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Effects of Some *Bacillus* sp. Isolates on *Fusarium* spp. *in vitro* and Potato Tuber Dry Rot Development *in vivo*

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Abstract: Four *Bacillus* sp. isolates were individually essayed against *Fusarium solani, F. oxysporum* f.sp. *tuberosi*, *F. graminearum* and *F. sambucinum* following an *in vitro* dual culture plate technique and *in vivo* pre-inoculation tuber treatment. All tested bacterial isolates significantly reduced radial mycelial growth of *Fusarium* spp., on PDA after 3 days of incubation at 25°C, comparatively to the untreated controls. Light microscopic studies of antagonist x *Fusarium* spp. *in vitro* interaction showed several hyphal abnomalies traduced particularly by lesser mycelium density, severe hyphal lysis, lesser sporulation, mycelial cords formation and early chlamydospores induction observed only at the confrontation zone. Potato tubers, cv. Spunta, treated by *Bacillus* sp. 24 h before individually inoculation by *Fusarium* species, showed reduced dry rot development after 21 days of incubation at 25-27°C comparatively to untreated controls.

Key words: Biocontrol, *Bacillus* sp., *Fusarium* spp., interaction, *Solanum tuberosum* L.

INTRODUCTION

In Tunisia, most part of season potato production is stored for local market supplies from June to November. Tuber storage is particularly traditional (Rejeb El Gharbi and El Fahem, 2004) where many fungal mixed infections may occur. In fact, species of *Fusarium* spp. causal agents of dry rot and/or *Pythium* spp. and *Phytophthora erythroseptica* causing tuber leak and pink rot are frequently observed (Daami-Remadi and El Mahjoub, 1996a, 2004, 2006; Daami-Remadi *et al*., 2006; Priou and El Mahjoub, 1999; Chérif *et al*., 2001). Absence of resistant cultivars to *Fusarium* and to other tuber rot agents (Daami-Remadi and El Mahjoub, 1996b; Priou *et al*., 1997), absence of registered fungicides for control of these post-harvest problems (Anonymous, 2003), introduction of resistant isolates of *F. sambucinum* via contaminated seeds (Daami-Remadi and El Mahjoub, 2006; Daami-Remadi *et al*., 2006) and pathogen's soil borne origin justify necessity of searching for other alternatives for tuber protection.

Some benzimidazole fungicides, used in other countries for *Fusarium* dry rot control (Leach and Nielsen, 1975; Carnegie *et al*., 1990; Bang, 1992; Carnegie *et al*., 1998), showed varying interaction with Tunisian *Fusarium* sp. Isolates of *F. solani*, *F. oxysporum* f.sp. *tuberosi* and *F. graminearum* are sensitive to these fungicides but those of *F. sambucinum* are resistant (Daami-Remadi and El Mahjoub, 2006). Risk of fungicide resistance development concerns the entire fungal complex associated to several tuber rots such as *Fusarium* spp., *Pythium* spp. and *Phytophthora erythroseptica* (Triki *et al*., 1996; Daami-Remadi and El Mahjoub, 1997; Triki and Priou, 1997; Chérif *et al*., 2001; Daami-Remadi, 2001a, Peters *et al*., 2001; Peters *et al*., 2003; Taylor *et al*., 2004). Cultural methods alone are not sufficient to effectively control the disease.

In the *Fusarium* spp.- potato pathosystem, many authors studied bacterial antagonism against these tuber rot agents. Gram negative bacteria such as *Pseudomonas*, *Enterobacter* and *Pantoea*, isolated from suppressive soils or with less incidence of dry rot, are effective against thiabendazole resistant strains *F. sambucinum* (Schisler and Slininger, 1994, Schisler *et al*., 1994).

