato disease pathogen (Swain and Ray, 2007). Here, 20 isolates were grown in Trypticase Soy broth (TSB) and subsequently frozen in TSB: glycerol medium (70:30; v:v) at –20 °C. The origin and date of collection for each isolate are indicated in Table 1. FORL strain Tn2, which is pathogenic to tomato, was obtained from the Laboratoire de Phytopathologie, Institut National de la Recherche Agronomique de Tunisie (Hajlaoui *et al.,* 2001).

Screening in dual culture of antagonists for antifungal activity. Tests of antagonism were performed on potato dextrose agar (PDA) in 10-cm Petri plates using the dual culture technique (Swain and Ray, 2007). *Bacillus* isolates from an overnight culture lawn were streaked across the centre of the plate, with a second streak made at right angles to the first. Four agar plugs, each 5 mm in diameter, were cut from the edge of 7-day-old culture of *F. oxysporum* and placed at each side of the antagonist. The distance between the inoculation sites was 2.5 cm. Plates were incubated at 25 °C for one week and the percent growth inhibition (GI) of *F. oxysporum* calculated (Whipps, 1987): (R1–R2) R1 x 100, where R1 is the farthest radial distance (measured in mm) grown by *F. oxysporum*, in the direction of the antagonist (the control value), and R2 is the distance of fungal growth from the point of inoculation to the colony margin in the direction of the antagonist. Inhibition of growth was categorized on a 0 to 3 scale: 0 (no inhibition), 1 (1-25%), 2 (26-50%), and 3 (51-75%) (Korsten *et al.,* 1995).

Systematic identification of isolated antagonistic bacteria. The selected microbes were identified according to the method described in *Bergey's Manual of Determinative Bacteriology* (Sneath, 1986), based on morphological, physiological, and biochemical properties of the *Bacillus* genus. Morphologically, the isolated strains formed wrinkled colonies on TSA and vegetative cells or spores were found in liquid culture. Biochemically, the isolates were Gram positive, formed endospores, and were motile. Complementary biochemical traits were determined by using the API-50 CHB test (API System, Montalieu-Vericien, France).

Detection of antifungal volatiles. Production of volatile compounds by the selected *Bacillus* strains was assayed by the sealed plate method (Fiddaman and Rossal, 1995). From 72-h TSB cultures of *Bacillus*, a 200-µl portion was spread on a TSA plate. After incubation at 37 °C, a second Petri dish (containing PDA), was inoculated with 6-mm plug of the test fungus in the center of the plate, inverted, and placed over the bacterial culture. The two plates were sealed together with Parafilm (Pechiney Parafilm M PM996 SKU: PH-LF) to prevent gas diffusion and further incubated at 25 °C. This incubation ensured that both organisms were growing in the same atmosphere although physically separated. As a control, a Petri dish containing TSA without bacteria was placed over the fungal pathogen. Fungal growth was measured as increases in radial growth of the test fungus at 24-h intervals for 5 days. Each test was replicated 3 times.

Cyanide production. Cyanide production was detected using the assay method of Bakker and Schippers (1987), where 10% TSA containing 4.4 g glycine liter^{-1} was inoculated with bacterial cultures. The lid of each Petri dish contained filter paper impregnated with a picric acid solution (0.5% picric acid and 2% sodium carbonate), which was sealed to the bottom Petri dish with Parafilm (Pechiney Parafilm M PM996) and incubated at 28 °C for 3 to 5 days. A change in colour from yellow to orange-brown of the impregnated filter paper indicated cyanide production.

Antibiotic assays. *Bacillus* isolates were streaked on TSA and incubated at 30 °C for 24 h. A loop of inoculum from the overnight culture was introduced into 100 ml production medium (20 g glucose, 5 g DL-glutamic acid, 1.02 g Mg $SO_4 \cdot 7H_2O$, 1.0 g K_2HPO_4, 1.5 g KCl, and 1 ml trace element solution containing 0.5 g $MnSO_4 \cdot H_2O$; 0.16 g $CuSO_4 \cdot 5H_2O$ and 0.015 g $FeSO_4 \cdot 7H_2O$ in 100 ml water; pH adjusted to 6.0-6.2) according to McKeen *et al.* (1986). The inoculated medium was incubated for 60 h in an incubator shaker maintained at 30 °C and 170 rpm. Bacterial suspensions were centrifuged at 10000 *x g* at 4 °C for 10 min. Each supernatant was filtered through a sterile 0.45-µm filter membrane. The cell filtrates were assayed for their ability to inhibit mycelial growth of *F. oxysporum* strain Fo2 by using an agar well diffusion method (Tagg and McGiven, 1971). Molten PDA kept at 45 °C was seeded with conidia of *F. oxysporum* and a 5-ml portion spread uniformly over nutrient agar medium (NA, Oxoid). After the seeded layer solidified, three wells were aseptically made using a cork borer, and filled with 100 µl of the test filtrate. The control consisted of 50 µl filter, sterilized distilled water. The samples were allowed to diffuse into the agar and the plate was inverted and incubated at 28 °C for 24h. The plates were examined for halos of inhibition around the wells (Tagg and McGiven, 1971).

