EVALUATION OF BACTERIAL ISOLATES FROM SALTY SOILS AND *BACILLUS THURINGIENSIS* STRAINS FOR THE BIOCONTROL OF FUSARIUM DRY ROT OF POTATO TUBERS

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SUMMARY

A total of 83 spore-forming bacteria belonging to the genus *Bacillus* was isolated from Tunisian salty soils. These isolates as well as five additional strains of *Bacillus thuringiensis*, previously selected for their efficiency against insects, were tested *in vitro* and *in vivo* against *Fusarium roseum* var. *sambucinum*, the causal agent of dry rot of potato tubers. Results of the *in vitro* dual culture screening revealed that more than 50% of *Bacillus* spp. isolated from salty soils inhibited the growth of the pathogen *in vitro*. By contrast, all five *B. thuringiensis* strains failed to inhibit the growth of the pathogen *in vitro*. On wounded potato tubers, the most effective isolates obtained from salty soils were X7, X9, X16, I32 and G7, with a percentage of dry rot reduction ranging from 66 to 89%. These effective *Bacillus* isolates were identified as belonging to one of the species *B. cereus* (X9, X16 and G7), *B. lentimorbus* (X7) or *B. licheniformis* (I32). Although ineffective *in vitro, B. thuringiensis* strains inhibited dry rot development *in vivo*, with percentage inhibition scores ranging from 41 to 52%. While *Bacillus* isolates selected from salty soils best inhibited dry rot development when applied as young cultures (24 h), *B. thuringiensis* strains generally performed better as older cultures (48-72 h). The cell-filtrates of *Bacillus* spp. were unable to inhibit the growth of *Fusarium*. By contrast, volatiles liberated by the antagonists seem to contribute to the inhibition of the pathogen. The two isolates X16 of *B. cereus* and I32 of *B. licheniformis* as well as all 3 tested strains of *B. thuringiensis* (1T, 10T and 55T) were able to degrade colloidal chitin. Our experiments with the chromogenic chito-oligosaccharides indicated also that *B. cereus* (X16) and *B. thuringiensis* (55T) are able to produce N-acetyl-β-D-glucosaminidases, chitobiosidases and endochitinases. The isolate X16 of *B. cereus* consistently showed a chitinase activity 2 to 3 fold higher than that of strain 55T of *B. thuringiensis.* The hydrolysis of chromogenic chito-oligosaccharide analogs correlated well with the release of reducing sugars from chitin or the formation of clearing zones on chitin agar. The diversity and complexity of chitinases produced by our selected strains may contribute significantly to their antagonistic activity towards *F. roseum* var. *sambucinum*.

Key words: Bacillus, potato, biocontrol, dry rot, chitinolytic enzymes.

INTRODUCTION

Fusarium dry rot of potato tubers is particularly prominent in Tunisia, resulting in partial or almost complete loss of stored potatoes, especially under traditional storage conditions (Daami-Remadi and El Mahjoub, 1996). Although these losses can be greatly reduced by storage at low temperatures (1-5°C) and high relative humidity (95%), *Fusarium* species are the prevalent fungi found on potato tubers with infections reaching 30-50% (Daami-Remadi and El Mahjoub, 1996). In addition to destroying tuber tissues, *Fusarium* spp. can produce toxins that have been implicated in mycotoxicoses of humans and animals (Senter *et al*., 1991; Schisler *et al*., 1997). *F. solani* var. *coeruleum*, *F. roseum* var. *sambucinum* and *F. oxysporum* are reported as common causes of dry rot of potatoes in Europe, North America and South Africa (Boyd, 1972; Tivoli *et al*., 1985; Carnegie *et al*., 1998; Venter and Steyen, 1998). In Tunisia, the investigations of Daami-Remadi and El Mahjoub, (1996) revealed that three varieties of *F. roseum*, *F. roseum* var. *sambucinum*, *F. roseum* var. *culmorum* and *F. roseum* var. *graminearum*, are the most prevalent and the most pathogenic to stored potatoes. More recently, we have also demonstrated that *F. roseum* var. *sambucinum* is the dominant fungus found on potatoes during traditional and cold storage and the main reason for losses (Chérif *et al*., 2000).

To prevent *Fusarium* spoilage and dry rot development it is common practice in different countries to dip harvested potatoes in fungicide solutions prior to storage (Kawchuck *et al*., 1994; Carnegie *et al*., 1998). An

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effective control of Fusarium dry rot has been obtained with the fungicide Fenpiclonil and the mixture of thiabendazole and imizalil (Carnegie *et al.*, 1998). Nevertheless, besides the problems relative to environmental pollution and chemical toxicity to humans and animals, resistance to the few chemicals registered for use on potato tubers for human consumption seems to be widespread among strains of *Fusarium* spp. (Kawchuck *et al.*, 1994; Secor *et al.*, 1994). This problem is compounded by the fact that all commonly grown potato cultivars are susceptible to Fusarium dry rot, and high levels of resistance in breeding stocks are not available (Pawlak *et al.*, 1987; Schisler *et al.*, 1997). An interesting approach to post-harvest disease control that has gained attention is the use of bacterial antagonists such as the members of the genera *Pseudomonas* and *Bacillus* (Wilson and Wisniewski, 1989). *Bacillus* spp. as compared to *Pseudomonas* spp., offer the great advantage of producing endospores, which are particularly amenable to formulation and long-term storage, and allows these bacterial antagonists to withstand harsh environmental conditions (Powell *et al.*, 1990; Fiddman and Rossall, 1995). Among the *Bacillus* group, *B. cereus*, *B. subtilis*, *B. mycoides* and others were used successfully against different fungal pathogens belonging to the genera *Rhizoctonia*, *Sclerotinia*, *Fusarium*, *Gaeumanomyces*, *Nectria*, *Pythium*, *Phytophthora* (Cook and Baker, 1983; McKnight, 1993; Fiddman and Rossall, 1995). Nevertheless, to our knowledge, no studies have evaluated the bioeffect of *Bacillus* spp. on dry rot of stored potatoes.

Bacillus species may assume their antagonistic effects against fungal pathogens by antibiosis, competition or exploitation, which is subdivided into predation and direct parasitism (Pleban *et al.*, 1997; Muninbazi and Bullerman, 1998; Walker *et al.*, 1998). Parasitism operating by degradation of cell walls of pathogenic fungi is one kind of exploitation and relies on extracellular lytic enzymes. Several *Bacillus* species produce enzymes that degrade chitin, an insoluble linear polymer of β-1,4-N-acetylglucosamine (GlcNAc), which is the second most abundant polysaccharide in nature and the major component of most fungal cell walls. Among these species, *B. circulans* (Watanabe *et al.*, 1990), *B. licheniformis* (Takayanagi *et al.*, 1991; Trachuk *et al.*, 1996), *B. cereus* (Trachuk *et al.*, 1996; Pleban *et al.*,1997), *B. pabuli* (Frändberg and Schnürer, 1994b) and *B. thuringiensis* (Chigaleichik, 1976), all of which have been cited as potential biocontrol agents, have been reported to secrete chitinases. Evidence that these chitinolytic enzymes play a major role in the biocontrol of fungal pathogens has been demonstrated in many systems involving bacterial and fungal antagonists (Shapira *et al.*, 1989; Chérif and Benhamou, 1990; Lorito *et al.*, 1993).

The aims of this study were to isolate *Bacillus* species from salty soils, screen these isolates *in vitro* and *in vivo* for antagonism against *F. roseum* var. *sambucinum*, select and identify isolates showing promising antagonism and determine the mode of action of the antagonists with special emphasis on the role of the chitinolytic enzymes in suppressing growth of the pathogen. Five additional isolates of *B. thuringiensis* previously selected for their ability to protect plants from insect attacks, by producing toxic crystal proteins, were included in the present studies in an attempt to select *Bacillus* isolates effective against both fungi and insects.

