The present studies were undertaken to investigate the interaction of the bacterial antagonists Bacillus cereus X16 and B. thuringiensis 55T with Fusarium roseum var. sambucinum, the causal agent of potato dry rot. On wounded potato tubers, both bacilli effectively suppressed the development of Fusarium dry rot. Nevertheless, confrontation of the fungal pathogen with the antagonists on nutrient agar revealed that B. cereus X16, induced a strong visible inhibition zone but B. thuringiensis 55T did not. Light microscopy of the interaction regions after confrontation with B. cereus X16 on nutrient agar generally showed apparently intact fungal cells with densely stained protoplasm, indicating that the inhibition was due to stasis rather than toxicity. By contrast, in presence of B. thuringiensis, Fusarium cells appeared markedly damaged, with partial to complete cell wall disintegration and disorganization and generally complete loss of protoplasm. These observations suggest that, contrary to our expectations, antibiotic and chitinolytic activities of B. thuringiensis 55T may be highly significant in the parasitism of the pathogen. Confrontation in liquid medium revealed that both bacteria completely inhibited the germination of Fusarium macroconidia and resulted in their destruction. This suggests that, to produce their fungitoxic and hydrolytic effects, the two bacteria should make intimate contact with Fusarium cells.

Key words: Bacillus, biocontrol, Fusarium dry rot, microscopy, cytochemistry.
da oleophila 182), gram-negative bacteria (Pseudomonas fluorescens EG1053, P. syringae ESC-10 and ESC-11, Burkholderia cepacia) and gram-positive bacteria such as Bacillus subtilis GB03 and MBI 600 (Kim et al., 1997; El-Ghaouth et al., 1998). Bacillus species, as a group offer several advantages over other gram-negative bacteria, including longer shelf life because of their ability to form endospores and the broad-spectrum activity of their antibiotics (Fiedman and Rossall, 1995; Kim et al., 1997). Bacillus species are generally soil-inhabitating or exist as epiphytes and endophytes in the spermosphere and rhizosphere (Walker et al., 1998). They are also found in many environments as their survival is aided by their ability to form endospores resistant to UV irradiation, dessication, heat and organic solvents. Some Bacillus species, such as B. thuringiensis and B. sphaericus, are entomopathogenic as they produce toxins effective against the larvae of a wide range of insects. The success of B. thuringiensis as an insecticide has promoted research and development for additional Bacillus-based products. There is a growing list of reports of postharvest fungal disease control with Bacillus species, including B. subtilis for control of green mold, sour rot, and stem end rot on citrus (Singh and Deverall, 1984), brown rot on stone fruit (Utkhede and Sholberg, 1986), and several diseases of apple (Sholberg et al., 1995) and avocado (Korsten et al., 1997).

Recently, 83 Bacillus isolates, obtained from salty soils, and five B. thuringiensis strains were screened in our laboratory for ability to suppress growth of F. roseum var. sambucinum in vitro and to control dry rot on potato tubers (Sadfi et al., 2001). Among these isolates, strains X16 of B. cereus and 55T of B. thuringiensis showed high levels of control in vivo. Nevertheless, while B. cereus inhibited the growth of the fungal pathogen in vitro by forming inhibition zones in dual cultures, B. thuringiensis (55T) failed to produce such inhibition zones and seemed to be ineffective on agar plates. According to the literature, Bacillus spp., may cause their antagonistic effects against fungal pathogens by antibiosis, nutrient competition, site exclusion, parasitism and/or induced resistance (Kehlenbeck et al., 1994; Muninbazi and Bullerman, 1998; Walker et al., 1998). The current study was undertaken to investigate more closely the interaction of B. cereus (X16) and B. thuringiensis (55T) with F. roseum var. sambucinum in vitro by means of light and transmission electron microscopy with gold cytochemistry.

MATERIALS AND METHODS

Fungal and bacterial isolates and growth conditions. The isolate of F. roseum var. sambucinum used was obtained from infected potato tubers with typical symptoms of Fusarium dry rot and reported to be virulent on potato tubers (Chérif et al., 2000). The fungal pathogen was grown on potato-dextrose agar (PDA) medium at 25°C.