Phosphate solubilisation. P-solubilisation was quantified as described by Gupta *et al.* (1994). One loop of spore suspension was inoculated onto Pikovaskey medium containing bromophenol (in g 1000 ml^{-1}): glucose (10.00), $Ca(PO_4)_2$ (5.00), $(NH_3)_2SO_4$ (0.50), NaCl (0.20), $MgSO_4 \cdot 7H_2O$ (0.10), KCl (0.20), yeast extract (0.50), $MnSO_4$ (0.005), $FeSO_4 \cdot 7H_2O$ (0.005), and 15 g agar). A 25-mL portion of bromophenol (0.5%) was added to the medium and the pH adjusted to 7.0-7.2. Each plate was incubated for 96 h at room temperature. After incubation, yellow-colored halos were observed. These halos formed around the individual bacterial colony in response to the pH drop produced by the release of organic acids by microorganisms and were responsible for P-solubilisation.

Greenhouse experiments. Tomato seeds (*Lycopersicum esculentum* cv *Chourouk*) were surface-sterilized by immersion in 2.5% sodium hypochlorite solution for 2-3 min, then washed thoroughly in three changes of sterile distilled water. The seeds were pre-germinated for three days in Petri dishes containing sterile distilled water. Four seedlings were transplanted into each plastic pot (200 cm^3, with four pots for each treatment). The soil was a clay loam from the Mornag experimental farm near Tunis, Tunisia and contained: clay (27%), silt (62%), sand (11%), C (0.87%), N (< 0.01%), and C/N (9.15). The soil was air-dried and passed through a 2-mm sieve. The positive control was a FORL-inoculated soil with 10^5 spores g^{-1} soil dry weight while the soil of the negative control was mixed with 2 ml of a non-inoculated solution. For each treatment involving a *Bacillus* sp., the seeds were coated with a liquid suspension (~10^7 CFU ml^{-1}) of the appropriate isolate.

The study was conducted in triplicate in a greenhouse at 25 ± 5 °C at 60-90% relative humidity for 6 weeks. Plants were watered as needed and fertilized weekly with 100 ml Hoagland's nutrient solution. Samples were harvested to assess the effect of the *Bacillus* on growth parameters. Shoots and roots of each plant were separated and dried at 70 °C. Shoot elongation was also recorded. Plant N was determined with a Kjeldahl procedure and distilled with NaOH (30%; NF EN 25663, January 1994), total and Olsen-extractable P was determined according to standard methods (APHA, 1989), and total and water-extractable K was determined with an atomic absorption spectrophotometer.

Enumeration of *Bacillus* sp. in root of tomato. To determine whether each *Bacillus* sp. applied to tomato seeds grew systemically in the root system, the number of *Bacillus* cells was determined 6 weeks after sowing from a random sample of three replicates. Enumeration of bacilli was determined from samples of tomato roots by dilution and plating on a TSA medium. A 5-g sample of root (fresh weight) was ground in 10 ml 0.85% NaCl solution. The mixture was agitated mechanically at 240 rpm for 1 h, serially diluted in sterile 0.85% NaCl solution, and heated to 80 °C for 10 min. After cooling, a volume of 0.5 ml from the dilution was plated on TSA medium and incubated at 30 °C for 48 h. Bacilli were counted and $\log_{10}$-transformed before expressing the result as CFU cm^{-1} root (log CFU cm^{-1}). Dry weight was determined by cutting the root into 1-cm long pieces and drying at 100 °C to constant weight.

Disease assessment and data analyses. All experiments were replicated in completely randomized blocks with three replicates. Statistical analyses were performed with proprietary software (SPSS Statistics v 10, SPSS, Chicago, IL, USA), and means were separated by the least significant difference according to the Student-Newman-Keuls test.