Microorganisms, naturally present on potato tubers, at harvest or during refrigerated storage, are tested *in vitro* against potato pathogens development. Harbour and Wastie (1996) held 35, on about 800 microorganisms, able of inhibiting mycelial growth of *Rhizoctonia solani*,

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Phoma foveata and *F. coeruleum* by more than 5 mm and their post-harvest incidence. Furthermore, other authors found that some strains of *Pseudomonas fluorescens* and *Enterobacter cloacae* inhibited *F. sambucinum in vivo* in comparison to untreated and fungicide treated controls. However, these strains are ineffective against *F. coeruleum* showing existence of an interaction between *Fusarium* and bacterial antagonists in the *Fusarium* spp. potato pathosystem (Schisler *et al*., 1996; Schisler *et al*., 2000). Some bacterial antagonists showed synergistic effects when used in mixtures. In fact, Schisler *et al*. (1997) found that *Enterobacter cloaca*, *Pantoea agglomerans*, *Pseudomonas corrugata* and *P. fluorescens*, tested individually or in dual mixtures, reduced dry rot development induced by *F. sambucinum* by 38 to 76%, respectively.

All studies dealing with biological control of potato tuber dry rot concerned in their majority one *Fusarium* species or two (Schisler *et al*., 2000). Furthermore, tuber incubation during *in vivo* studies of *Fusarium* x bacterial antagonist interaction was realized at 15°C, optimal temperature for dry rot and antagonist development. However, in Tunisia, *F. solani*, *F. oxysporum* f. sp. *tuberosi*, *F. sambucinum* and *F. graminearum* are the causal agents of this disease and they are frequently present as mixed infections (Daami-Remadi and El Mahjoub, 2004, 2006, Daami-Remadi *et al*., 2006). Furthermore, traditional tuber storage occurs at environmental temperatures exceeding 25°C and reaching sometimes 40°C and more. Consequently, our research is concentrated to bacterial antagonists supporting these thermal conditions of a traditional storage.

Bacteria belonging to the genus *Bacillus* form endospores that tolerate extremes conditions of pH, temperature and osmotic pression (McSpadden Gardener, 2004; Dricks, 2004; Fritze, 2004). *Bacillus* species are able of colonizing root surfaces, promoting plant growth and causing mycelium lysis of several fungal agents (Turner and Backman, 1991; Walker *et al*., 1998; Emmert and Handelsman, 1999; Kiewnick *et al*., 2001; Basha and Ulaganathan, 2002). Furthermore, *Bacillus* are used against pre- and post-harvest pathogens in several pathosystems (Korsten *et al*., 1997; Mari and Guizzardi, 1998; Kiewnick *et al*., 2001).

In Tunisia, Sadfi *et al*. (2001) and Chérif *et al*. (2002) tested *in vitro* and *in vivo* antagonism of several *Bacillus* isolates, collected from salty soils, against *F. sambucinum* and determined their mechanisms of action. However, interaction of *Bacillus* sp. with *F. solani*, *F. graminearum* and *F. oxysporum* f.sp. *tuberosi*, the other pathogens causing tuber dry rot, has never been investigated in Tunisia. As, in our natural conditions, potato dry rot is caused by a *Fusarium* species complex, as mixed infections, the present study concerns antagonists supporting conditions of a traditional storage and able of inhibiting the entire *Fusarium* complex.

MATERIALS AND METHODS

Isolates of *Bacillus* sp.: *Bacillus* sp. isolates are obtained during tuber dry rot agent isolations on PDA and are tested against *Fusarium* spp. following other technique of confrontation (Daami-Remadi, 2001b). They induced on PDA formation of inhibition zone against *Fusarium* spp. These bacteria are resistant to streptomycin-sulphate at 300 mg L^{-1}. Four isolates B1, B2, B3 and B7 are chosen within twenty collected showing antagonism against *Fusarium* spp.

Bacterial isolates are cultured on Nutrient Agar (NA) for two days before use. They are maintained at -20°C in NA containing 40% of glycerol for long term storage (Kim *et al*., 1997).

Pathogens: *F. solani*, *F. graminearum*, *F. sambucinum* and *F. oxysporum* f.sp. *tuberosi* are isolated on 2003 and 2004 from tubers of cv. Spunta showing typical symptoms of dry rot. These *Fusarium* spp. are grown at 25°C on PDA for one week. They are stored at-20°C in 20% glycerol solution for long term preservation.

