# PURIFICATION AND CHARACTERIZATION OF THE NOVEL BACTERIOCIN BAC IH7 WITH ANTIFUNGAL AND ANTIBACTERIAL PROPERTIES

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## SUMMARY

*Bacillus subtilis* strain IH7 was isolated from the rhizosphere of Tunisian healthy plants and selected for its antimicrobial activities against bacteria and fungi. This strain produced a novel antimicrobial peptide (bacteriocin) called Bac IH7. The pure bacteriocin was obtained after precipitation by ammonium sulphate, chromatography on Sephadex G-25 and C18 reverse-phase HPLC. The SDS-PAGE analysis of purified Bac IH7 revealed a single band with an estimated molecular mass of approximately 14 kDa. Bac IH7 was sensitive to various proteases, thus proving to have protein nature and displayed a wide inhibitory spectrum towards Gram-positive and Gram-negative bacteria and fungal pathogens. Purified Bac IH7 exhibited a bactericidal mode of action against *Agrobacterium tumefaciens* C58 and a fungistatic mode of action against *Candida tropicalis* R2 CIP203. Interestingly, based on conventional agronomic parameters for seed vigour, the application of Bac IH7 (800 AU) showed it to be a potent exogenous enhancer of growth that enhanced the vigour of tomato and muskmelon seedlings. Compared to the control, the germination percentage, shoot weight, shoot height, and root length were all significantly enhanced in the Bac IH7-treated plant seeds. Bac IH7 also exhibited effective disinfectant properties against seed-borne diseases. In treated seeds, Bac IH7 had significant effects on the control of damping-off disease groups at the pre-germination stage, of root rot caused by *Alternaria solani*, as well as of wilt diseases and other bacterial seed-borne pathogens.

*Key words*: *Bacillus subtilis*, bacteriocin, Bac IH7, agronomic seed vigour response, seed disinfectant, biocontrol.

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## INTRODUCTION

The use of biological control based on natural microorganisms offers a powerful alternative to chemical control of plant diseases. In fact, the abuse of pesticides and fungicides to cure or prevent plant diseases has often been reported to bring about a wide array of pernicious effects, particularly on plants, soil, environment and, ultimately, humans. Fungicide and pesticide seed treatments have often been used in conventional agriculture to protect against soil-borne diseases, to prevent or reduce plant mortality and losses, to enhance plant emergence and, thus, to improve their overall production (Tu and Zheng, 1993). Nevertheless, disinfectants, such as sodium hypochlorite, or fumigants, such as methyl bromide, can cause toxicity problems to young plants and serious occupational and environmental risks to handlers and the environment (Soriano *et al*., 2006). They can also pose irreparable damage to the metallic structure of greenhouses (Ciafardini and Zullo, 2003). Physical methods, such as heat treatment, are not always adequately appropriate for application and often produce large amounts of unviable seeds (Soriano *et al*., 2006).

Due to the limitations associated with conventional chemical and physical control systems, recent research has been directed primarily towards the search for alternative control strategies. The literature seems to emphasize that in order for a potential alternative control agent to be effective, it should meet at least three main criteria., i.e. to be highly specific against the target pathogens, easily degradable after use, and sufficiently cost-effective for wide scale application. Accordingly, extensive interests in developing control strategies with maximum effectiveness and minimum occupational and environment risks have emerged in the last few decades (Montesinos, 2007). This new line of research has, in fact, come to the conclusion that effective solutions for the management of soil-borne diseases are more likely to involve biocontrol agents rather than conventional chemicals.

Current research provides strong evidence that biological control offers one of the most promising, environmentally safe, and cost-effective tactics. In fact, the

use of bacteria as biocontrol agents has been extensively investigated, and a wide array of bioactive metabolites, such as bacteriocins and antibiotics, and others with antifungal, antiviral, insecticide and herbicide properties have been described in the literature. Bacteriocins, the prevailing group of antimicrobial agents, are defined as proteins or peptide antibiotics able to kill closely related bacterial populations without causing harm to the producer strain (Montesinos, 2007).

It is well documented that bacteriocins or bacteriocin-like substances can be produced by different *Bacillus* species. The latter include Bac 14B (Hammami *et al*., 2009) produced by *B. subtilis* 14B, BLIS (Risoen *et al*., 2004) produced by *B. cereus* ATCC 4579, coagulin (Le Marrec *et al*., 2000) produced by *B. coagulans*, megacin (Von Tersch and Carlton, 1983) produced by *B. megaterium*, tochicin (Paik *et al*., 1997), thuricin 7 (Cherif *et al*., 2001), thuricin 439 (Ahern *et al*., 2003), entomocin 9 (Cherif *et al*., 2003) and entomocin 110 (Cherif *et al*., 2008) produced by *B. thuringiensis*. However, despite their promising properties as potential antibacterial agents, only a few *Bacillus* bacteriocins have been fully characterized.

In fact, *B. subtilis* is known to produce many antimicrobial peptide substances, such as bacilysin (Walker and Abraham, 1970), mycobacillin (Sengupta *et al*., 1971), subtilin (Gross *et al*., 1973), and subtilosin A (Shelburne *et al*., 2007), antifungal substances antagonistic to *Aspergillus flavus* (Moyne *et al*., 2001), biosurfactants, chitinases and other fungal wall-degrading enzymes, as well as volatiles and compounds that elicit plant-resistance mechanisms (Leifert *et al*., 1995). Moreover, *B. subtilis* NB22 has been reported to produce iturin, an antifungal-antibiotic peptide that can effectively be used to control a broad range of soil-borne diseases of tomato. Last but not least, a recent study that involved the screening of bacteriocin-producing strains with anti-*Agrobacterium* spp. activity has found that *B. subtilis* 14B is able produce a novel bacteriocin (denoted Bac 14B) that is highly effective as a BCA against crown-gall disease (Hammami *et al*., 2009).