RESULTS AND DISCUSSION

Dual culture screening

Of the 20 *Bacillus* isolates tested in dual culture, 12 inhibited fungal growth > 30%; isolates B11, B13, and B17 inhibited fungal growth >50%. Fifteen isolates produced volatiles that inhibited growth of pathogens (Table 1). Most *Bacillus* isolates were unable to solubilise phosphorus. Only B3, B6, B11, B14, and B17 produced a halo on agar plates containing $CaHPHO_3 \cdot 2H_2O$. Eleven isolates capable of producing antibiotics were positive for cyanide production. B12 and B17 were positive for phosphorus solubilisation, antibiotic production, cyanide production, and produced the highest level of inhibition when tested for volatiles.

All *Bacillus* strains were Gram-positive, required oxygen, and formed spores. All strains produced catalase and were indole-negative (Sneath, 1986). Based on the API system identification, all strains were *Bacillus* sp.

Different studies have implicated antifungal secondary metabolites produced by *Bacillus* in the control of plant diseases caused by pathogenic fungi (Duijff *et al.,* 1998; Lucas-García *et al.,* 2004; Szcrech and Shoda*,* 2004). In our study, reduction of fungal growth in dual culture by certain *Bacillus* isolates and formation of inhibition zones were presumably due to the metabolites released by the

TABLE 1 - Effect of *Bacillus* sp. isolates in dual culture with *Fusarium oxysporum* f. sp. *radicis-lycopersici* and ability to solubilize phosphate, to produce cyanide, volatiles and antibiotic

Bacillus isolates	Origin	Collection date	Identification of the isolates	Dual culture assay					
				Mycelial inhibition (%)	GI category*	Fungal inhibition by volatiles (%)	Cyanide production	Antibiotic production	Phosphate solubilisation
B1	compost	1999	*B. stearothermophilus*	40.73[a]	2	9.92[a]	-	+	-
B2	compost	1999	*B. cereus*	37.44[b]	2	8.67[bc]	-	-	-
B3	compost	1999	*B. cereus*	38.54[c]	2	8.90[c]	+	-	+
B4	compost	1999	*B. licheniformis*	33.83[d]	2	5.34[d]	+	+	-
B5	compost	2002	*B. mycoides*	18.06[e]	1	4.66[e]	-	+	-
B6	compost	2002	*B. sphaericus*	0.00[f]	0	0.00[f]	-	+	-
B7	compost	2002	*B. sphaericus*	0.00[f]	0	0.00[f]	+	-	-
B8	compost	2003	*B. cereus*	42.73[h]	2	7.68[g]	+	+	-
B9	compost	2003	*B. cereus*	42.30[j]	2	8.99[b]	-	+	-
B10	Biosolids	2003	*B. sphaericus*	0.00[f]	0	0.00[f]	+	-	-
B11	Biosolids	2003	*B. subtilis*	66.33[g]	3	44.77[h]	+	+	+
B12	Biosolids	2003	*B. sphaericus*	0.00[f]	0	0.00[f]	+	-	-
B13	Biosolids	2003	*B. licheniformis*	0.00[f]	0	0.00[f]	+	-	-
B14	Biosolids	2003	*B. subtilis*	46.80[l]	2	11,55[f]	+	-	-
B15	Biosolids	2003	*B. sphaericus*	0.00[f]	0	0.00[f]	-	-	-
B16	Biosolids	2003	*B. stearothermophilus*	41.00[l]	2	7.07[k]	-	-	-
B17	Biosolids	2003	*B. sphaericus*	66.33[g]	3	52.78[l]	+	+	+
B18	Biosolids	2003	*B. cereus*	7.06[m]	1	1.08[m]	-	+	-
B19	Biosolids	2003	*B. cereus*	41.00[i]	2	6.67[n]	-	+	-
B20	Biosolids	2003	*B. cereus*	15.56[n]	1	4.79[e]	-	+	-
Total inhibitory bacteria					15				

(+): Presence of inhibition zone (-): Absence of zone inhibition. Percent growth inhibition was determined after 7 days incubation using Whipps (1987) formula. Inhibition of growth was categorised on a 0 to 3 scale: 0 (no inhibition), 1 (1-25%), 2 (26-50%) and 3 (51-75%) (Korsten *et al.*, 1995). Values followed by the same letter are not significantly different according to students Newmans Keuls test (P≤0.05). The results of the column indicating the identify of the selected isolates were achieved by API system and that the named species are only an indication.