Potato cultivars: Tubers cv. Spunta, the most cultivated in Tunisia, are used in this current study. They are obtained, on 2004, from the Technical Centre of Potato of Tunisia, stored in darkness at 6°C and bought to room temperature three hours before use.

***In vitro* activity of *Bacillus* sp. isolates against *Fusarium* spp.:** Isolates of *Bacillus* sp. are applied on PDA (containing 300 mg L^{-1} of streptomycin-sulphate) following two perpendicular axes by injecting 10 μL of bacterial suspension (10^7 bacteria mL^{-1}). Pathogen is placed at four equidistant sites of a petri dish. Control cultures are treated similarly by replacing bacterial suspension with sterile distilled water. Every elementary treatment is repeated eight times.

Mean diameter of pathogen colonies is measured after 3 days of incubation at 25°C and any morphological alteration of colonies, in comparison to untreated control, is also noted. Damage caused by the bacterium to the fungal mycelium, removed from confrontation zone of both microorganisms (pathogen and antagonist), is observed under light microscope, in comparison to untreated controls.

Statistical analyses (ANOVA) are performed following a completely randomised factorial design where treatments (bacterial isolates and untreated control) and *Fusarium* sp. are both fixed factors. Means are separated using Fisher's protected LSD test ($p \leq 0.05$).

***In vivo* inhibitory activity of *Bacillus* sp. isolates against *Fusarium* spp.:** Tubers are superficially disinfected with a solution of 10% sodium hypochlorite, for 5 min and then rinsed abundantly with sterile distilled water. After air-drying, tubers are dipped in an alcohol solution (at 70%) than briefly blazed for elimination of surface pathogens (*Rhizoctonia solani* and others).

Container and alveolus plaques used for inoculated tubers incubation, are washed before use, dipped for 24 h in sodium hypochlorite solution then rinsed with sterile distilled water.

As *Fusarium* sp. are wound tuber pathogens, *Bacillus* sp. isolates are applied by injecting 100 µL of a bacterial suspension (10^7 bacteria mL^{-1}) at sites of inoculation 24 h before pathogen application. Dimension of inoculation sites is of 6 mm diameter and depth. Inoculation technique consists of deposing an agar disc (6 mm diameter) colonized by pathogen at occasioned wounds. Tuber incubation is realized, in a growth chamber, at 25-27°C for 21 days at high relative humidity. Every elementary treatment is repeated twenty times (ten tubers x two wounds).

After incubation period, tubers were cut longitudinally via sites of inoculation. Parameters of dry rot induced (maximal width (w) and depth (d)) are noted. The pathogen penetration into tubers is calculated following formula of Lapwood *et al.* (1984) where:

$$\text{Penetration (mm)} = (w/2 + (d - 6))/2$$

Statistical analyses (ANOVA) are performed following a completely randomised factorial design where treatments (bacterial isolates and untreated control) and *Fusarium* sp. are both fixed factors. Means are separated using Fisher's protected LSD test ($p \leq 0.05$).

RESULTS

***In vitro* interaction of *Bacillus* isolates with *Fusarium* spp.:** Mean colony diameter of four *Fusarium* species, noted after 3 days of incubation at 25°C, depends upon pathogens tested and treatments realized. A significant interaction is observed between both fixed factors ($p \leq 0.05$).

Bacillus sp. isolates applied, in dual culture with pathogen on PDA, have significantly inhibited, with variable degrees, mycelial growth of *Fusarium* spp. comparatively to the untreated control (Table 1).