In view of the persistent search for natural alternatives to chemical control practices, the present study was undertaken to probe into the promising properties of the genus *Bacillus* in the attempt to identify potential new BCAs that can help inhibit fungal and bacterial soil- borne pathogens. During a search program for bioactive antifungal and antibacterial compounds from *Bacillus* conducted by our research team, a new strain called IH7 was isolated from the rhizosphere of almond trees cultivated in the nursery soils of Chbika (Kairouan, Tunisia), historically known to be highly contaminated by *A. tumefaciens* (Rhouma *et al*., 2004), and selected for its antimicrobial activity against bacteria and fungi. The present paper describes the selection and identification of IH7 and reports the purification and characterisation of a bacteriocin-like substance denoted Bac IH7.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *B. subtilis* strain IH7 was used as the source of chromosomal DNA to amplify the 16S rRNA gene. Strains of *E. coli* DH5α (Hanahan *et al*., 1983) served as host strains and the following bacterial strains were used as indicators for antibacterial activity assays: Gram-positive bacteria (*Micrococcus luteus* LB 14110 and *Staphylococcus aureus* ATCC 6538) and Gram-negative bacteria (*A. tumefaciens* C58, *A. rhizogenes* CFBP 2408, *A. vitis* CFBP 2678$^{T}$, *Pseudomonas savastanoi* pv *savastanoi* IVIA 1628, *E. coli* ATCC 8739 *Pseudomonas aeruginosa* CIP 82.118, *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* (kindly provided from the culture collection of The Centre of Biotechnology of Sfax). Antifungal activity was determined against *Fusarium* spp., *Phytium* spp, *Rhizoctonia solani* (kindly provided by Dr. M.A. Triki, Olive Tree Institute of Sfax), *Alternaria citri, Alternaria solani, Phytophtora capsici, Rhizoctonia solani, Aspergillus oryzae* (kindly provided from the culture collection of The Centre of Biotechnology of Sfax), *Verticillium dahliae* V4i, *Botrytis cinerea* SAS56 and *Candida tropicalis* R2 CIP20. The pCR2.1 vector (Invitrogen, USA) was used as cloning vector. Thus, pIH7 is a derivative of pCR2.1 vector carrying a 1540 bp DNA fragment corresponding to the whole 16S rRNA gene of the IH7 strain.

**Culture conditions.** *E. coli* DH5α was grown on Luria Bertani (LB) agar medium plates [containing g $l^{-1}$ of yeast extract (5), peptone (10), NaCl (10) and bacteriological agar (20)] supplemented with ampicillin (50 g $l^{-1}$) and 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (40 g $l^{-1}$) when appropriate (Sambrook *et al*., 1989). Transformation of *E. coli* DH5α with the pIH7 derivative was carried out according to Hanahan *et al*. (1983).

Indicator microorganisms were grown overnight in LB broth to determine the antibacterial activity of the isolate. Strains of *A. tumefaciens* C58, *A. rhizogenes* CFBP 2408, *A. vitis* CFBP 2678$^{T}$, *P. savastanoi* pv. *savastanoi* IVIA 1628, *P. syringae*, *E. coli* ATCC39 and *X. campestris* were grown in aerobic conditions at 30°C and pH 7.2. Strains of *E. coli* ATCC 8739 and *S. aureus* ATCC 6538 were grown in aerobic conditions at 37°C. As to the determination of antifungal activities, fungi were grown in PDA at 30°C for 7 days. Spores were collected in sterile distilled water and concentrated to produce a suspension with approximately a $10^4$ spore $ml^{-1}$.

*C. tropicalis* R2 CIP203 was grown in YP10 medium [containing g $l^{-1}$ of yeast extract (10), peptone (10), glucose (100), plus 15 ml of 2 g $l^{-1}$ adenine solution] in an orbital shaking incubator set at 200 rpm and 30°C for

24 h. The IH7 strain was maintained as frozen stock at -80°C in LB broth containing 20% (v/v) glycerol. Before use, the cultures were propagated twice in LB medium at 30°C for 12 h. Transfer inoculum was 1% (v/v).

**Identification of antagonistic strain IH7.** Strain IH7 was identified by carrying out biochemical and physiological tests as described in the Bergey's Manual of Systematic Bacteriology. Amplification of 16S rDNA was conducted by PCR with two universal primers, designed from the conserved zones within the rRNA operon of *E. coli* (Gurtler and Stanisich, 1996). The forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') extended from base position 8 to 27; the reverse primer (5'-AAGGAGGTGATCCAAGCC-3') extended from base position 1541 to 1525. Genomic DNA of strain IH7 was then used as a template for PCR amplification that consisted of 35 cycles, with denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min. The amplified product *ca.* 1.5 kb in size was cloned in the pCR-2.1 vector (Invitrogen, USA), leading to the pIH7 plasmid. All recombinant clones of *E. coli* DH5α were grown in LB media with the addition of ampicillin, IPTG and X-gal for screening. DNA electrophoresis, purification, restriction, ligation, and transformation were carried out in accordance with Sambrook *et al.* (1989). The nucleotide sequence of 16S rRNA gene was determined on both strands with BigDye Terminator v3.1 Cycle Sequencing Kit and the automated DNA sequencer ABI Prism 3100-Avant Genetic Analyser (Applied Biosystems, USA). Computer analysis of the 16S rRNA sequence was performed with the Sequence match software package through the Ribosomal database project (http://rdp.cme.msu.edu/html).

**Biological assay of antimicrobial activities.** *Antibacterial activity assay.* The antibacterial activity of bacteriocin was ascertained by the agar-well diffusion assay (AWDA) (Tagg and McGiven, 1971). After an adequate time of incubation, the diameter of the inhibition zones was measured. The bacteriocin samples to be spotted were serially diluted two-folds, and the reciprocal of the highest inhibitory dilution was used to calculate the arbitrary activity units (AU) per millilitre. The uninoculated media were also tested for inhibitory zones and served as a control.