MATERIALS AND METHODS

Origin of microorganisms

Isolation of spore-forming bacteria from salty soils. Four samples of natural salty soils collected from different locations in the South of Tunisia were used as sources of spore-forming bacteria. Some chemical and physical characteristics of these soils are presented in Table 1. Each soil sample (*ca* 1 g) was thoroughly mixed in 9 ml volumes of sterile distilled water in sterile flasks. Aerobic, Gram-positive spore-forming bacteria were isolated after heating the soil suspensions at 90°C for 10 min in order to kill vegetative cells. Single bacterial colonies were isolated by plating of serial dilutions of soil samples on Nutrient Agar (NA, Oxoid). Then colonies were streaked on successive NA media to obtain pure cultures which were maintained on NA slants at +4°C and subcultured every two-months. Different tests were performed for initial identification of bacterial isolates. The following characteristics were retained: Gram-positive rods, endopsore producing, motile, catalase and oxydase positive. *Bacillus* isolates, the most effective *in vitro* and *in vivo* against the pathogen, were identified down to species level by Dr. James J. Germida (Department of Soil Science, University of Saskachewan, Saskatoon, Sk.) based on chromatography of fatty acids methyl esters (MIDJ Inc., Newark, DE).

B. thuringiensis *isolates.* Five strains of *B. thuringiensis* (1T: *B. thuringiensis* serotype 1; 10T: *B. thuringiensis* var. *darmstadiensis* serotype 10; 14T: *B. thuringiensis* var. *israelensis* serotype 14; 33T: *B. thuringiensis* var. *kurstaki* serotype 3a 3b; 55T: *B. thuringiensis* var. *galleriae* serotype 5a 5b) previously selected for their antagonism against insects in the laboratory of Microbiology (Faculté des Sciences de Tunis), were also tested for their ability to suppress the growth of *F. sambucinum* and dry rot development.

Fungal pathogen. The fungal pathogen was isolated from infected potato tubers with typical symptoms of Fusarium dry rot and reported to be virulent on potato tubers (Chérif *et al.*, 2000). It was cultivated on plates of potato dextrose agar (PDA) for 7 to 10 days at 25°C until sporulation. The fungal pathogen was maintained on PDA at +4°C and was subcultured onto fresh PDA plates within a period of 3 months.

In vitro screening. *In vitro* antagonism tests were performed on NA in 9 cm Petri plates by applying a dual culture technique. *Bacillus* isolates and *B. thuringiensis* strains were streaked across the center of the plate. A second streak of the bacterium was made perpendicularly to the first. Four discs of 5 mm in diameter cut from the edge of a 7 day-old culture of *F. sambucinum* were placed at each side of the antagonist. The distance between the two microorganisms was 2.5 cm. Plates were then incubated at 25°C for one week. Percent growth inhibition of *F. sambucinum* was calculated by the formula of Whipps (1987): $(R_1 - R_2)/R_1*100$, where R_1 is the farthest radial distance (measured in mm) grown by *F. sambucinum,* after 7 days of incubation, in the direction of the antagonist (a control value), and R_2 is the distance of fungal growth from the point of inoculation to the colony margin in the direction of the antagonist, and GI the percentage of growth inhibition. Growth inhibition was categorized on a scale (Korsten *et al.*, 1995) from 0 to 3, where 0 = no growth inhibition, 1 = 1 to 25% growth inhibition, 2 = 26 to 50% growth inhibition and 3 = 51 to 75% growth inhibition.

Antagonistic activity on potato tubers. After *in vitro* screening, 42 *Bacillus* isolates that inhibited the growth of *F. sambucinum* were retained for a further screening made *in vivo* on potato tubers. This screening included also the 6 strains of *B. thuringiensis*. Potato tubers cv. 'Spunta' were surface-sterilized by soaking in 2% aqueous sodium hypochlorite for 10 min. They were then thoroughly rinsed, dried with sterile filter paper, and then wounded by removing 3 plugs, 3 mm in diameter and 3 mm in depth, from the surface using a sterile cork borer. For each experiment, fresh cultures of the pathogen and the bacterial antagonists were used. *Bacillus* isolates were tested after 24 h, 48 h and 72 h of culturing at 10^6 CFU ml^{-1}. Bacterial concentration was determined by dilution plating on NA. Conidial suspensions of *F. sambucinum* were adjusted to 10^5 spores ml^{-1} by counting with a heamacytometer. Each potato tuber was wounded three times along a line joining the two ends. The wounds were immediately co-inoculated with 20 µl of a bacterial antagonistic candidate and 20 µl of a conidial suspension of the pathogen. As positive and negative controls, tubers were either inoculated with the pathogen alone, with the bacterial candidate alone or with distilled water. For each treatment 3 potato tubers were assayed and the experiment was repeated at least twice. The treated wounds were sealed with Scotch tape and potato tubers were placed in plastic bags to maintain a high humidity and then incubated at 15°C for 2 weeks. Diameters of lesions were determined regularly until the end of the experiment. By that time we had also estimated the extent of rot penetration as well as the loss of weight of potato tubers due to *Fusarium* rot. Estimation of the loss of weight has enabled us to calculate the percentage of disease reduction according to the formula of Elphinstone (1987): disease reduction (%) = $L_p - L_A/L_p*100$, where L_p is the loss of weight due to *Fusarium* infection in untreated potato tubers and L_A is the loss of weight in infected potato tubers treated with the antagonist.

Detection of antifungal activity of volatiles. The production of volatile compounds, by the selected *Bacillus* strains was assayed by a sealed plate method as described by Fiddman and Rossall (1993). From a 72-h NA culture of *Bacillus*, 200 µl were spread on an agar medium in a Petri dish (two media were tested: NA and PDA). After incubation at 37°C for 24 h, a second Petri dish (containing PDA), was inoculated with a 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 and further incubated at 25°C. This ensured that both organisms were growing in the same atmosphere though physically separated. As a control, a Petri dish containing agar medium without bacteria was placed over the PDA medium inoculated with the fungal pathogen. Fungal growth was measured as increases in radial growth of the test fungus over 24 h intervals for a period of 5 days. Each test was replicated 3 times.

Detection of antibiotic substances

Antibiotic production. Bacillus isolates (X7, X9 and X16) were streaked on NA slants and then incubated at 30°C for 24 h. A loopful of inoculum from the overnight slant culture was introduced into 100 ml of the production medium. Two media were tested for production of antifungal metabolites. The first is the minimal defined medium (MD) described by Mckeen *et al.* (1986). The medium contained 20 g dextrose; 5 g DL-glutamic acid; 1.02 g $MgSO_4.7H_2O$; 1.0 g K_2HPO_4; 0.5 g KCl; and 1 ml of trace element solution (0.5 g $MnSO_4.H_2O$; 0.16 g $CuSO_4.5H_2O$ and 0.015 g $FeSO_4.7H_2O$ in 100 ml of water). The pH of the medi-

um was adjusted to 6.0-6.2 with 5 N NaOH. The second medium used was the Beef extract-peptone-sodium chloride (BPS, Difco), pH 6.5. The inoculated media were then incubated for 60 h in an incubator shaker maintained at 30°C and 170 rpm. The bacterial suspension was centrifuged at 10,000 ***g*** for 10 min at 4°C. The supernatants were filtered through sterile 0.45 µm acrodisc filters.

Antibiotic assay. The cell-free filtrates were assayed for their ability to inhibit mycelial growth of *F. sambucinum* by using an agar-well diffusion method (Tagg and McGiven, 1971). Five milliliters of molten potato dextrose agar kept at 45°C were seeded with conidia of *F. sambucinum* and spread uniformly over the NA medium. After the solidification of the seeded layer, 3 wells were made using a no. 3 cork borer, and filled with 50 µl of the test fluid. The control consisted of 50 µl of filter-sterilized distilled water. The samples were allowed to diffuse into the agar, and the plate was inverted and incubated at 28°C for 24 h. The plates were examined for halos of inhibition around the wells.

Detection of chitinolytic activity

Screening for chitinolytic bacteria. The selected isolates of *Bacillus* spp. (X16, X7, X9, I32 and G7) and *B. thuringiensis* strains (1T, 10T and 55T) exhibiting antifungal activity against *F. sambucinum* were cultured on an agar medium containing: 0.5% colloidal chitin, prepared from crab shell (Practical grade, Sigma) according to the method of Rodriguez-Kabana *et al.*, (1983). This medium contained per liter of water: 7 g $(NH_4)_2SO_4$; 1 g K_2HPO_4; 1 g NaCl; 0.1 g $MgSO_4.7H_2O$; 0.5 g yeast extract and 15 g agar. Bacteria showing clearing zones after 7 days of incubation at 30°C on colloidal chitin agar were maintained for further tests.