Isolate X16 of B. cereus was selected among a collection of 83 Bacillus spp. isolated from samples of salty soils collected from different locations in the south of Tunisia (Sadfi et al., 2001). Strain 55T of B. thuringiensis was selected among six strains kindly provided by Dr. A. Boudabbous from the laboratory of Microbiology of the Faculté des Sciences de Tunis. Bacterial isolates were maintained on slants of nutrient agar (NA, Oxoid) at 4°C and subcultured at two-month intervals.

Antagonistic activity on solid medium. Two-day-old cultures of B. cereus X16 and B. thuringiensis 55T were studied in their interaction with F. roseum var. sambucinum on NA in 9-cm Petri plates using a dual culture technique. Bacteria were streaked across the center of each agar plate with a loopful of pure bacterial culture. Two 5-mm disks cut from a 7-day-old culture of F. roseum var. sambucinum, were placed 2.5 cm apart on each side of the bacteria. The plates were then incubated at 25°C. Mycelial samples were collected from the interaction region 2 to 6 days after inoculation and processed for light and transmission electron microscopy (TEM).

Antagonistic activity in liquid medium. These tests were performed in 250 ml-Erlenmeyer flasks containing 100 ml of sterile nutrient broth (NB). Fungal spore suspensions and bacterial suspensions were obtained by flooding 10-day-old PDA cultures of F. roseum var. sambucinum and 2-day-old NA cultures of Bacillus spp., respectively with sterile distilled water. The antagonists were added to the flasks containing NB and adjusted to obtain a final concentration of 10^6 CFU ml^-1 of the bacteria and 10^5 spores ml^-1 of the fungus. Bacterial and fungal concentrations were determined, respectively by dilution plating and counting with a hemocytometer. The flasks were then incubated at 25°C and 100 rev min^-1 on a rotary shaker. Samples were collected from 2 to 6 days after incubation. Fungal and bacterial cells were pelleted by centrifugation at 7000 g for 15 min. The pellets were embedded in 2% water agar and processed for light microscopy and TEM.

In order to determine the effect of the bacterial antagonists on the fungal mycelium, the same procedure
was adopted with the only exception that bacteria were added to the liquid medium 5 days after inoculation with fungal macroconidia. By that time, fungal spores germinated and developed a dense mycelium.

**Antagonistic activity on potato tubers.** Potato tubers cv. ‘Spunta’ were surface-sterilized by soaking in 2% aqueous sodium hypochlorite for 10 min. They were then thoroughly rinsed, dried by using sterile filter papers, and then wounded by removing a plug 3 mm in diameter and 3 mm in depth with a sterile cork-borer. *B. cereus* X16 and *B. thuringiensis* 55T isolates were used after, respectively, 24 h and 48 h of culturing at 10⁶ CFU ml⁻¹, and bacterized potato wounds received 20 µl of bacterial suspension. Potato wounds were challenged with the pathogen immediately after bacterial inoculation and received 20 µl of a conidial suspension adjusted to 10⁵ spores ml⁻¹. 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 20°C for one week. For each treatment 20 potato tubers were assayed and the experiment was repeated at least twice.

**Light microscopy and TEM.** Before fixation and processing for TEM, samples were always examined by light microscopy and photographed with a Zeiss Axioskop microscope (Carl Zeiss, Thorwood, NY). Samples collected from the interaction regions of solid dual cultures, from liquid cultures, as well as samples from pure cultures of the fungus and each bacterium, were fixed in 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer and post-fixed in 1% (w/v) osmium tetroxide in the same buffer. They were then dehydrated in a graded series of ethanol and embedded in Epon 812 resin. For light microscopy, thin sections (0.25 to 0.5 µm) were collected on microscope slides and stained with ethylene blue. For TEM, ultrathin sections were collected on 200-mesh nickel grids coated with Formvar and stained with uranyl acetate and lead citrate. Grids were examined with a JEOL 1200 EX transmission microscope (JEOL, Tokyo) operating at 80 kV.

For each treatment, samples were collected from at least four replicates to obtain a representative sampling of the interactions. For each sample, more than 10 thin or ultrathin sections were examined by light microscopy and TEM, respectively.