*Antifungal activity assay.* Petri dishes (9 cm diameter) containing 20 ml PDA were used for antifungal activity assay, performed according to Duru *et al.* (2003). Sterile Whatman paper discs 6 mm in diameter were pierced in the agar, equidistant and near the border, where an aliquot of 20 µl of the bacteriocin Bac IH7 was introduced. An agar plug of fungal inoculums (6 mm diameter) was removed from cultures of all the fungal strains to be tested and placed upside down in the center of the Petri dishes. The plates were incubated at 25°C for 2-7 days, until the fungal growth in the control plates reached the edge of the plates. Plates without Bac IH7 were used as negative controls. The plates were used in triplicate for each treatment. The relative growth inhibition of the treatment compared to the negative control was calculated as a percentage, using the following formula:

Inhibition (%) = [1– radial growth of treatment (mm)/ radial growth of control (mm)] × 100

**Culture conditions.** To determine the optimal nutritional and culture conditions for active compounds production, $10^4$ CFU $ml^{-1}$ of the IH7 strain were used to inoculate 1000 ml Erlenmeyer flasks with four indents, containing 200 ml of LB medium supplemented with 1% (w/v) of one of the five tested carbon sources (maltose, glucose, fructose, glycerol and saccharose). After incubation in an orbital shaking incubator at 200 rpm and 30°C for 72 h, each culture supernatant was assayed for its biological activities. In order to optimize the concentration of the carbon source, eight different concentrations of glucose were tested: 2, 2.5, 3, 3.5, 4, 4.5, 5 and 5.5 g $l^{-1}$. To investigate the effect of different concentrations of $MgSO_4.7H_2O$ (0, 0.123, 0.246, 0.369, 0.492, 0.615, 0.738, 0.861, 0.984 g $l^{-1}$), $K_2HPO_4$ (0.174 and 0.348 g $l^{-1}$) and trace mineral oligoelements (0.04 g $ZnCl_2$, 0.2 g $FeSO_4.7H_2O$, 0.0065 g $H_3BO_3$ and $MoNa_2O_4.2H_2O$ per 100 ml of distilled water) on the production of active molecules, 1.5 ml of the latter solution were added to 200 ml of the growth medium. The effects of culture conditions, such as those pertaining to the different values of initial pH (5.5, 6, 6.5, 7, 7.5 and 8), incubation temperature (25, 30, 35 and 40°C), incubation time (6, 14, 24, 48, 60, 72, 96, 120 and 144 h) and agitation rates (100, 150, 200, 250 and 300 rpm), on growth and production of antimicrobial substances were also investigated. The growth of IH7 strain was monitored in terms of colony-forming units (CFU $ml^{-1}$) at different time intervals. The influence of adding heat-killed fungi to the culture media on the production of active compounds was determined using three different concentrations (0.01, 0.05 and 0.1 g $l^{-1}$) of heat-killed fungi. The latter were prepared using $10^7$ spores $ml^{-1}$ of *Verticillium dahliae* V4i, *Fusarium* sp. or *Aspergillus oryzae* to inoculate 1000 ml Erlenmeyer flasks with four indents containing 200 ml of Sabouraud medium [g $l^{-1}$ of peptone (10), glucose (10) and pH adjusted to 5.6]. After incubation in an orbital shaking incubator at 200 rpm and 30°C for 48 h, the cultivation medium was filtered in a sterile environment using a non-absorbent cotton filter tube for the separation of spore suspensions and hyphal fragments. The hyphal fragments retained in the cotton wool were washed twice with distilled water, mixed with 50 ml of distilled water, then autoclaved twice at 120°C for 20 min. After cooling, the

hyphal suspension was placed at 80°C for 2 h and freeze-dried overnight. The agar PDA plates were inoculated with a sample suspension from this dry extract to confirm the absence of surviving hyphae.

**Bacteriocin purification, molecular weight determination and zymogram analysis.** One litre of culture medium, obtained after 72 h cultivation was centrifuged at 9,000 *g* for 30 min to remove the cells. The supernatant containing extracellular active compounds was designated as the crude bacteriocin preparation. Proteins from the clear supernatant were precipitated with 40, 60 and 80% saturated ammonium sulphate, gradually added by slow stirring at 4°C for 4 h. After centrifugation at 9,000 *g* and 4°C for 30 min, the precipitate was suspended in 10 ml of 20 mM sodium phosphate buffer pH 7.0 and dialyzed overnight against repeated changes of the same buffer. The protein solution (designated Fraction I) was stored at -20°C for further use. A 2.5 ml aliquot of the resulting solution (Fraction I) was loaded on a column (70 × 1.5 cm) of gel filtration Sephadex G-25 equilibrated with 20 mM sodium phosphate buffer (pH 7). The proteins were eluted with the same buffer at 0.5 ml $min^{-1}$. Using absorption measurements at 280 nm, a total of 135 fractions (F1-F135) with 5 ml each were detected and then eluted and automatically collected from the Sephadex G-25 column. They were then fractioned into six samples (I1-I6): I1 (F1-F40), I2 (F41-F70), I3 (F71-F76), I4 (F77-F81), I5 (F82-F93) and I6 (F94-F134) and tested for their antimicrobial activity using *A. tumefaciens* C58, *S. aureus* ATCC 6538 and *Fusarium* sp. as indicator strains. The active fraction was then subjected to a second round of purification by a reverse phase HPLC using a C18 column 201 (300 × 4.6 mm) at a flow rate of 0.5 ml $min^{-1}$. Bacteriocin was eluted from the column with two mobile phases: A (99.9% water, 0.1% trifluoroacetic acid "TFA") and B (99.9% acetonitrile, 0.1% TFA); from 0 to 5 min (90% A, 10% B), from 5 to 30 min (50% A, 50% B), from 30 to 35 min (20% A, 80% B), from 35 to 50 min (10% A, 90% B) and from 50 to 60 min (90% A, 10% B). The proteins were monitored at 280 nm. The pooled biological active fraction obtained from HPLC elution was concentrated and stored at -20°C. The estimated molecular weight of the purified bacteriocin was determined by SDS-PAGE according to Laemmli (1970) with 20% acrylamide gel. Protein concentration was measured using BSA as a reference following Bradforf (1976). Protein marker was from Pharmacia (Sweden) with a broad range of 14-97 kDa. Protein bands were visualized by Coomassie brilliant blue R-250 (Bio-Rad, USA) staining. Following SDS-PAGE, a zymogram analysis was done and the gel was washed twice for 1 h in water containing 100 ml of 2.5% Triton X-100 to replace SDS and the separation buffer in the gel. The washed gel was then incubated at 30°C for 4 h in sodium phosphate buffer and the antimicrobial activity was identified as a clear zone at the location of the bacteriocin band by overlaying it with an LB soft agar containing $10^8$ cells of *A. tumefaciens* C58 as an indicator.