Preparation of crude enzymes. Selected bacteria were cultivated in a liquid medium of the same composition with 0.5% colloidal chitin and incubated at 30°C for 60 h on a rotary shaker (200 rpm). The cultures were centrifuged at 10,000 ***g*** for 10 min to remove cells. Ammonium sulfate was added to supernatants to achieve 80% saturation, with stirring at 5°C for 2 h and the 80% precipitates were collected by centrifugation (18,000 ***g*** for 10 min). The resulting precipitates were then suspended in 50 mM potassium phosphate buffer, pH 6.1 and dialyzed against distilled water for 12 h. The dialyzates were lyophilized and finally resuspended in potassium phosphate buffer. Intracellular proteins were extracted from cells with a French pressure cell press at 10,350 kPa. Debris was separated by centrifugation (10,000 ***g***, 20 min) and the enzyme extracts filtered through 0.22 µm filter (Scleicher and Shuell). Protease inhibitors were added to the extracts. Crude enzymes from extracellular or intracellular proteins were used as assay solutions for the chitin tests.

Protein determination. Protein measurements on crude enzyme samples were performed according to Bradford (1976) and bovine serum albumin was used as a standard.

Enzyme assays

Hydrolysis of chito-oligosaccharides. Chitinase activity was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-*N*-acetyl-β-D-*N*,*N'*-diacetylchitobiose [*p*NP-$(GlcNAc)_2$; Sigma] as described by Roberts and Selitrennikoff (1988). The *p*NP-$(GlcNAc)_2$ was dissolved in 50 mM potassium phosphate buffer (KPB) (pH 6.0). Enzyme samples (10 µl) were added to 90 µl of 0.18 mM *p*-nitrophenyl reagent in a microtiter plate and incubated at 50°C for an appropriate period. The reaction was terminated by adding 10 µl of 1 M NaOH and the absorption was measured at 450 nm in a microplate autoreader. One unit (1 U) of enzymatic activity was defined as 1 µmol of pNp mg^{-1} of protein min^{-1}. The hydrolysis of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (pNP-GlcNAc; Sigma) and *p*-nitrophenyl-*N*-acetyl-β-D-*N*,*N*',*N*''-triacetylchitotriose [pNP-$(GlcNAc)_3$; Sigma] were both measured in an identical way.

Hydrolysis of colloidal chitin. Chitinolytic activity was also determined by the release of reducing sugars from chitin. The reaction mixture contained 0.5 ml of 0.5% colloidal chitin suspension in 0.1 M sodium acetate buffer (pH 5.0) and 0.3 ml of an enzyme solution. The mixture was incubated for 30 min at 50°C. The reaction was terminated by adding 0.75 ml DNSA (10 g NaOH, 10 g dinitrosalicyclic acid, 2 g phenol, 0.5 g Na_2SO_3 and 200 g sodium potassium tartrate in total volume of 500 ml; Trachuk *et al.*, 1996). Color was developed by incubation of the mixture for 10 min at 100°C. After centrifugation (8000 rpm, 10 min), the adsorption of the supernatant was measured at 540 nm. A calibration curve was plotted using N-acetylglucosamine (NAG, Sigma). In this case, 1 U of chitinolytic activity was expressed as 1 µmol of GlcNAc mg^{-1} of protein min^{-1}.

Clearing zones. Fifty µl portions of enzyme samples were added to wells (6 mm diam.) in chitin agar containing (g l^{-1}) 1.5 g chitin; 15 g agar and 0.2 g NaN_3 in 50 mM potassium phosphate buffer (pH 6.1). Plates were placed at 4°C for 2 h and then incubated at 37°C for 48 h, and the diameter of clearing zones measured.

RESULTS

Isolation and *in vitro* testing of bacterial antagonists. A total of 83 spore-forming bacteria were isolated from Tunisian salty soils, which are characterized by a high electric conductivity and a low level of organic matter (Table 1). The results of the *in vitro* dual culture screening revealed that 42 of these bacterial isolates reduced the mycelial growth of *F. roseum* var. *sambucinum* by forming an inhibition zone (Fig. 1A). All these isolates were identified as belonging to the genus *Bacillus*. Of these isolates, 16 were isolated from Chott-El Jerid (location 1), 17 from Foum El Khanga (location 2), 7 from Chott-Er-Rahim (location 3), and 2 from Deggache (location 4). The five *B. thuringiensis* strains tested in our *in vitro* screening were unable to inhibit the growth of the fungal pathogen (Table 2).

***In vivo* testing of bacterial antagonists.** The 42 *Bacillus* isolates showing antagonistic activity against *Fusarium in vitro* and the five *B. thuringiensis* strains were further tested for their inhibitory effect on wounded potato tubers by using 24 h cultures of these bacteria. The identity and *in vivo* inhibitory effect of these bacteria against dry rot development are presented in Table 3. Of the 42 *Bacillus* isolates obtained from Tunisian salty soils, the most effective ones were X7, X9, X16, I32 and G7, with a percentage of dry rot reduction ranging from 66 to 89% (Table 3, Fig. 1B). Although ineffective *in vitro*, *B. thuringiensis* strains inhibited dry rot development *in vivo* (Table 3). Similar results were obtained with the 4 strains of *B. thuringiensis* 1T, 14T, 33T and 55T, which gave percentage inhibition scores ranging from 41 to 52% (Table 3). By contrast, the strain 10T of *B. thuringiensis* was unable to effectively control Fusarium dry rot when applied as a 24 h bacterial culture (Table 3). Nevertheless, application of this strain as a 48 h or 72 h bacterial culture almost completely inhibited the growth of the pathogen and resulted in percentage dry rot reduction by respectively 94 and 84% (Fig. 2).

Better percentages of disease reduction were also obtained with strains 1T and 55T when applied as 48-h cultures (Fig. 2). By contrast, the use of more aged cultures of *Bacillus* isolates X7, X9, X16, I32 and G7 has generally significantly increased dry rot symptoms as compared to 24 h cultures (Fig. 3).

Besides evaluating bacterial antagonistic effects on percentage disease reduction, which is determined based on the weight of rotted potato tissues, we also determined lesion diameters over time and the extent of rot penetration by the end of the experiment, 14 days after inoculation (Tables 4 and 5). Results based on lesion diameter development revealed that the 5 selected *Bacillus* isolates X7, X9, X16, I32 and G7 significantly reduced lesion extension, with the exceptions of X7, X9 and I32 when applied as a 24 h bacterial culture, particularly after 14 days of inoculation (Table 4). All these strains, except X7 and G7 when applied as 72 h cultures, showed also a significant decrease in the extent of dry rot penetration within potato tubers (Table 4). From Table 4 it appears also that the isolate X16 consistently performed best during the *in vivo* experiments. These results indicate also, in concordance with percentage disease reduction data (Fig. 3), that the least penetration values were often obtained with the bacteria applied as a 24 h culture (Table 4). This was usually not the case when we consider lesion diameters (Table 4). The same discrepancy between lesion diameter data of *B. thuringiensis* strains (Table 5) and those of percentage disease reduction (Fig. 2) may be noticed, where application of the antagonists as a 24 h cultures resulted in lesion diameters similar or lower than those of the 48 h and 72 h cultures. This discrepancy was not observed as far as the extent of rot penetration was concerned (Table 5). In this case, *B. thuringiensis* isolates always

Table 1. Some chemical and physical characteristics of soil samples.