Table 1: Effect of some *Bacillus* sp. isolates on mean radial growth (cm) of *Fusarium* spp. causing potato dry rot (incubation at 25°C for 3 days, PDA, means of eight replicates per elementary treatment)

Treatments	Control	B1	B2	B3	B7
F. graminearum	3.01	2.70	2.52	2.35	1.08
F. sambucinum	1.56	1.36	1.30	1.22	0.90
F. oxysporum	1.55	1.53	1.41	1.26	1.30
F. solani	1.07	0.91	0.97	0.62	0.80

LSD (Treatments x *Fusarium* spp.) = 0.20 cm ($p \leq 0.05$). B1, B2, B3 and B7: *Bacillus* sp. isolates

Table 2: Effect of *Bacillus* sp. isolates, applied 24 h prior tuber inoculation, on development of dry rot occasioned by *Fusarium* spp. in comparison to untreated controls as measured by the mean pathogen penetration (mm) into tubers

Treatments	Control	B1	B2	B3	B7
F. graminearum	18.4	7.3	3.9	4.0	5.8
F. sambucinum	8.4	8.0	9.2	7.7	6.6
F. oxysporum	5.7	4.8	3.9	3.6	4.3
F. solani	8.4	4.5	4.1	4.0	4.5

LSD (Treatments x *Fusarium* spp.) = 1.54 mm ($p \leq 0.05$). B1, B2, B3 and B7: *Bacillus* sp. isolates, (Tubers cv. Spunta, incubation at 25-27°C for 21 days, 20 replicates per elementary treatment)

Mycelial growth reduction reached 64 and 42% for *F. graminearum* and *F. sambucinum* treated by isolate B7 and *F. solani* treated by B3 isolate, respectively in comparison to untreated control (treated similarly with sterile distilled water).

This reduction of mycelial growth of most *Fusarium* spp., induced by *Bacillus* sp. isolates, is also accompanied by morphological alterations of mycelial filaments. In fact, treated pathogen colonies showed lesser mycelial density at bacterium confrontation zone (Fig. 1).

Furthermore, light microscopic observations realized for elementary treatment on pathogen mycelium developed at the confrontation zone, showed several mechanisms of action of these antagonists. In fact, reduction of mycelial density, diminution of *Fusarium* spp. sporulation, generalized mycelium lysis, vacuolisation and early chlamydospores formation were observed only on treated colonies. Some hyphal filaments form mycelial cords via an anastomose mechanism showing stressed behaviour of treated pathogens (Fig. 2).

***In vivo* interaction of *Bacillus* isolates with *Fusarium* spp.:** The effect of treatment, 24 h before, of inoculation sites of tubers (cv. Spunta) with suspensions of *Bacillus* sp. is assessed on dry rot development. Mean penetration, noted after 21 days of incubation at 25-27°C, depends upon treatments applied and *Fusarium* species used for tuber inoculation (Table 2). A significant interaction is observed between both fixed factors ($p \leq 0.05$). For *F. graminearum* and *F. solani* (Fig. 3), *in vivo* development was limited, respectively, by more than 60 and 46% by isolates of *Bacillus* sp. but lesser inhibition was observed in the case of *F. sambucinum* that seems to be more sensitive to isolate B7 of *Bacillus* sp.

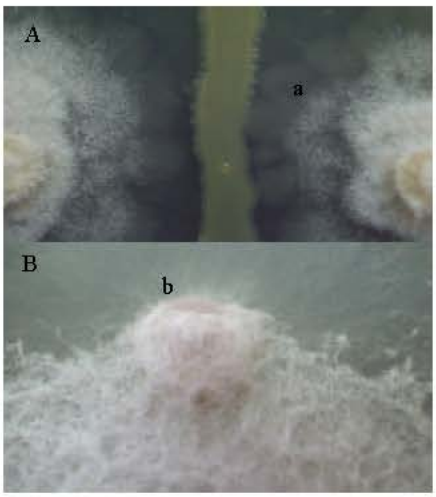

Fig. 1: Reduced mycelial growth and density (a, b) observed on PDA at the confrontation zone of *F. sambucinum* (A) and *F. oxysporum* f. sp. *tuberosi* (B) with *Bacillus* sp. (After 3 days of incubation at 25°C)