**Effect of pH, temperature and enzymes.** Bac IH7 (800 AU $ml^{-1}$) was resuspended in different buffer solutions at 50 mM with pH values ranging from pH 2.0 to 9.0 (citrate, for pH 2.0-6.0; phosphate for pH 7.0; Tris-HCl for pH 8.0-9.0) and incubated at 4°C for 2 h. Antimicrobial activity was expressed in terms of AU $ml^{-1}$ and compared with the untreated controls. The thermal stability of bacteriocin was assessed by determining the residual activity after incubation of Bac IH7 at different temperature levels ranging from 40°C to 100°C for 30 and 60 min and to 120°C for 20 min. The sensitivity of Bac IH7 to proteolytic, lipolytic and α-amylase enzymes was investigated by the addition of trypsin, pronase E (Sigma-Aldrich, USA), proteinase K (Boehringer, Germany), lipase A (Sigma, USA), α-amylase and endo H (New England Labs, France) at final concentrations of 1 mg $ml^{-1}$ to 800 AU $ml^{-1}$ of Bac IH7. After 2 h of incubation at 37°C, enzyme activity was stopped by heating at 100°C for 5 min and the residual activity was determined immediately afterwards, as described above. Antimicrobial activity was assayed using *A. tumefaciens* C58. The untreated protein samples were used as controls.

**Inhibitory spectrum and mode of action of Bac IH7.** The inhibitory spectrum was checked by AWDA (Tagg and McGiven, 1971) for antibacterial activity and by the disc diffusion method (Duru et *al*., 2003) for antifungal activity. Pure bacteriocin Bac IH7 was added to 200 ml LB culture of *A. tumefaciens* C58 and 200 ml YP10 of *C. tropicalis* at an early exponential phase ($10^7$ CFU $ml^{-1}$) so as to obtain a final concentration of 800 AU $ml^{-1}$. The two indicator microorganisms grown in LB (*A. tumefaciens* C58) and YP10 (*C. tropicalis)* in the absence of bacteriocin were used as controls. The changes that the cultures underwent in terms of turbidity were recorded at an O.D. of the 600 nm and the number of viable cells (CFU $ml^{-1}$) was determined by plating the samples on LB or YP10 agar at different time intervals.

**Phytotoxicity.** Phytotoxicity was assessed by determining the germination index at 36-72 h of tomato (*Lycopersicon esculentum*) and 25-50 h of muskmelon (*Cucumis melo*) seeds following the standard method of Zucconi *et al*. (1981). Tomato seeds were treated by water (negative control), sodium hypochlorite (positive control) and three different doses of Bac IH7 (1200, 1000 and 800 AU $ml^{-1}$). The post-germinated seeds were transplanted in a sterile potting mix as described below. Traditional seed vigour biomarkers were determined based on the total number of fully emerged

seedlings i.e. shoot height, shoot weight and root length.

**Seeds disinfection bioassays.** Seed disinfection was carried out in two steps. The first step consisted to contaminate seeds with phytopathogenic fungi (*Alternaria solani)* or phytopathogenic bacteria (*X. campestris* or *P. syringae*) in Petri dishes; the second involved the transplanting of germinated seeds that exhibited symptoms of damping-off, root rot and wilt at the juvenile stage of tomato growth in plug trays.

For each microorganism, 50 g of bleached seed were surface contaminated with 1% (v/w) of microbial suspensions that contained approximately $10^6$ spores $ml^{-1}$ for phytopathogenic fungi (*Alternaria solani*) or $10^8$ CFU $ml^{-1}$ for phytopathogenic bacteria (*X. campestris* or *P. syringae*). Seeds were then dried at room temperature for about 8 h and were then divided into three groups. The first was disinfected by placing the seeds in contact with a 10% (dw/v) sodium hypochlorite solution for 30 min and then rinsed three times with sterile distilled water. The second lot was mixed with Bac IH7 at 800 AU $ml^{-1}$ for the fungi and bacteria infected seeds. The third group was untreated and served as control.

After 24 h incubation at room temperature, the seeds were transplanted into Petri dishes (10 seeds/Petri dish of 9 cm in diameter) containing a twice autoclaved sterile potting mix. Petri dishes containing muskmelon and tomato seeds were then incubated at 27°C for 52 and 72 h, respectively. Contaminated seeds were then counted and the germination index calculated according to Zucconi *et al*. (1981). This was done to determine whether Bac IH7 had disinfestation or disinfection properties. Accordingly, germinated tomato seeds were planted in a sterile potting mix and grown in seedling plug trays (plug size 3 cm/3 cm/5 cm, 120 plugs per tray). Plug trays were placed in a greenhouse, monitored for four weeks, counting the plants showing characteristic disease symptoms.

**Statistical analysis.** The trial was established as randomised plots experimental design with three triplicates, including 30 plants in each replicate. The data were subjected to analysis of variance using the Statistical Package for the Social Sciences (SPSS V.11, USA). Mean values among treatments were compared by the Duncan's multiple range test at the 5% ($p = 0.05$) level of significance.

## RESULTS

**Identification of antagonistic IH7 strain.** Classical taxonomic findings showed the newly isolated bacterium (IH7) to be a *Bacillus*, i.e. a Gram-positive, catalase+, oxydase+, aerobic, motile, rod shaped and spore forming bacterium. DNA similarity searches against bacterial databases revealed that the 1450 bp fragment amplified by PCR 16S rRNA sequence of the isolate was 99% identical to different *B. subtilis* strains. Based on the data obtained, we propose the provisional assignment of this bacterium as *B. subtilis* strain IH7.