TABLE 2 - Effect of *Bacillus* isolates on Fusarium crown and rot of tomato inidence in tomato cultivar *Chourouk* inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici* 6 weeks after sowing

Treatment	Healty plants (%)	log CFU/cm roots
Control	98.54[a]	2.04[a]
FORL (positive control)	0.00[b]	1.04[b]
B1	66.33[c]	5.72[c]
B2	66.33[c]	6.12[d]
B3	80.29[d]	5.83[c]
B4	54.21[e]	6.09[d]
B5	83.46[d]	4.36[e]
B6	0.00[b]	1.04[b]
B7	0.00[b]	1.23[b]
B8	78.21[f]	5.34[c]
B9	66.37[c]	6.13[d]
B10	0.00[b]	1.22[b]
B11	96.85[a]	8.25[f]
B12	0.00[b]	1.34[b]
B13	70.83[c]	9.28[g]
B14	33.36[g]	6.89[h]
B15	0.00[b]	5.99[i]
B16	70.86[c]	5.99[i]
B17	83.36[d]	9.22[g]
B18	83.33[d]	4.32[e]
B19	54.63[e]	6.48[j]
B20	79.23[f]	4.47[e]

log CFU/cm roots: expressed the numbers of *Bacillus* quantified at the level of tomato root by dilution plating on TSA medium and expresed as logarithmic transformed CFu per cm root (log CFU/cm).
Column with a treatment values are averages ot three observations. Means followed by the same letter are not significantly different according to the Student Newmans Keuls test (P<0.05).

TABLE 3 - Effect of *Bacillus* isolates on growth parameters in tomato plant and seedlings inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici* under greenhouse conditions 6 weeks after sowing

	N (g Kg^{-1})	P (g Kg^{-1})	K (g Kg^{-1})	Root (g)	Shoot (cm)
Control	7.2[a]	5.5[a]	20.3[a]	4.90[a]	20.60[a]
B1	8.4[b]	5.8[b]	14.7[b]	8.14[b]	29.80[b]
B2	8.4[b]	5.8[b]	14.7[b]	8.67[c]	29.70[b]
B3	8.4[b]	5.8[b]	14.7[b]	8.73[d]	31.37[c]
B4	7.2[a]	4.5[c]	15.5[c]	8.67[c]	31.11[c]
B5	8.4[b]	5.8[b]	14.7[b]	6.33[e]	24.60[d]
B6	7.2[a]	5.5[a]	20.3[a]	5.34[f]	22.00[e]
B7	7.2[a]	5.5[a]	20.3[a]	5.23[g]	22.00[e]
B8	8.4[b]	5.8[b]	14.7[b]	8.67[c]	30.40[f]
B9	8.4[b]	5.8[b]	14.7[b]	8.76[h]	29.60[b]
B10	7.2[a]	5.5[a]	20.3[a]	5.23[g]	23.20[g]
B11	9.2[c]	6.0[b]	13.5[d]	9.89[i]	34.60[h]
B12	7.2[a]	5.5[a]	20.3[a]	5.23[j]	21.90[e]
B13	8.4[b]	5.8[b]	14.7[b]	9.70[k]	33.20[i]
B14	7.2[a]	4.5[c]	15.5[c]	8.14[b]	29.80[b]
B15	7.2[a]	5.5[a]	20.3[a]	9.89[l]	22.70[j]
B16	8.4[b]	5.8[b]	14.7[b]	8.67[c]	28.50[k]
B17	8.4[b]	5.8[b]	14.7[b]	10.13[m]	34.60[h]
B18	8.4[b]	5.8[b]	14.7[b]	6.34[e]	24.60[d]
B19	7.2[a]	4.5[c]	15.5[c]	8.67[c]	24.60[d]
B20	8.4[b]	5.8[b]	14.7[b]	6.34[e]	28.37[k]

Shoots and roots of each plant were separated and dried at 70 °C. Shoot dry weight and shoot elongation were recorded 6 weeks after sowing. Roots expressed in g. Shoot expressed in cm. Means of replication three replicates. Value within treatment envisaged, data followed by the same letter are statically identical according to students Newmans Keuls test (P<0.050).

bacteria into the culture medium. These metabolites may include antibiotics and/or cell-wall degrading enzymes.

In our study, among *Bacillus* tested strains, only one was effective against *Fusarium* tomato infestation. Moreover, *Bacillus* strains were tested for volatiles, cyanide production, and solubilisation of phosphorus because of the potential implication of such traits in promoting plant growth (Bakker and Schippers, 1987; Glick *et al.,* 1995). Our experiments showed that B17 strain was able to produce cyanide and antibiotics at the same time; while, some other isolates (B6 and B15) were not effective against FORL and did not produce cyanide.

Similarly, some of *Bacillus* spp. strain solubilise phosphorus in soil, thereby increasing crop yield (Rodriguez and Fraga, 1999). In our study, the B17 isolate gave an indication of P-solubilisation.