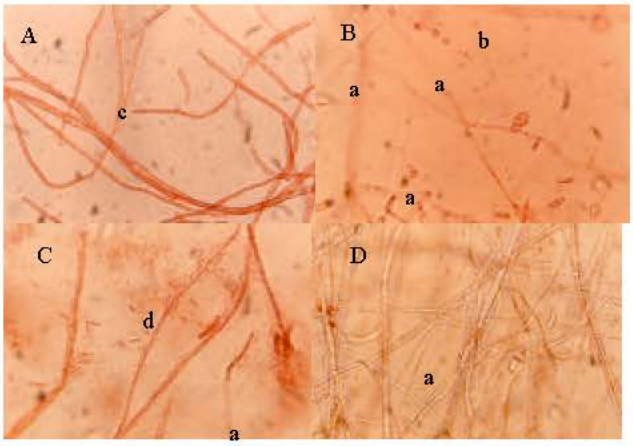

Fig. 2: *In vitro* interaction of *F. graminearum* (A), *F. oxysporum* f. sp. *tuberosi* (B), *F. sambucinum* (C) and *F. solani* (D) with *Bacillus* sp. observed at the confrontation zone after 3 days of incubation at 25°C: (a) mycelium lysis, (b) early chlamydospores formation, (c) formation of mycelial cords via anastomose mechanism, (d) *Bacillus* sp. around a lytic mycelium

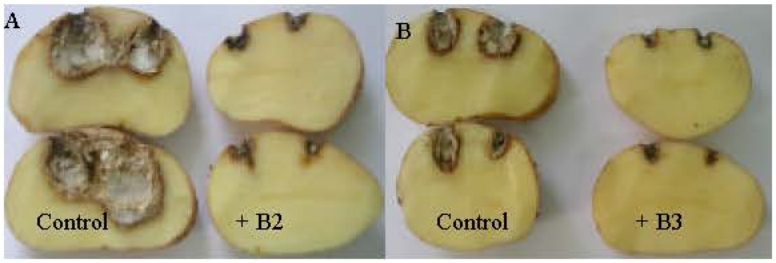

Fig. 3: Effect of *Bacillus* sp. isolates (B2 and B3) on potato dry rot development occasioned by *F. graminearum* (A) and *F. solani* (B) after 21 days of incubation at 25-27°C (inoculated tubers, cv. Spunta)

DISCUSSION

According to tunisian conditions, involvement of a *Fusarium* complex in development of potato dry rot, absence of resistant potato cultivars, together with the exploitable pathogen etiology, its resistance to several traditional strategies of control and its capacity of causing serious losses mentioned by Slininger *et al*. (2003), are the most important factors that incited us to begin the present biocontrol experiments.

The present study revealed existence of several interactions, *in vitro* and *in vivo*, between tested *Fusarium* species and bacterial antagonists. Activity of *Bacillus* sp. isolates is translated not only by reduction of *in vitro* development but several mechanisms including severe lysis, morphological alteration, early chlamydospores formation, mycelial cords and reduction of mycelial density include the whole atypical phenomena observed on untreated control and showing stress occasioned by *Bacillus* sp. on *Fusarium* spp. This result joins our past results where similar phenomena were observed on other isolates of *Fusarium* species treated by the same *Bacillus* isolates, when incorporated into PDA, and their efficacy was superior to that obtained by a fungicide treatment such as Thiabendazole (Daami-Remadi, 2001b).

Inhibition of *F. sambucinum* by *Bacillus* sp. and hyphal alterations noted during their interaction joins, in part, results of Chérif *et al*. (2002) who showed, via ultrastructural observations and cytochemical localization of N-acetylglucosamine residues, involvement of chitinolytic and antibiotic activity of *B. thuringiensis* 55T in *F. sambucinum* parasitism. They also observed hyphal damage associated with several alterations such as generalized cytoplasm disorganization, complete protoplasm loss, fungal cell wall disintegration and cytoplasm disorganization. In the same way, Sadfi *et al*. (2001) found, following ultra-structural studies, that both varieties of *Bacillus cereus* 65B and X16, showed antagonistic activity against *F. sambucinum* on potato tubers, are able of degrading chitine suggesting production of chitinases.