**Optimization of nutritional and cultural conditions.** Strain IH7 was able to grow in all the carbon sources tested and a maximum biological activity production was obtained when glucose was used as a carbon and energy source. To further optimize the culture conditions, trace mineral oligoelement, potassium, and magnesium salts were tested using glucose as carbon sources. Results showed that antimicrobial activity against the indicator microorganisms used were observed under all three conditions; however, the combination of glucose and magnesium produced the best results. In the latter case, the secretion of bioactive compound was related with biomass production. This biomass increased with increasing glucose and magnesium concentrations up to 2.5 g $l^{-1}$ and 0.86 g $l^{-1}$, respectively. Further increases in the level of both substrates resulted in a decrease of biomass and of production of active molecules.

Strain IH7 showed a narrow range of incubation temperature for good growth and production of active molecules. According to its optimum temperature of growth (30°C), this strain appeared to be a mesophilic bacterium. The highest antimicrobial activity was obtained at pH 7, which indicated the neutrophilic nature of the strain. With regards to the incubation time, the biological activity was pronounced after 36 h of growth, with a maximum at 72 h incubation. It remained stable between 72 and 84 h, to decrease and disappear after 140 h incubation. As far as agitation rates are concerned, low active molecule production was obtained at 100, 150 and 250 rpm, the best results being achieved at 200 rpm.

Antifungal tests showed that each of the killed fungi at each of the concentration levels studied, were able to enhance antifungal production. According to the diameter of the inhibition zones, for all the three fungi tested, the highest increase of antifungal activity caused by the heat-killed fungi was towards the same fungus, the best results being obtained with heat-killed *Fusarium* sp cells at a concentration of 0.05 g $l^{-1}$. However, although the secretion of antifungal activity was increased in the presence of heat-killed fungal cells, no significant difference was observed with respect to the growth of strain IH7 under these conditions.

**Bacteriocin purification and molecular weight.** Bac IH7 was purified to homogeneity from a cell-free culture supernatant of strain IH7 by a three-step protocol (Table 1). Maximum of antimicrobial activity was obtained when the cell-free culture supernatant was pre-

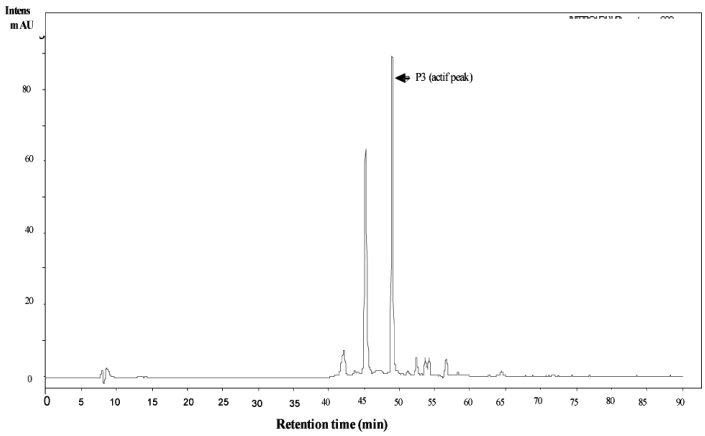


**Fig. 1.** Elution profile of bacteriocin using HPLC reverse phase chromatography on C18 column monitoring by absorbance at 280 nm.

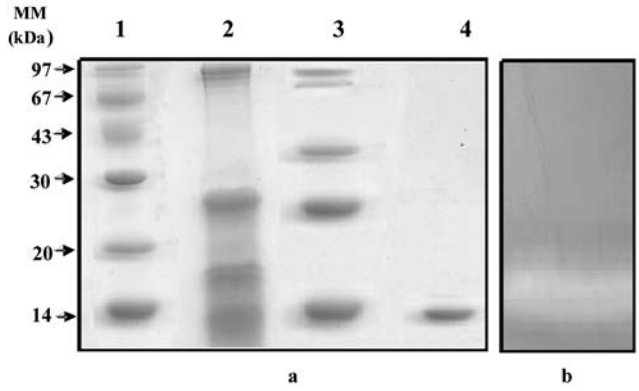


**Fig. 2.** Electrophoretic and zymogram analyses of the purified Bac IH7. (a) Coomassie brilliant blue-stained SDS-PAGE gel showing different steps in the purification procedure. Lane 1, molecular mass protein markers; Lane 2, Fraction I; Lane 3, Fraction II; Lane 4, Fraction III. (b) Portion of the renaturated SDS-PAGE, overlaid with the *A. tumefaciens* C58.

cipitated with 80% ammonium sulphate. After dialysis, the protein solution (fraction I) was separated by gel filtration chromatography in a Sephadex G-25 column. The active fraction obtained (fraction II) was then applied to another round of purification using the HPLC technique. Only the fraction of the peak P3 (Fraction III), which had a retention time of P3: 48.31 min, exhibited antimicrobial activity against the four indicator microorganisms, while no activity was observed for the other fractions (Fig. 1). The yield, activity and purification factor obtained for Bac IH7 with the three different purification steps are summarized in Table 1. SDS-PAGE analysis of purified Bac IH7 revealed a single band with an estimated $M_r$ of *ca.* 14 kDa, which was taken as an indication that this bacteriocin was purified to homogeneity (Fig. 2a). The determination of antibacterial activity against *A. tumefaciens* strain C58 revealed a growth inhibitory zone at the same position of that visualized in the stained gel (Fig. 2b)

**Effect of pH, temperature and enzymes.** To determine the thermostability of Bac IH7, the inhibitory activity was examined following exposure to a variety of different temperatures. Bac IH7 was completely stable at temperatures up to 121°C for 15 min. With regard to pH sensitivity, the antibacterial activity of Bac IH7 was highest at pH 8.0 and the activity was maintained at a high level within the pH range of 6.0-9.0. It lost only 30% of its activity when exposed to a pH value below 6.0. Complete inactivation was observed after treatment with all proteolytic enzymes, a key criterion for the characterization of a bacteriocin. However, Bac IH7 showed no loss of activity when treated with α-amylase, lipase and endo H enzymes. Indicating that the bacteriocin did not require a glucosidic or a lipidic moiety for its biological activity.