Greenhouse experiment

Seeds coated with *Bacillus* (15 of the 20 isolates tested) significantly increased the population of healthy plants from 33 to 96.8% (Table 2). The best disease control occurred with isolates B4, B5, B17, and B18, which reduced the Fusarium crown and root rot of tomato incidence to < 20%. The highest growth response (root dry weight and shoot) was obtained with the strains B11 and B17. The initial counts for all *Bacillus* strains were higher than the control. Overall, the B11 strain showed the highest level of protection; indeed, 96.8% of the plants inoculated with this strain remained healthy. In second position was the B17 strain with 83.3% of the inoculated plants remaining healthy.

Tomato seeds treated with B11 and B17 strains showed dramatic increases in growth parameters compared with the control. Values recorded for all growth parameters except K were significantly higher (Table 3). On the other hand, inoculation significantly decreased K content in shoots. This result is not in agreement with Jaizme-Vega *et al.* (2004), who reported that inoculation with *Bacillus* significantly increased K content. Inoculation of seeds with *Bacillus* increased root dry weight significantly by > 1.2 times compared with the control treatment. Shoot length was almost double when inoculated with the B11 strain.

Screening in dual culture positively correlated with *in vivo* results, excepting for the strain B18, which was effective under greenhouse conditions but showed weak inhibition of radial growth fungi. Our results were not in agreement with those of

TABLE 4 - Correlation between disease incidence DI with all others studied parameters (N, P, K; root, shoot colonisation and disease incidence

	N	P	K	Shoot	Disease incidence	Root
N	1.000	0.718*	0.802*	0.694*	0.882*	0.572*
P	-	1.000	-0.182	0.181	0.351	0.027
K	-	-	1.000	-0.814*	-0.967*	-0.778*
Shoot	-	-	-	1.000	0.772*	0.857*
Disease Incidence	-	-	-	-	1.000	0.717*
Root colonisation	-	-	-	-	-	1.000

* Correlation is significant at the 0.01 level.

Chérif *et al.* (2002), who reported that antagonistic microorganisms performing best in dual culture are not necessarily the most effective in the host plant, and vice versa.

Greenhouse experiments showed that adding FORL (positive controls) to soil at 10^5 spores g^{-1} soil (dry weight) reduced plant growth and caused severe wilting in the susceptible cultivar. Interestingly, application of most *Bacillus* isolates significantly reduced the wilting index and increased plant growth. These bacteria also increased N content and dry weight of roots and shoots. Beneficial effects of *Bacillus* on plant growth and reduction of diseases was also reported by Raj *et al.* (2003) and Rodríguez-Romero *et al.* (2005).

Relationship between nutrition parameters and incidence of disease

The incidence of the disease, shown in Table 4, correlates with growth parameters. A significant correlation was observed between incidence of disease with N (r = 0.88), shoot elongation (r = 0.77), and root colonization by *Bacillus* strains (r = 0.71). The negative correlation (r = –0.96) was recorded for potassium content. No significant correlations were found between phosphorus content and other parameters, including nitrogen, shoot tomato elongation, and colonization of roots by *Bacillus*.

Bacillus isolates B3, B5, B11, B17, B18, and B11 were effective in promoting tomato growth, leading to an increase in shoot and root dry weights compared to the controls. These benefits are likely to result from disease control in the presence of the *Bacillus* strains and their by-products, including additional nutrients (McSpadden-Gardener, 2004). For many other plants like, tomato, bell pepper, muskmelon, watermelon, sugar beet, tobacco, *Arabidopsis* sp., cucumber, loblolly pine, and two tropical crops, different *Bacillus* species were reported to be effective biocontrol agents in greenhouse or field trials (Stabb *et al.,* 1994; Kloepper *et al.,* 2004). Considering these data, our results highlight the widespread of biocontrol potential among *Bacillus* strains and may enable to the use of the selected isolates for Fusarium crown and root rot of tomato biocontrol.

CONCLUSION

Biological control agents offer an attractive alternative to synthetic chemical fungicides. These agents can be safer and less expensive to develop than synthetic chemical fungicides. Disease control in this study is attributed to beneficial/antagonistic microorganisms (*Bacillus* spp.) and their by-products, the metabolites and antibiotics that suppress plant pathogens and activate natural plant defence responses.

Our results reported the selection of two *Bacillus* strains (B11 and B17) for the biological control of Fusarium crown and root rot of tomato. They could be introduced within an integrated disease management package that includes moderately resistant cultivars, limited fungicide application, and effective cultural practices.

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