	Location			
	(1) Chott-El Jerid	(2) Foum El Khanga	(3) Chott-Er-Rahim	(4) Deggache
pH	7.3	7.8	8.0	8.1
Electric conductivity (mmho cm^{-1})	45.9	38.7	43.5	18.9
Organic matter (%)	0.3	0.2	0.2	0.9
Carbon (%)	0.2	0.1	0.1	0.5

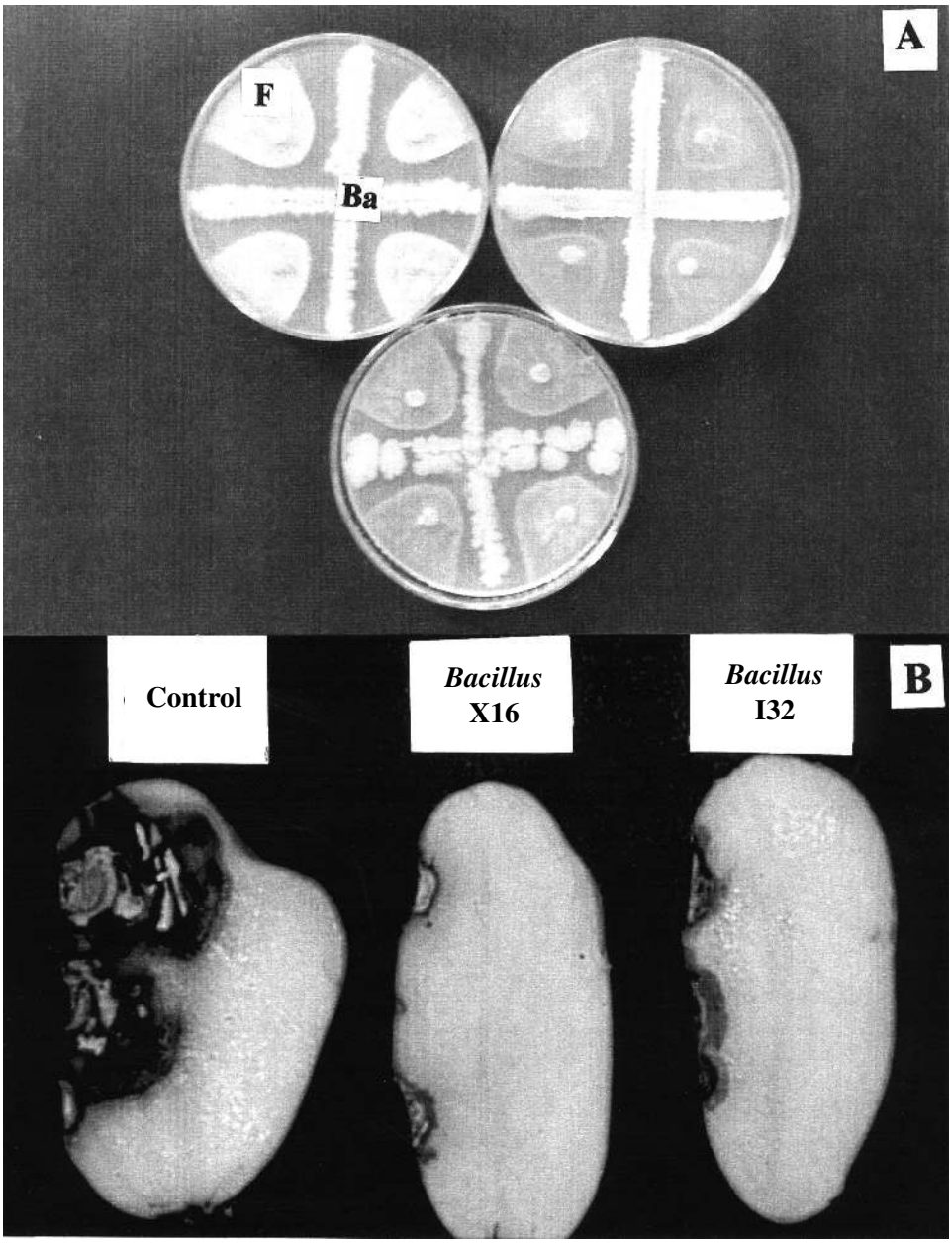

Fig. 1. Effect of *Bacillus* spp. isolated from salty soils on **A** mycelial growth of *F. roseum* var. *sambucinum in vitro* and on **B** dry rot development *in vivo* on potato tubers cv. 'Spunta'. F: fungus ; Ba: *Bacillus* spp.

behaved better when applied as 48 or 72 h bacterial cultures, resulting in lower penetration (Table 5). Table 5 indicates also that the best results are obtained with *B. thuringiensis* isolates 55T and 1T when applied as 48 h cultures. These treatments significantly reduced ($P < 0.01$) lesion diameter from more than 27 mm in the control to respectively 11 and 13.1 mm, and rot penetration from 15.6 mm in the control to respectively 3 and 4 mm (Table 5). Inoculation of wounded potato tubers by the different tested bacterial antagonists in the absence of the pathogen resulted in no disease development (results not shown).

Antifungal activity of *Bacillus* volatiles. The results of experiments on the effect of *Bacillus* isolates volatiles on the growth of the pathogen are presented in Fig. 4. These experiments showed variable fungal growth inhibition according to the tested isolate and to the medium on which the bacterial isolate was grown. On PDA, 8 *Bacillus* isolates out of 9 showed an inhibitory effect on *Fusarium* growth (Fig. 4A), while on NA, X16, 1T and 10T seem to be the only tested *Bacillus* isolates capable of producing volatiles effective against the pathogen (Fig. 4B). These isolates resulted in more than 11.8% reduction in fungal growth after 96 h of incubation (Fig. 4B).

Table 2. Effect of bacteria isolated from salty soils and of *B. thuringiensis* strains on *in vitro* growth of the rot pathogen.

Bacillus spp. isolates	GI category*	*Bacillus* spp. isolates	GI category*
Chott-El Jerid		Foum El Khanga	
X1	1	I1	0
X2	3	I2	2
X3	2	I3	2
X4	1	I4	0
X5	0	I5	0
X6	0	I6	3
X7	2	I8	2
X8	2	I9	0
X9	2	I10	2
X10	1	I12	2
X11	2	I13	0
X11b	0	I14	0
X12	1	I15	0
X13	2	I16	0
X14	0	I17	0
X15	0	I18	2
X16	3	I19	0
X17	2	I20	3
X18	0	I21	0
X19	3	I23	3
X20	0	I24	0
X21	2	I25	3
X22	1	I27	2
X23	0	I28	2
X24	0	I29	0
Mean GI category score	1.20	I30	0
Total inhibitory bacteria	16	I31	3
		I32	3
Chott-Er-Rahim		I33	2
G1	0	I34	2
G2	3	I35	2
G4	0	Mean GI category score	1.29
G5	3	Total inhibitory bacteria	17
G6	0		
G7	2	Deggache	
G8	0	E1	0
G9	0	E2	1
G10	3	E4	0
G11	2	E6	1
G12	0	E7	0
G13	2	E8	0
G14	0	E9	0
G31	2	E11	0
Mean GI category score	1.21	E13	0
Total inhibitory bacteria	7	E14	0
		E15	0
B. thuringensis strains		E19	0
1T	0	E20	0
10T	0	Mean GI category score	0.15
14T	0	Total inhibitory bacteria	2
33T	0		
55T	0		
Mean GI category score	0		
Total inhibitory bacteria	0		

* Percent growth inhibition was determined after 7 days of incubation using Whipps' (1987) formula. Values were categorized on a scale from 0 to 4, where 0: no growth inhibition; 1: 1 to 25%; 2: 26 to 50%; 3: 51 to 75%.

Table 3. Identity and effect of *Bacillus* isolates and *B. thuringiensis* strains on dry rot development on wounded potato tubers cv. 'Spunta' inoculated with *F. roseum* var. *sambucinum*. Only the most effective isolates were identified to the species level.