**Inhibitory spectrum and mode of action of Bac IH7.** Bac IH7 showed a broad spectrum of antibacterial activity (Table 2 and 3) that was effective against Gram-positive, Gram- negative bacteria as well as against fungi. The addition of purified Bac IH7 (800 AU $ml^{-1}$) to cells of *A. tumefaciens* C58 (3 h old) and *C. tropicalis* R2 CIP203 (9 h old) in their early logarithmic growth phas-

**Table 1.** Purification and properties of bacteriocin Bac IH7.

| Purification step | Volume (ml) | Total activity (AU)* | Total protein (mg) *b | Specific activity (AU $mg^{-1}$) | Yield (%) | Purification factor |
|---|---|---|---|---|---|---|
| Crude extract | 1000 | 16,384 ± 200 | 50 ± 4 | 327.68 | 100 | 1 |
| $(NH_4)_2SO_4$ fractionation (60-80%) | 10 | 9,000 ± 157 | 1.5 ± 0.8 | 6,000 | 18.34 | 54.9 |
| Sephadex G25 | 2.5 | 3,600 ± 98 | 0.52 ± 0.2 | 6,923.07 | 21.12 | 21.9 |
| HPLC | 1.25 | 1,550 ± 50 | 0.15 ± 0.07 | 10,333.33 | 31.53 | 9.48 |

Antibacterial activity (in arbitrary units [AU]) was assayed by agar well diffusion assay using *A. tumefaciens* C58 as an indicator strain. [b]Proteins were estimated by Bradford method. *The experiments were conducted three times and standard errors are reported.

**Table 2.** Antifungal activity of the bacteriocin Bac IH7.

| Fungal strain | Mycelium growth inhibition [a] | |
|---|---|---|
| | mm | % |
| *Rhizoctonia solani* | 21.0 ± 1.0 | 54.0 ± 2.0 |
| *Alternaria citri* | 27.3 ± 1.1 | 39.6 ± 2.8 |
| *Alternaria solani* | 14.6 ± 0.5 | 67.6 ± 0.2 |
| *Phytophtora capsici* | 18.0 ± 1.0 | 60.3 ± 2.5 |
| *Botrytis cineria* SAS56 | 21.3 ± 1.5 | 53.0 ± 3.6 |
| *Verticillium dahliae* V4i | 15.6 ± 0.5 | 65.6 ± 1.1 |
| *Candida tropicalis* R2 CIP20 | 19.0 ± 1.0 | 58.0 ± 2.0 |
| *Phytium* sp. | 17.6 ± 1.1 | 61.3 ± 2.8 |
| *Fusarium* sp. | 18.3 ± 1.5 | 59.6 ± 3.5 |

Values are given as mean + S.D. of three experiments.
[a] mm, radial growth; %, percentage of radial growth inhibition.

es resulted in a rapid decrease in the number of *A. tumefaciens* C58 viable cells (from $10^7$ CFU ml$^{-1}$ to less than $10^2$ CFU ml$^{-1}$) over a period of 5 h (Fig. 3a). The optical density readings of this indicator microorganism remained constant from the time when Bac IH7 was added, indicating that the bacteriocin exhibited a bactericidal effect against *A. tumefaciens* C58. As to *C. tropicalis* R2 CIP203, the optical density readings were found to be very similar in the presence and absence of Bac IH7 (Fig. 3b). However, an increase in the number of the viable cells grown in the absence of the bacteriocin was observed at 42 h of growth. This indicates that Bac IH7 had a fungistatic effect against *C. tropicalis* R2 CIP203 (Fig. 3b).

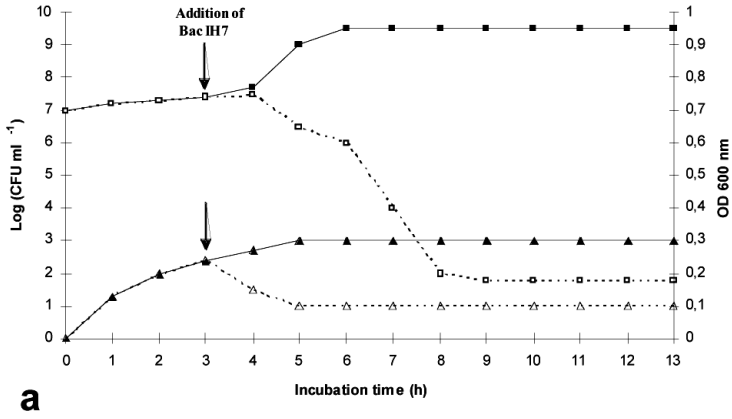


**Fig. 3.** a) The effect of Bac IH7 on the growth of *A. tumefaciens* C58. Optical density at 600 nm in absence (▲) and in the presence (△) of Bac IH7. Viable cell counts (CFU ml$^{-1}$) in the absence (□) and presence (■) of Bac IH7. b) The effect of Bac IH7 on the growth of *C. tropicalis* R2 CIP203. Optical density at 600 nm in the absence (▲) and presence (△) of Bac IH7. Viable cell counts (CFU ml$^{-1}$) in the absence (■) and presence (□) of Bac IH7 (b).

**Phytotoxicity effect of Bac IH7 on plant species.** The results of seed gemination experiments indicated that it was strongly inhibited in both species studied when treated with sodium hypochlorite and Bac IH7 (1000 and 1200 AU ml$^{-1}$). Bac IH7 (800 AU ml$^{-1}$) did not show any inhibitory effect on seed germination as the two test plants showed germination rates close to those of the control (Fig. 4a,b). Actually, the application of Bac IH7 (800 AU ml$^{-1}$) improved the germinative energy of both seed lots. The germination index at the half-period of germination was 36 and 25 h for tomato and muskmelon, respectively, and was significantly