**Cytochemical labeling.** Colloidal gold suspension was prepared as described by Grandmaison *et al.* (1988). A lectin with N-acetylglucosamine binding specificity, was used for localizing fungal chitin according to a previously described procedure (Benhamou, 1989; Chérif and Benhamou, 1990). Because of its low molecular weight, this lectin could not be directly complexed to colloidal gold. It was used in a two-step procedure, using ovomucoid as a second step reagent. Ovomucoid was conjugated to gold at pH 5.4.

Sections were first incubated on a drop of WGA [25 µg ml⁻¹ in phosphate-buffered saline (PBS), pH 7.4] for 60 min at room temperature, rinsed with PBS, and transferred to a drop of ovomucoid gold-complex for 30 min at room temperature. After washing with PBS and rinsing with double-distilled water, sections were contrasted with uranyl acetate and lead citrate, and examined by TEM.

Specificity of labeling was assessed by the following controls: (i) incubation with WGA to which was previously added N,N',N'' triacetyl-chitotriose (1 mg ml⁻¹ in PBS); (ii) incubation with WGA, followed by unlabeled ovomucoid and finally by ovomucoid gold-complex; and (iii) direct incubation with the ovomucoid gold-complex, the lectin step being omitted.

**Reagents.** Tetrachloroauric acid was purchased from BDH chemicals, Montreal. All other reagents for electron microscopy were obtained from JBEM company, Pointe-Claire, QB, Canada.

**RESULTS**

**Macroscopic observations.** Coinoculation of *F. roseum* var. *sambucinum* with bacterial antagonists on NA revealed that *B. thuringiensis* 55T was apparently unable to inhibit growth of the fungus after 5 days of incubation (Fig. 1A). By that time, however, the fungus was strongly inhibited by *B. cereus* X16, with the appearance of inhibition zones (Fig. 1B). While the first contact between *B. thuringiensis* 55T and the pathogen was observed by 3 days after coinoculation, intimate contact between *F. roseum* var. *sambucinum* and *B. cereus* X16 was never observed even after many weeks of incubation.

Potato wounds inoculated with *F. roseum* var. *sambucinum* alone started to show typical dry rot symptoms by the third day of incubation at 20°C. By the seventh day, all potato tubers infected with the pathogen were diseased and showed lesions of more than 20 mm in diameter (Fig. 1C).

Application of both *Bacillus* isolates to potato wounds before challenge with the pathogen, significantly reduced development of dry rot (Fig. 1D and E). Tubers treated with *B. cereus* X16 and inoculated with the
pathogen showed no symptoms of infection after 7 days at 20°C (Fig. 1D). Although some small, brownish lesions could be seen in potatoes bacterized with *B. thuringiensis* 55T and inoculated with the pathogen after 7 days of incubation (Fig. 1E), their frequency and diameter never reached those in the infected controls. Tubers inoculated with either *B. cereus* X16 (Fig. 1F) or *B. thuringiensis* 55T alone were free of disease symptoms.

**Light microscopy observations.** Mycelial samples from pure cultures of *F. roseum var. sambucinum* revealed densely stained hyphal cells (Fig. 2A). After 3 days of confrontation, examination of sections from the edge of *Fusarium* colonies in contact with the inhibition zones caused by *B. cereus* X16 on NA generally showed apparently intact fungal cells with densely stained protoplasm (Fig. 2B). A relatively low percentage of *Fusarium* cells showed signs of cell damage, even after a much longer period of exposure to *B. cereus* X16. By contrast, in presence of *B. thuringiensis* 55T, where contact between the two protagonists was macroscopically observed, *Fusarium* cells appeared markedly damaged, as evidenced by disorganization of the cytoplasm and generally complete loss of protoplasm (Fig. 2C, D).