Isolate	Identity of the selected isolates	Dry rot reduction (%)
X1	*Bacillus* spp.	16
X2	*Bacillus* spp.	34
X3	*Bacillus* spp.	26
X4	*Bacillus* spp.	2
X7	*B. lentimorbus*	66
X8	*Bacillus* spp.	14
X9	*B. cereus*	78
X10	*Bacillus* spp.	57
X11	*Bacillus* spp.	34
X12	*Bacillus* spp.	58
X13	*Bacillus* spp.	27
X16	*B. cereus*	79
X17	*Bacillus* spp.	34
X19	*Bacillus* spp.	38
X21	*Bacillus* spp.	11
X22	*Bacillus* spp.	17
I2	*Bacillus* spp.	26
I3	*Bacillus* spp.	5
I6	*Bacillus* spp.	39
I8	*Bacillus* spp.	30
I10	*Bacillus* spp.	48
I12	*Bacillus* spp.	56
I18	*Bacillus* spp.	3
I20	*Bacillus* spp.	43
I23	*Bacillus* spp.	23
I25	*Bacillus* spp.	48
I27	*Bacillus* spp.	5
I28	*Bacillus* spp.	28
I31	*Bacillus* spp.	49
I32	*B. licheniformis*	89
I33	*Bacillus* spp.	28
I34	*Bacillus* spp.	37
I35	*Bacillus* spp.	28
G2	*Bacillus* spp.	58
G5	*Bacillus* spp.	18
G7	*B. cereus*	69
G10	*Bacillus* spp.	10
G11	*Bacillus* spp.	5
G13	*Bacillus* spp.	2
G31	*Bacillus* spp.	7
E2	*Bacillus* spp.	2
E6	*Bacillus* spp.	5
1T	*B. thuringiensis*	52
10T	*B. thuringiensis*	1
14T	*B. thuringiensis*	41
33T	*B. thuringiensis*	50
55T	*B. thuringiensis*	50

Detection of antibiotic substances. None of the used media (MD and BPS) promoted the production of an antifungal compound by the tested bacteria (X7, X9 and X16). Bacterial cell-free extracts were not bioactive against *F. roseum* var. *sambucinum* since no inhibition zones were detected around the wells.

Detection of chitinolytic activity. Of the five *Bacillus* isolates selected from salty soils, only X16 and I32 were able to hydrolyze colloidal chitin and to form large zones of clearing around the inoculated wells (Table 6). All three *B. thuringiensis* strains 1T, 10T and 55T hydrolyzed colloidal chitin when they were grown on this compound as the sole carbon source (Table 6). While X16 and 55T hydrolyzed chitin after 7 days of incubation at 30°C, clearing halos were not observed before 10 days of incubation in the case of isolates I32, 1T and 10T. Clearing zone formation suggested the presence of chitinolytic activity in the proteins secreted into the growth medium. To confirm this, X16 and 55T were grown for 96 h in a synthetic medium (SM) amended with 0.5% colloidal chitin, and the presence of chitinolytic enzymes in extra- and intracellular proteins was examined in reaction mixtures with chromogenic oligomers of GlcNAc, by the release of *p*-nitrophenol (*p*NP), and by the release of GlcNAc from colloidal chitin. From Fig. 5 it appears that both isolates 55T and X16 released the chromophore pNP from dimeric pNP-GlcNAc, trimeric *p*NP-$(GlcNAc)_2$ and from tetrameric pNP-$(GlcNAc)_3$ derivatives of chitin. As shown for strain 55T of *B. thuringiensis*, the activity was detected not only extracellularly, but also in intracellular proteins and with a comparable level of chitinolytic activity regardless of the nature of the chromogenic oligomer used (Fig. 5). The same figure indicates also that as far as extracellular activity is concerned the highest activity is obtained in the reaction with the monosaccharide derivative pNP-GlcNAc. With all three chromogenic oligomers, strain X16 showed a chitinolytic activity 2 to 3 fold higher than that of isolate 55T of *B. thuringiensis* (Fig. 5). Comparison of the activities of chitinases from the two bacteria showed an agreement between the release of pNP from pNP- oligomers and the release of reducing sugars from chitin (Fig. 5 and 6a). In fact, while isolate X16 produced a chitinolytic activity of approximately 2.5 U, strain 55T showed an activity of less than 1 U (Fig. 6a). Similar results were obtained when chitinase activity was measured as the diameters of clearing zones produced by crude enzyme samples on chitin agar (Fig. 6b). After 48 h of incubation in the presence of enzyme extracts, enzyme samples of strains X16 and 55T gave respectively clearing zones of 40 and 12 mm mg^{-1} protein.

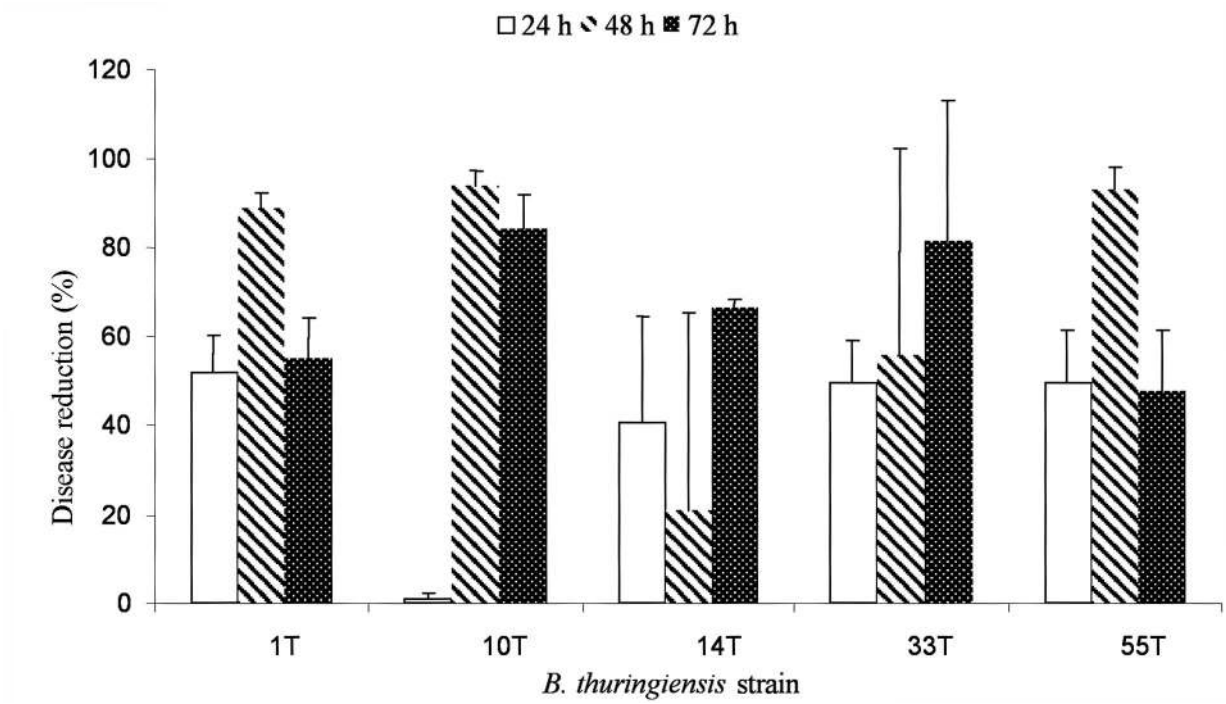

Fig. 2. Effect of *B. thuringiensis* strains, applied as 24, 48 or 72 h bacterial cultures on the percentage of disease reduction. Bars indicate standard deviation.

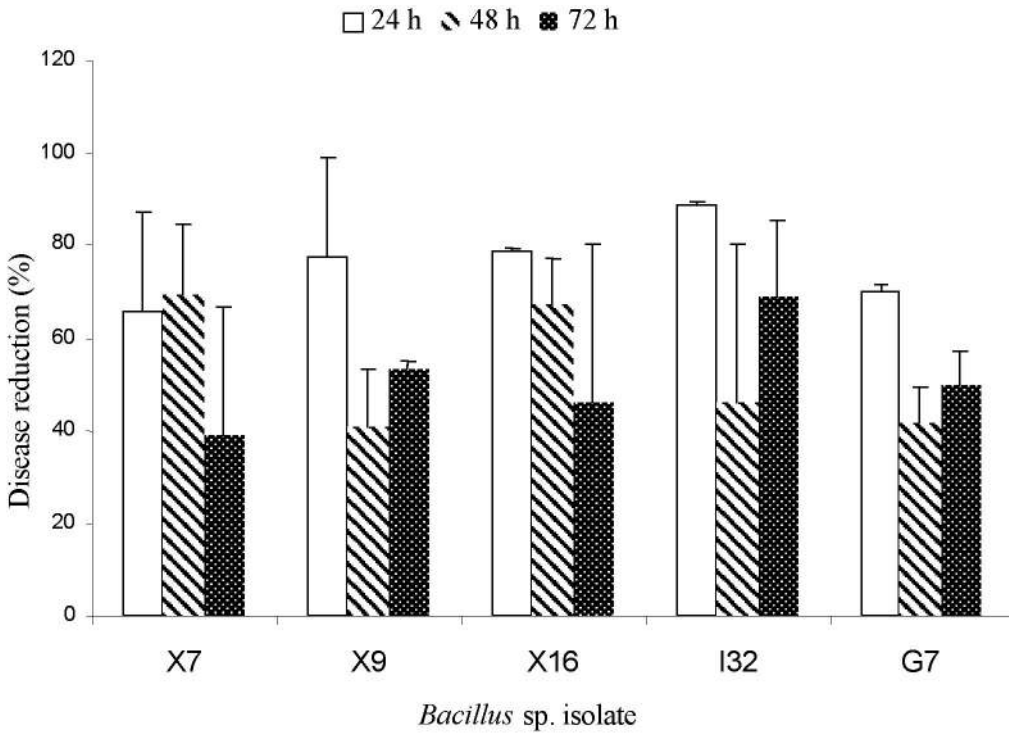

Fig. 3. Effect of the most effcetive *Bacillus* isolates *in vitro* applied as 24, 48 or 72 h bacterial cultures, on the percentage of disease reduction. Bars indicate standard deviation.