**Table 3.** Antibacterial activity of Bac IH7 against indicator strains.

| Indicator strains | Diameter of inhibition zone (mm) |
|---|---|
| Gram positive bacteria | |
| *Micrococcus luteus* LB 14110 | 17 ± 1.5 |
| *Staphylococcus aureus* ATCC 6538 | 18 ± 0.5 |
| Gram negative bacteria | |
| *Escherchia coli* ATCC 8739 | 19 ± 1.0 |
| *Pseudomonas savastanoi* pv. *savastanoi* IVIA 1628 | 22 ± 1.0 |
| *Pseudomonas syringae* pv. *tomato* | 24 ± 1.0 |
| *Pseudomonas aeruginosa* CIP 82.118 | 20 ± 1.5 |
| *Xanthomonas campestris* | 23 ± 0.5 |
| *Agrobacterium tumefaciens* C58 | 24 ± 1.0 |
| *Agrobacterium rhizogenes* CFBP 2408 | 22 ± 1.0 |
| *Agrobacterium vitis* CFBP 2678$^T$ | 23 ± 1.0 |

Values in the table are means of three independent experiments and the standard deviation is reported.

higher than that of the negative controls (Fig. 4a,b). On the other hand, germinative energy yields obtained for treatment with Bac IH7 (1000 and 1200 AU $ml^{-1}$) and sodium hypochlorite, were lower than the optimum (ratio <50%). The initial vigour response was measured using traditional agronomic parameters, namely germination percentage, root length and shoot weight and height. In fact, the results indicated that vigour response in response to Bac IH7 (800 AU $ml^{-1}$) was higher than that obtained with the other treatments (Fig. 4a and Fig. 4b). Nevertheless, Bac IH7 (1000 and 1200 AU $ml^{-1}$) had a negative affect on the vigour response of the seeds, bringing about a significant reduction in root heights as well as shoot heights and weights (Fig. 5). These results were comparable to those obtained with sodium hypochlorite treatment, where seed vigour biomarkers were drastically affected (not shown).

**Disinfectant property.** Bac IH7 (800 AU $ml^{-1}$) was able to inhibit the growth of seed-borne diseases investigated as it afforded a highly significant control of the damping-off disease groups on seeds treated in the pre-germination stage (Fig. 6). A higher damping-off control was observed in the seeds exposed to Bac IH7 + *X. campestris* than in those treated with Bac IH7 + *P. syringae* pv. *tomato* (Fig. 6). Furthermore, the trials carried out in plug trays showed that Bac IH7 was able to control root rot caused by *A. solani* (Fig. 7).

## DISCUSSION

Recently, a variety of bacteria, such as *Bacillus* and other Gram-positive bacteria have attracted attention for their production of promising compounds with potential uses in various fields and processes. Among these bioactive compounds, bacteriocins have increasingly gained attention particularly for their astoundingly high diversity and industrial applicability. In view of this continuing search for new natural alternatives to chemical

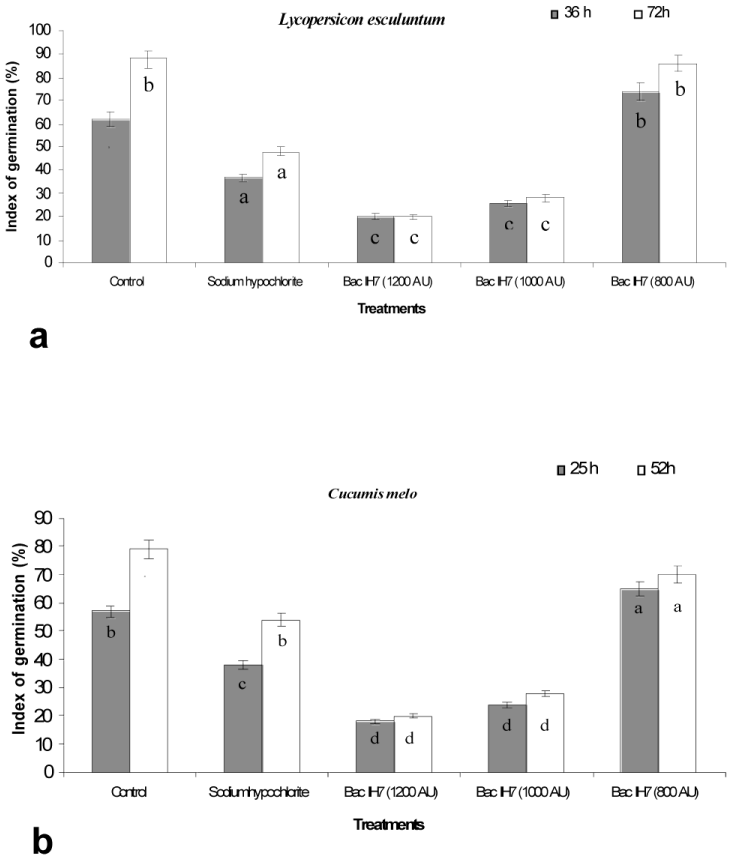


**Fig. 4.** Germination index of *Lycopersicon esculintum* (a) and *Cucumis melo* (b) determined on distilled water (control), sodium hypochlorite and Bac IH7 at different concentrations (1200, 1000 and 800 AU). Histograms with different letters are significantly different according to the test of Duncan (p = 0.05). Values are means of three independent experiments and the standard deviation is reported. Error bars indicate standard deviation.