In pure liquid cultures (NB), fungal macroconidia germinated after 24 h of incubation (Fig. 3A) and gave a very dense mycelium with numerous newly formed macroconidia 5 days later (Fig. 3B). Coinoculation with fungal spores and either *B. cereus* X16 or *B. thuringiensis* 55T completely inhibited the germination of macroconidia after 24 h (Fig. 3C, D) and 6 days (Fig. 3E-G) of incubation. By the end of the experiment, the *Bacillus* spp. had abundantly colonized the medium and fungal macroconidia, resulting in destruction of a high percentage of macroconidia, which appeared brightly stained (Fig. 3E-G). In some instances, bacterial cells of *B. thuringiensis* 55T seemed to be inside macroconidia (Fig. 3G, arrow). This was never observed with *B. cereus* X16. Nevertheless, this bacterial isolate caused severe damage to the macroconidia in liquid medium (Fig. 3F).
Ultrastructural and cytochemical observations

Interaction of B. cereus X16 with the pathogen. The time course study of fungal growth in dual cultures in presence of B. cereus X16 revealed that colonization of the NA medium by Fusarium ceased 48-72h after confrontation. Up to that time, the majority of Fusarium hyphae were free of any sign of alteration, as judged by the integrity of the cytoplasm and cell wall (Fig. 4A). From 3 to 6 days after inoculation, some hyphae showed various degrees of cytoplasm disintegration, leading in some cases to complete depletion of their cytoplasm (Fig. 4B). Nevertheless, hyphae showing such severe alteration were very rarely observed and most cells of Fusarium var. sambucinum displayed apparently well-preserved organelles and cytoplasm. Labeling with the WGA/ovomucoid-gold complex revealed that even hyphae with advanced cytoplasm alteration preserved the integrity of their cell walls, which were intensely and regularly labeled with gold particles (Fig. 4C). In many instances, Fusarium cells developed cell wall thickenings, which were also intensively labeled (Fig. 4D). By contrast, coinoculation of Fusarium and B. cereus X16 in liquid medium (NB), resulted in much more damage to fungal cells (Fig. 4E, F). Under these conditions, the contact between the pathogen and B. cereus X16 was more intimate and direct (Fig. 4E). Six days after inoculation, most fungal cells appeared severely damaged and the main characteristic of these cells was partial to complete wall disintegration associated with depletion of cytoplasm contents (Fig. 4E). This observation was confirmed by labeling with the WGA/ovomucoid-gold complex, which showed that some fungal cells were reduced to traces that could only be identified by the presence of gold particles in remaining wall debris (Fig. 4F).

Interaction of B. thuringiensis 55T with the pathogen. Mycelial samples from the edge of growing colonies of F. roseum var. sambucinum examined two days after coinoculation with B. thuringiensis 55T on NA, revealed generally dense hyphal cells with apparently preserved cell walls, nucleus and organelles (Fig. 5A).

Fig. 2. Light microscope observations of F. roseum var. sambucinum cells from pure cultures (A), from the edge of Fusarium colonies in contact with the inhibition zone caused by B. cereus X16 (B), or from fungal colonies in contact with B. thuringiensis 55T (C, D), after 3 days of incubation on nutrient agar plates. F = Fungus.
Alterations in wall labeling with the WGA/ovomucoid-gold complex started to appear by 3 days after inoculation, at which time the two protagonists were very close to each other (Fig. 5B). These wall-labeling alterations were always associated with abundant presence of gold particles in NA medium (Fig. 5B, arrows). At this stage, fungal nuclei and vacuoles often showed obvious signs of malformation, as exemplified by contortion of their membranes (Fig. 5B, and C).

In many instances, fungal cells responded to *B. thuringiensis* 55T attack by the accumulation of cell wall appositions and thickenings (Fig. 5D). Such appositions were intensely labeled with the WGA/ovomucoid-gold complex indicating high accumulation of chitin at these sites (Fig. 5D). As early as 3 days after inoculation, significant alterations of the fungal cytoplasm were readily discernable, such as retraction, aggregation and organelle disintegration (Fig. 5C). By that time, fungal cell walls generally showed good structural preservation. Past this stage, from 4 to 6 days after inoculation, most *Fusarium* cells became affected (Fig. 6A), with pronounced damage, as judged by total loss of protoplasm (Fig. 6A-C) and partial or complete wall disintegration (Fig. 6B), leading to empty hyphal shells with many holes, corresponding to locally digested areas in the cell walls (Fig. 6C1, and C2). Such cell wall degradation was generally accompanied by the release of gold particles, which were scattered over the surrounding agar medium (Fig. 6C2).