Table 4. Effects of the most effective *Bacillus* isolates *in vitro*, on the evolution of lesion diameters, as a function of time, and on the extent of rot penetration, by the end of the experiment, in wounded potato tubers cv. 'Spunta' inoculated with *F. roseum* var. *sambucinum*.

Treatment		Lesion diameter (mm)			Rot penetration (mm)
Bacillus sp. isolate	Culture	7	10	14 (days)	
X7	24 h	13.22 ± 3.42**	19.22 ± 4.38**	22.89 ± 7.25	6.78 ± 2.95**
	48 h	11.22 ± 2.17**	11.89 ± 3.37**	12.78 ± 3.11**	7.56 ± 1.51**
	72 h	10.33 ± 1.73**	15.33 ± 2.55**	18.44 ± 6.00**	13.78 ± 3.73
X9	24 h	15.67 ± 2.78*	21.67 ± 1.32**	25.33 ± 1.80	7.00 ± 1.50**
	48 h	14.22 ± 1.30**	15.00 ± 4.21**	18.22 ± 4.32*	10.78 ± 2.17*
	72 h	11.00 ± 3.24**	12.78 ± 3.99**	13.78 ± 4.44**	9.11 ± 1.05**
X16	24 h	9.67 ± 2.65**	11.00 ± 5.27**	12.00 ± 4.58**	5.33 ± 2.00**
	48 h	9.89 ± 1.90**	10.78 ± 2.44**	13.78 ± 3.99**	8.89 ± 2.37**
	72 h	10.11 ± 3.02**	11.56 ± 2.96**	14.78 ± 5.63**	10.11 ± 2.98*
I32	24 h	21.67 ± 1.32	25.33 ± 3.61	26.00 ± 3.12	6.67 ± 2.18**
	48 h	12.78 ± 3.93**	12.44 ± 4.07**	14.89 ± 6.49**	11.22 ± 1.3*
	72 h	12.67 ± 4.09**	13.89 ± 4.14**	15.78 ± 4.71**	8.11 ± 3.10**
G7	24 h	9.00 ± 6.76**	11.33 ± 7.26**	14.5 ± 9.31**	9.33 ± 4.44*
	48 h	10.44 ± 2.01**	13.44 ± 2.70**	16.22 ± 2.82**	11.00 ± 2.96*
	72 h	14.78 ± 3.49*	19.44 ± 5.79*	19.78 ± 5.02*	11.44 ± 3.64
Control		20.78 ± 5.17	25.89 ± 3.44	27.25 ± 5.42	15.56 ± 5.25

Note: data are the average of three replications ± the standard error of the means . * and **, statistically significant from the control at the 0.05 and the 0.01 probability level respectively.

Table 5. Effects of *B. thuringiensis* strains on the evolution of lesion diameters, as a function of time, and on the extent of rot penetration, by the end of the experiment, in wounded potato tubers cv. 'Spunta' inoculated with *F. roseum* var. *sambucinum*.

Treatment		Lesion diameter (mm)			Rot penetration (mm)
Bacillus sp. isolate	Culture	7	10	14 (days)	
1T	24 h	11.22 ± 4.94**	13.22 ± 5.02**	13.67 ± 6.28**	7.89 ± 1.96**
	48 h	11.33 ± 1.00**	12.00 ± 1.22**	13.11 ± 1.90**	3.99 ± 0.71**
	72 h	11.67 ± 1.00**	18.00 ± 4.58**	23.00 ± 9.37	4.00 ± 1.50**
10T	24 h	10.22 ± 2.39**	11.11 ± 3.95**	12.00 ± 1.50**	8.00 ± 2.29**
	48 h	9.00 ± 1.00**	14.78 ± 4.35**	16.00 ± 4.58**	6.33 ± 1.00**
	72 h	10.33 ± 5.00**	12.67 ± 5.22**	14.00 ± 5.12**	5.33 ± 1.00**
14T	24 h	12.11 ± 1.90**	13.78 ± 4.68**	18.67 ± 5.74**	8.33 ± 2.74**
	48 h	12.33 ± 1.87**	15.78 ± 3.49**	17.56 ± 5.5**	7.44 ± 2.70**
	72 h	15.67 ± 2.18*	19.00 ± 1.50**	20.33 ± 4.18*	7.00 ± 1.50**
33T	24 h	10.67 ± 1.12**	15.22 ± 2.39**	16.33 ± 3.71**	10.22 ± 1.92*
	48 h	13.11 ± 1.62**	17.00 ± 5.36**	20.44 ± 8.22**	8.00 ± 3.94**
	72 h	11.67 ± 1.80**	12.78 ± 2.73**	13.22 ± 3.31**	7.56 ± 1.88**
55T	24 h	10.89 ± 2.76**	12.67 ± 6.08**	14.33 ± 6.61**	8.67 ± 3.5**
	48 h	10.22 ± 0.67**	10.56 ± 1.01**	11.00 ± 1.58**	3.00 ± 0.71**
	72 h	14.67 ± 1.32**	18.67 ± 1.32**	23.56 ± 2.60	8.00 ± 1.50**
Control		20.78 ± 5.17	25.89 ± 3.44	27.25 ± 5.42	15.56 ± 5.25

Note: data are the average of three replications ± the standard error of the means . * and **, statistically significant from the control at the 0.05 and the 0.01 probability level respectively.