Similar phenomena are observed by Basha and Ulaganathan (2002) which also found, via light microscopic studies of *Bacillus* sp. strain BC121 x *Curvularia lunata in vitro* interaction, presence of abnormal hyphae, with condensation and deformation and occurrence of extensive malformation and damages to the mycelium. Emmert and Handelsman (1999) reported that *Bacillus cereus* modifies the ionic composition of the medium in which it grows. It raises the pH, sequesters calcium, and excretes ammonia. This combination is highly toxic to zoospores of Oomycetes pathogens, causing rapid swelling of the expulsion vacuole, followed by zoospore lysis. In fact, *Bacillus* sp. acts via antibiosis, nutrient competition, exclusion of infection sites, parasitism and/or induced resistance (Jacobsen and Backman, 1993; Kloepper *et al*., 2004). *B. subtilis* produced metabolic antibiotics such as subtilin, bacitracin, bacillin, subtenolin and bacilomycin (Muhammad and Amusa, 2003) and also antifungal volatile compounds active against *Rhizoctonia solani* and *Pythium ultimum*; morphological hyphal anomalies were also observed showing distortion and vacuolisation.

Tested *Bacillus* isolates were obtained during isolations of causal agents of tuber dry rot and this phenomenon is justified by the fact that *Bacillus* sp. develops as epiphyte and endophyte in the spermosphere and rhizosphere (Sturz *et al*., 1999). Furthermore, Shekhawat *et al*. (1984) found both pathogenic and non-pathogenic endophytic bacteria coexisting within potato tubers.

A whole tuber essay was used to assess ability of these *Bacillus* sp. isolates to decrease dry rot development in presence of an active host resistance. *In vivo* studies showed that *Bacillus* application into sites of infection, 24 h before inoculation by pathogen, reduced dry rot development occasioned by the four tested *Fusarium* species, their efficacy is higher than that obtained with the same bacterial isolates when applied simultaneously with fungal inoculum (Daami-Remadi, 2001b). Present results also agree, in part, with those of Chérif *et al*. (2002) for *B. cereus* X16 and *B. thuringiensis* 55T tested against *F. sambucinum*. Burkhead *et al*. (1995) explained for *P. seudomonas. cepacia* B37w, tested against *F. sambucinum*, that this antagonist produced, within wounds, antifungal pyrrolnitrines responsible of dry rot development inhibition.

Bacillus sp. isolates reduced tuber dry rot incidence occasioned by *Fusarium* species sensitive to benzimidazoles fungicides such as *F. oxysporum* f. sp. *tuberosi*, *F. solani* and *F. graminearum* but also *F. sambucinum* resistant to these fungicides (Daami-Remadi and El Mahjoub, 2006). These results agree with those obtained by Daami-Remadi (2001b) with the same isolates of *Bacillus* sp. where *in vivo* efficacy was superior to that obtained with a thiabendazole treatment and those of Schisler *et al*. (1998) which found five bacterial antagonists able of controlling 10 strains of *Gibberella pulicaris* (teleomorph of *F. sambucinum*) including those resistant to Thiabendazole. In the same way, Jacobsen *et al*., (2004) reported that *Bacillus* sp. have modes of action different of those of synthetic chemical fungicides so that they can be used in fungicide resistance management programs.

Diversity of *Fusarium* spp. species causes tuber dry rot in Tunisia and this present study showed that at least one *Bacillus* sp. isolate is able of inhibiting most of species tested *in vitro* and *in vivo*. This study revealed the important antagonistic potential employed, naturally present at potato environment and applicable to the post-harvest phase. Furthermore, *Bacillus* sp. supported tunisian local conditions of a traditional potato storage and as, an interaction was noted *in vivo* between *Fusarium* and isolates of *Bacillus* tested, mixtures of several isolates will be tested in the future for optimising their efficacy.

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