Examination of ultrathin sections from samples of *Fusarium* macroconidia and mycelium confronted with *B. thuringiensis* 55T in liquid medium (NB) revealed the same events of fungal cytoplasm disintegration and cell wall degradation (Fig. 6D), with the exception that alterations started to appear by 24 h after inoculation, thus earlier than for solid cultures, where the first contact between the two protagonists was not observed before 3 days of incubation.
Specificity of labeling with the WGA/ovomucoid-gold complex was assessed by the negative results obtained with all control tests including the previous adsorption of the WGA with N,N',N''-triacetylchitotriose (data not shown).

**DISCUSSION**

Our results based on ultrastructural observations and cytochemical localization of N-acetylglucosamine residues showed that contrary to our expectations, antibiotic and chitinolytic activities of *B. thuringiensis* 55T may be highly significant in the parasitism of *F. roseum* var. *sambucinum*. In fact, although *in vitro* antagonism tests performed on NA showed no apparent inhibition of *Fusarium* growth in presence of *B. thuringiensis* 55T, microscopic observations revealed that this antagonist was very destructive to fungal hyphae, on both solid and liquid media. Moreover, although apparently ineffective *in vitro* on NA, *B. thuringiensis* 55T effectively inhibited dry rot *in vivo* on wounded tubers. This confirms the idea that *Bacillus* isolates unable to form inhibition zones on solid medium are not necessarily incapable of killing the pathogen *in vitro* and inhibiting disease development *in vivo*.

Confrontation of *B. cereus* X16 with the pathogen on solid medium (NA) demonstrated that *Fusarium* hyphae appeared generally intact with a very low percentage of damaged cells, indicating that the inhibition zone observed exerted a fungistatic rather than a fungitoxic role. These conclusions were confirmed by ultrastructural observations, which generally revealed fungal hyphae with preserved cytoplasm, organelles and cell walls. Even the few pathogen cells showing damaged protoplasm preserved the integrity of their cell walls as evidenced by the intense and regular labeling for chitin. By contrast, confrontation of the pathogen with *B. thuringiensis* 55T...
Thuringiensis 55T resulted in serious damage to a high percentage of hyphae, associated with a series of degradation events, including alteration of chitin macromolecules and visible cell wall disruption, retraction of the plasmalemma, distortion of the nucleus, generalized disorganization of the cytoplasm, and, ultimately, complete loss of protoplasm. Fungal cell wall disintegration and cytoplasm disorganization were observed as soon as 3 days after confrontation, just before first contact between the two protagonists was established. This suggests that fungitoxic compounds and hydrolytic enzymes produced by *B. thuringiensis* 55T diffuse a short distance into the agar to cause the observed disturbances in advance of physical contact.

Cell wall appositions and thickenings in *Fusarium* cells were observed in presence of both bacteria. Such deposits contained large amounts of chitin and are similar to wall appositions observed in the cells of different fungal pathogens submitted to the activity of antagonistic fungi (Benyagoub et al., 1998; Benhamou et al., 1999) and to treatment with fungicides (Robertson and Fuller, 1990), chitosan (Benhamou, 1992) or 2-deoxy-D-glucose (El-Ghaoouth et al., 1997). The mechanisms that control the process of chitin deposition at such sites and the exact biological function played by these deposits are still unclear. Nevertheless, from the literature the most accepted explanations indicate that the deposited material may be laid down as newly synthesized molecules via deregulation of fungal membrane-bound enzymes involved in the synthesis of structural compounds (Benhamou et al., 1999). Accordingly, the massive accumulation of these structural compounds as abnormal wall-like deposits reflects a defense strategy elaborated by the fungal pathogen for preventing penetration of mycoparasites and fungitoxic compounds. Whatever the role played by the deposited material, our *Bacillus* antagonists, when in direct contact with the pathogen, i.e. in liquid medium, were able to circumvent such barriers and cause severe damage to fungal cells, which were rapidly reduced to empty shells.