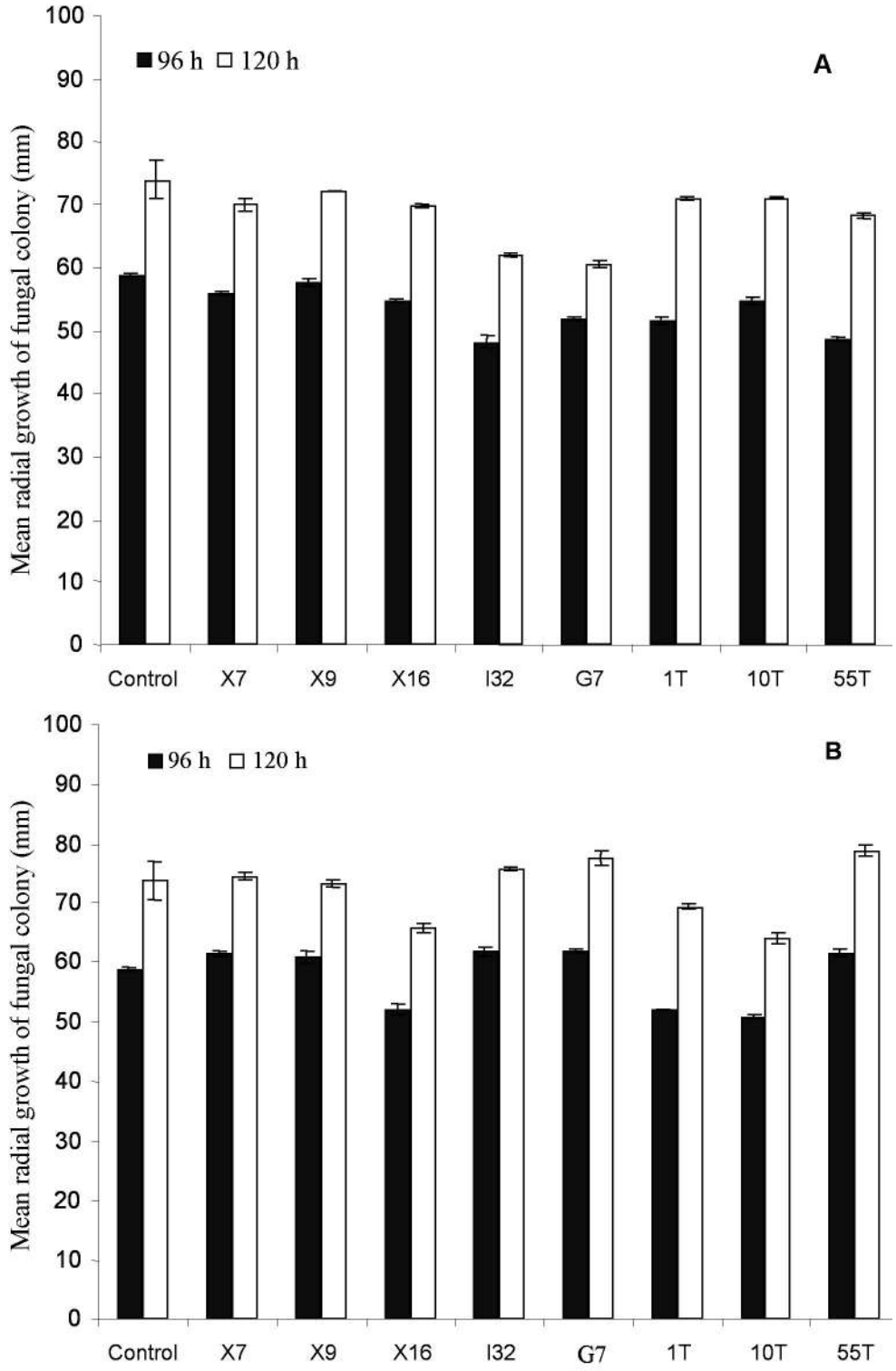

Fig. 4. Effect of volatiles of *Bacillus* isolates on radial growth of *F. roseum* var. s*ambucinum*. *Bacillus* isolates were grown either on **A** potato dextrose agar (PDA) or on **B** nutrient agar (NA).

Table 6. Effect of *Bacillus* spp. on the degradation of colloidal chitin agar.

Isolate	Species	Degradation of colloidal chitin agar
X7	*B. lentimorbus*	–
X9	*B. cereus*	–
X16	*B. cereus*	+
G7	*B. cereus*	–
I32	*B. licheniformis*	+
1T	*B. thuringiensis*	+
10T	*B. thuringiensis*	+
55T	*B. thuringiensis*	+

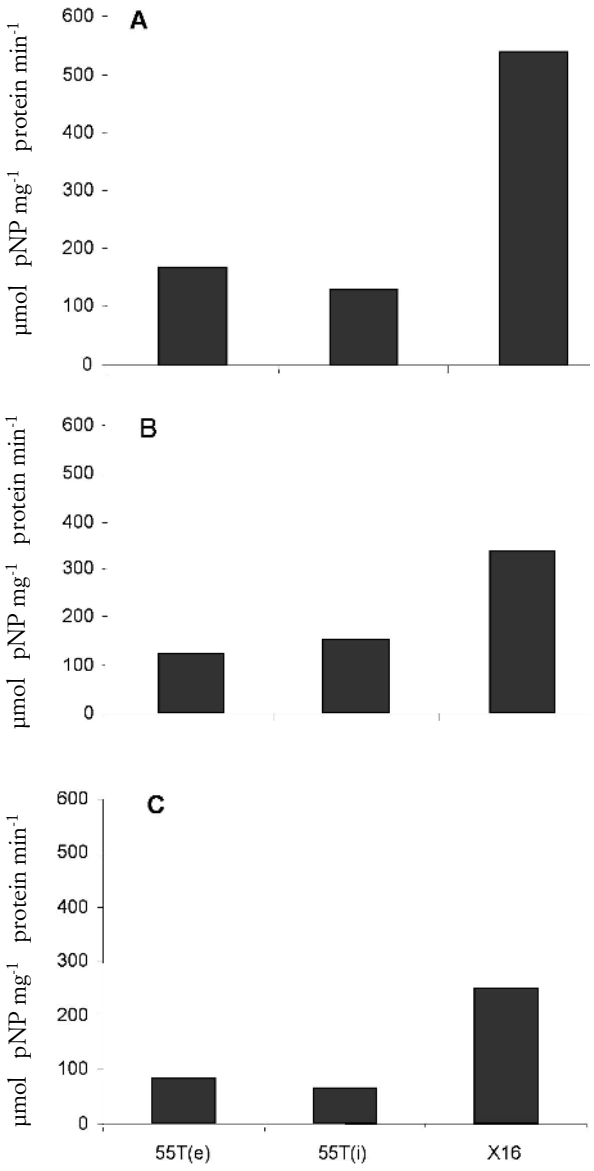

Fig. 5. Chitinase activity measured as (**A**) p-nitrophenol (pNP) released from p-nitrophenyl-N-acetyl-β-D-glucosaminide, (**B**) p-nitrophenyl-N-acetyl-β-D-N,N'-diacetylchitobiose, and (**C**) p-nitrophenyl-N-acetyl-β-D-N,N',N''-triacetylchitotriose by the halophilic *Bacillus* spp. isolate X16 and *B. thuringiensis* strain 55T(e): from extracellular proteins – 55T(i): from intracellular proteins.

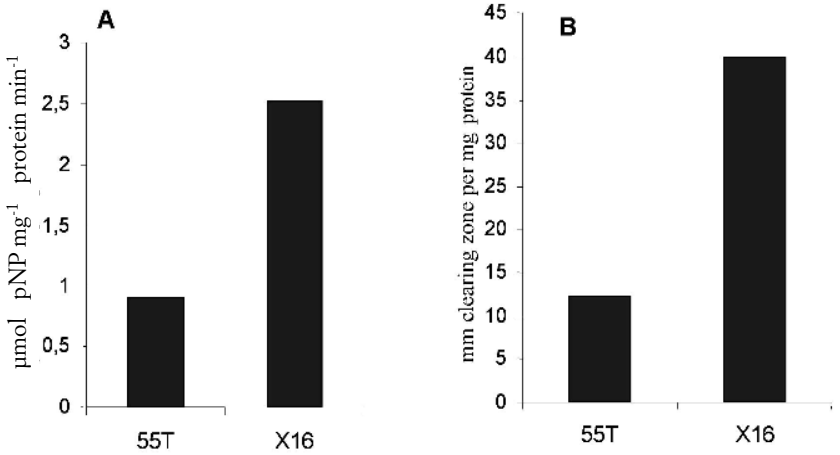

Fig. 6. Chitinase activity measured as (**A**) release of reducing sugars (NAG) from colloidal chitin and (**B**) formation of clearing zones on chitin agar using crude chitinase from X16 *Bacillus* sp. isolate and 55T *B. thuringiensis* strain.