The differences observed between *B. cereus* X16 and *B. thuringiensis* 55T on NA, relative to the percentage of fungal cells affected, cell wall degradation and the extent of cytoplasm disintegration, were not observed in liquid medium. In fact, *B. cereus* X16 was as effective as *B. thuringiensis* 55T and caused extensive cell wall disruption and cytoplasm disorganization of the
pathogen. This suggests that in order to exert its fungitoxic and hydrolytic effects, *B. cereus* X16 must be in intimate contact with the *Fusarium* cells. The absence of inhibition zones on solid medium after application of *B. thuringiensis* 55T may be explained by the fact that the fungal pathogen can counteract fungistatic effects and therefore reach intimate but deadly interaction with the bacterium. In contrast, fungistatic compounds produced by *B. cereus* X16 may halt fungal spread but avoid the lethal effects of close contact.

When intimate contact between the protagonists is achieved, fungal cell wall degradation by hydrolytic enzymes produced by both antagonists seems to play a major role in the outcome of their interaction with the pathogen. In a recent study, we have reported that *B. cereus* X16 and *B. thuringiensis* 55T exhibited strong chitinolytic activity as determined by the formation of clearing zones on chitin agar, the release of reducing sugars from colloidal chitin and by the release of p-nitrophenol (pNP) from dimeric, trimeric and tetrameric chromogenic chito-oligosaccharides, thus showing that these antagonists are able to produce N-acetyl-β-D-glucosaminidases, chitobiosidases and endochitinases (Sadfi et al., 2001). Our TEM observations revealed that while the alteration of chitin first occurred locally, as illustrated by the release of N-acetylglucosamine residues in the growing medium, it appeared to be more generalized later on and at more advanced stages of the interaction of the pathogen with the antagonistic bacilli. *Fusarium* cell walls were completely digested. From these observations, it can be speculated that the synergistic and coordinated action of chitinases with other polysaccharidases, such as β-1,3-glucanases, lipases and proteases may be an important determinant in the antagonistic process (Sivan and Chet, 1989; Chérif and Benhamou, 1990; Benhamou and Chet, 1996).

Confrontation of conidia and hyphae of *F. roseum* var. *sambucinum* to the bacilli generally resulted in severe alteration of their protoplasm. Such alteration may directly correlate with the toxic action of antifungal substances (*i.e.* antibiotics) produced by the antagonists. In

![Fig. 6. Transmission electron micrographs of *F. roseum* var. *sambucinum* hyphae grown on nutrient agar (A-C) or in liquid medium (D) in presence of the antagonistic bacterium *B. thuringiensis* 55T. (A, B) *Fusarium* hyphae showing pronounced damage after 4 days of incubation. (C1, C2) *Fusarium* hyphae labeled with the WGA/ovomucoid-gold complex. Note that cell wall degradation is accompanied with the release of gold particles (C2, a higher magnification of C1, arrow). (D) Confrontation of the pathogen with *B. thuringiensis* 55T in liquid medium. Bar = 1 µm. B = Bacterium; F = Fungus; W = Wall.
fact, fungal protoplasmic alterations have been reported in other antagonistic interactions involving antibiosis as the main mechanism of action (Hajlaoui et al., 1993; Bélanger et al., 1995). For instance, Benyagoub et al. (1996), showed that antibiotics from Sporothrix flocculosa caused protoplasm alteration and depletion in treated pathogenic fungi by promoting modification of the lipid composition of the plasmalemma. This raises the question as to what extent weakening of the cell wall of pathogenic fungi through the action of hydrolytic enzymes of the antagonist may facilitate the diffusion of toxic substances toward membrane receptors by increasing the wall permeability. A growing body of evidence indicates that the synergistic action of wall hydrolyases, especially chitinases, and antibiotics is required in the antagonistic process and that alteration of host cell walls are essential prerequisites for further antibiotic diffusion (Di Pietro et al., 1993). These findings are in line with our observations, which have demonstrated a high percentage of affected fungal cells with severely altered cytoplasm in liquid medium, where close contact between the fungus and B. cereus X16 was well established and cell wall alterations of the pathogen were evident. Such results were not observed on solid medium, where the fungal cell walls appeared intact, and chitin breakdown was not detected by cytochemical labeling.

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