DISCUSSION

The main aim of this research was to isolate and select bacterial biocontrol agents for controlling, or at least reducing, the effects of *F. roseum* var. *sambucinum* and dry rot development on potato tubers during storage. From the outset it was decided to isolate these bacteria from salty soils and to select for spore-forming bacterial antagonists (*Bacillus* spp.), since they would present the great advantage of being more resistant to harsh environmental conditions. *In vitro* and *in vivo* evaluation of *Bacillus* spp. as potential antagonists towards various phytopathogenic fungi has been described (Chang and Kommedhal, 1968; Kommedhal and Chang, 1975; Pusey and Wilson, 1984; Leifert *et al.*, 1993; Fiddman and Rossall, 1995; Pleban *et al.*, 1995; Sholberg *et al.,* 1995; Asaka and Shoda, 1996; Podile and Prakash, 1996; Korsten *et al.*, 1997; Sharga, 1997; Podile and Laxmi, 1998; Walker *et al.,* 1998; Chang *et al.*, 1999). This is, however, the first report in which *Bacillus* spp. from salty soils have been isolated and their antagonistic potential evaluated against the main agent of Fusarium dry rot of potatoes during storage. Five *B. thuringiensis* strains, previously selected for their antagonism towards insects, were also included in the present studies in an attempt to identify strains effective against both Fusarium dry rot and insect attacks, caused mainly by the potato tuber worm, *Phthorimaea operculella*, during storage. More than 50% of *Bacillus* spp. isolated from salty soils inhibited the mycelial growth of *F. sambucinum in vitro*. In contrast, all five *B. thuringiensis* strains failed to inhibit the growth of the pathogen during the *in vitro* dual culture screening. However, all these *B. thuringiensis* strains effectively inhibited dry rot development on wounded potato tubers, especially when they were applied at the right growth stage. This indicates that *Bacillus* isolates ineffective *in vitro* are not necessarily ineffective *in vivo.* We have also demonstrated that their activity *in vivo* is variable according to the age of bacterial preparation. The most inhibitory bacteria isolated from salty soils, were identified as belonging to one of the species *B. cereus* (X9, X16 and G7), *B. lentimorbus* (X7) or *B. licheniformis* (I32). While all these species best inhibited dry rot development when applied as young cultures (24 h), *B. thuringiensis* strains generally performed better as older cultures (48-72 h). This may indicates that the ability of these species to control Fusarium dry rot is correlated with their growth phase. A growing body of evidence indicates that production of antifungal metabolites by *Bacillus* species appears to be related to their physiological and development stages (vegetative growth, sporulation, germinating spore; Walker *et al.*, 1998). Identification of the growth stage under which the antagonist is the most effective will be useful to enhance the biocontrol potential of these *Bacillus* species against Fusarium dry rot development. In fact from our results it was evident that all *Bacillus* isolates selected from salty soils (X7, X9, X16, I32 and G7) and all the tested *B. thuringiensis* strains (1T, 10T, 14T, 33T and 55T) without exception significantly inhibited Fusarium rot development when applied at the right phase of growth (Figs 2 and 3, Tables 4 and 5). Our data indicate also that evaluation of rot development based on the measurement of the extent of rot penetration correlates better with the percentage of rotted potato tissues than lesion diameter determination. Dry rot severity determined by measuring the extent of rot penetration at the end of the experiment is a relatively rapid and easy method that would be very helpful in our studies of evaluation of *Bacillus* antagonists under commercial storage conditions. In fact, evaluation based on the percentage of rotted potato tissues is time consuming and too laborious.

Suppression of fungal growth *in vitro* by the selected *Bacillus* isolates and formation of inhibition zones were presumably due to the metabolites being released from bacteria into the culture medium. Different studies revealed that *Bacillus* spp. produce a wide range of diffusible metabolites including antibiotics (Walker *et al.*, 1998), biosurfactants (Edwards and Seddon, 1992), and cell-wall degrading enzymes (Priest, 1977; Pelletier and Sygusch, 1990; Frändberg and Schnürer, 1994a, b). The fact that *B. thuringiensis* strains are effective *in vivo* but unable to form inhibition zones in dual cultures may denotes a mode of action different from that performed by *Bacillus* isolates selected from salty soils. *B. thuringiensis* isolates may assume their antagonistic effect by mainly producing cell-bound antifungal compounds (Edwards, 1993; Walker *et al.*, 1998) or indirectly by inducing plant resistance mechanisms (Schönbeck *et al.*, 1980; Kehlenbeck *et al.*, 1994).

In the present studies neither the cell-filtrates of *Bacillus* isolates selected from salty soils nor those of *B. thuringiensis* strains inhibited the growth of *Fusarium*. While these results correlate with the dual culture assays in the case of *B. thuringiensis*, the ineffectiveness of culture supernatants of the other *Bacillus* isolates may merely indicates that the antifungal compounds were not produced in the culture media in the absence of the pathogen or were produced at low concentrations insufficient to inhibit the pathogen. The characteristic production and the release of antifungal metabolites by these *Bacillus* isolates require further study.

The results also showed that volatile substances may contribute to the inhibition of the growth of *F. roseum*

var. *sambucinum*. The activity of these antifungal volatiles varied greatly depending upon the medium on which the bacterium was cultivated. The number of *Bacillus* spp. producing inhibitory volatiles was more important on PDA than on NA. These results are in concordance with those of Fiddman and Rossall (1993), who showed that *B. subtilis* produced more antifungal volatiles when grown on PDA rather than on NA or triptych soy agar. Whether these antifungal volatiles produced by *Bacillus* spp. contribute to *Fusarium* rot suppression on potato tubers remains to be demonstrated. Nevertheless, several studies revealed the importance of antimicrobial volatiles in biocontrol of different plant diseases (Dymovich, 1960; Dennis and Webster, 1971).

Among the tested five isolates of *Bacillus* selected from salty soils, the two isolates X16 of *B. cereus* and I32 of *B. licheniformis* as well as all 3 tested strains of *B. thuringiensis* (1T, 10T and 55T) were able to degrade colloidal chitin. It has been demonstrated that various species of *Bacillus* such as *B. cereus*, *B. licheniformis*, *B. pabuli*, and *B. circulans* secrete chitinases (Watanabe *et al.*, 1990; Trachuk *et al.*, 1996; Pleban *et al.*, 1997). However, to our knowledge, little is known about crystal forming *B. thuringiensis* as far as these enzymes are concerned (Chigaleichik, 1976). There have also been no previous reports of halophilic *Bacillus* species producing chitinolytic enzymes. *B. cereus* X16 and *B. thuringiensis* 55T exhibited a strong chitinolytic activity as determined by the formation of clearing zones on chitin agar, the release of pNP from chromogenic analogs of chito-oligosaccharides and by the release of reducing sugars from colloidal chitin. When the activities of chitinases from the two bacteria X16 and 55T were compared, an evident agreement between these three methods was observed. The isolate X16 of *B. cereus* consistently showed a chitinase activity 2 to 3 times higher than that of strain 55T of *B. thuringiensis*. Such agreement between the different methods, was not observed by Frändberg and Schnürer (1994), when they compared the chitinolytic activities of 10 bacteria belonging to different genera. Our experiments with the chromogenic chito-oligosaccharides indicated also that *B. cereus* (X16) and *B. thuringiensis* (55T) are able to produce multiple chitinases. The current nomenclature divides chitinolytic enzymes into three main types: *N*-acetyl-β-D-glucosaminidases, which split the chitin polymer in an exo-type fashion; endochitinase, which randomly cleave the molecule of chitin at internal sites; exochitinases or chitin-1,4-β-chitobiosidases, which catalyses the release of diacetylchitobiose units from the chitin chain, such that no monosaccharides or other oligosaccharides are formed during the course of the reaction. Based on this nomenclature *B. cereus* X16 and *B. thuringiensis* 55T produced the three types of chitinases with exo- and endo-activities. The highest activities were generally observed with pNP-GlcNAc as a substrate when extracellular extracts were assayed. In contrast, Pleban *et al.* (1997), working with a strain of *B. cereus* showed only one chitinolytic activity of the chitobiosidase type produced by that strain. In fact, the strain of *B. cereus* used by these authors showed a chitinolytic activity with trimeric *p*NP-$(GlcNAc)_2$ but not with dimeric or tetrameric derivatives as a substrate. Our data are, however, in agreement with previous data demonstrating the presence of multiple chitinases in *Bacillus* strains, as well as in other bacterial genera such as *Streptomyces* (Broadway *et al.*, 1995), *Enterobacter* (Chernin *et al.*, 1995) and *Serratia* (Brurberg *et al.*, 1996). For example, Watanabe *et al.* (1990) indicated that *B. circulans* WL-12 produced six distinct chitinases. Strain 55T of *B. thuringiensis* used in our studies also showed significant levels of *N*-acetyl-β-D-glucosaminidase, endochitinase, and chitobiosidase in intracellular proteins. The diversity and complexity of chitinases produced by our selected strains may contribute significantly to their antagonistic activity towards *F. roseum* var. *sambucinum*. However, this could not be clearly demonstrated before isolation of mutants of these selected strains deficient in chitinases production. Chernin *et al.* (1995), demonstrated the importance of the ability to produce and excrete chitinolytic enzymes for biocontrol of *Rhizocotonia solani* by using Tn5 mutants of the antagonistic bacteria *Enterobacter agglomerans*. Mutants, which lost only chitinolytic activity, but not antibiotic or proteolytic activities, were unable to inhibit the growth of the pathogen and to reduce disease development.

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