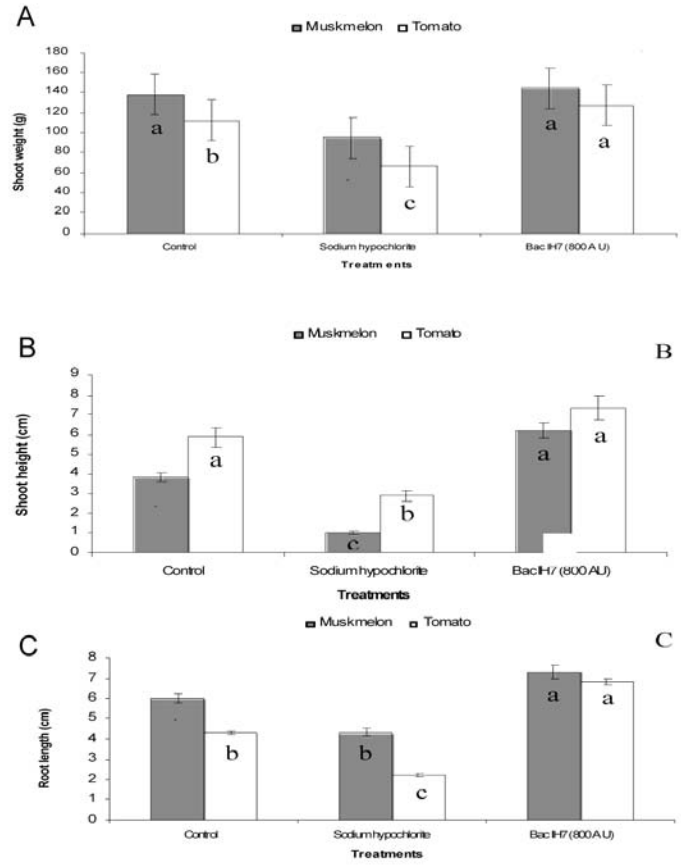


**Fig. 5.** The effect of germination index of distilled water (control), sodium hypochloride and Bac IH7 (800 AU) on (A) shoot weight; (B) shoot height; and (C) root length at 2 months after transplantation of tomato (*Lycopersicon esculentum*) and muskmelon (*Cucumis melo*) plants. Histograms with different letters are significantly different according to the test of Duncan (p = 0.05).Values are means of three independent experiments and the standard deviation is reported.

control agents, the present study was undertaken to investigate the production and purification processes of a novel bacteriocin produced by *B. subtilis* IH7. It also reports on the characterization of the purified bacteriocin in terms of stability under different culture conditions and the evaluation of its antibacterial and antifungal action against a series of pathogenic microorganisms.

The findings from the optimization of the components and physical parameters of the culture medium showed that highest levels of antimicrobial activity were obtained at 2.5 g $l^{-1}$ and 0.86 g $l^{-1}$ for glucose and magnesium, respectively. The optimum environmental factors for the maximum production of antimicrobial activity consisted of a growth temperature of 30°C, a pH value of 7, an incubation time of 72 h, and an agitation rate of 200 rpm.

The effect of the addition of heat-killed fungi to the culture media was also investigated. The findings indicated that the addition of those fungi brought about an increase in the secretion of the bioactive compound. In fact, the few studies currently available on this topic seem to have treated the issue at two different contexts. Slattery *et al.* (2001) reported on the induction of the production of antibiotics istamycin A and B in the marine bacterium *Streptomyces tenjimariensis* in the presence of some live bacterial species. Ben Fguira *et al.* (2008), on the other hand, reported on the induction of the biosynthesis of three antifungal molecules (irumamycin, X-14952 B and 17-hydroxy-venturicidin A) by the addition of heat-killed fungi to the medium growth of the *Streptomyces* US80 strain. The findings of the present study corroborate the results presented in those previous studies and suggest that this may constitute an important tool for the biotechnological mass production of promising biological active molecules from bacteria and contribute to the development of new methods for the screening of novel active compounds.

The development of a three-step purification procedure allowed the purification of the novel bacteriocin, termed Bac IH7, to homogeneity. Bac IH7 was found to be a monomeric holoprotein with a molecular mass of 14 kDa. It was also observed to be stable up to 121°C for 15 min. This stands in contrast with, for instance, bacthuricin F4 that was previously reported to preserve only 20% of its activity after incubation at 90°C for 30 min (Kamoun *et al.*, 2005). Many bacteriocins have also previously been reported to lose their heat stability upon purification (Barefoot and Klaenhammer, 1983; Stoffels *et al.*, 1992). In contrast, the purified Bac IH7 retained its full heat stability, which was also the case for nisin (Hurst, 1981).

Moreover, being proteinaceous in nature, the purified Bac IH7 was able to achieve complete inactivation after treatment with different proteolytic enzymes, contrary to other bacteriocins produced by *Bacillus*, such as coagulin I4 and cerein 8A, are more resistant to proteolytic treatments (Hyroimus *et al.*, 1998; Bizani and Brandelli, 2002).The other enzymes tested, such as lipase, α-amylase or endo H, were not noted to cause any inactivation. This could be attributed to the lack of lipid or carbohydrate moiety in the bacteriocin. Similar results of enzyme treatments with lipases and amylases have been reported for Cerein 8A from *Bacillus cereus* (Bizani *et al.*, 2005) and Bacillocin from *Bacillus brevis* Bb (Salleem *et al.*, 2009). Similarly, the antimicrobial activity of the bacteriocin produced by *Pediococcus acidilactici* HA-6111-2 and HA- 5692-3 was not affected by the treatment with proteases, lipase and α-amylase (Albano *et al.*, 2007). In contrast, the *S. thermophilus* bacteriocin reported by Ward and Somokuti (1995), leuconocin S, produced by *Leuconostoc paramesenteroides* (Lewus *et al.,* 1992) and carnocin 54, produced by *Leuc.*

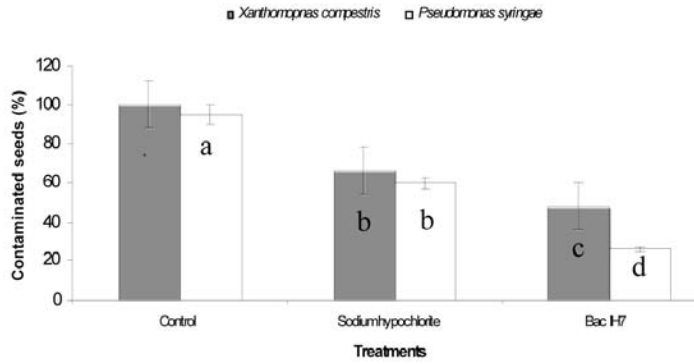


**Fig. 6.** Disinfection effect of water (control), sodium hypochloride, and Bac IH7 (800 AU) on the percentage of contaminated seeds by *X. campestris* and *Ps. syringae* pv. Tomato. Histograms with different letters are significantly different according to the test of Duncan (p = 0.05). Values are means of three independent experiments and the standard deviation is reported.

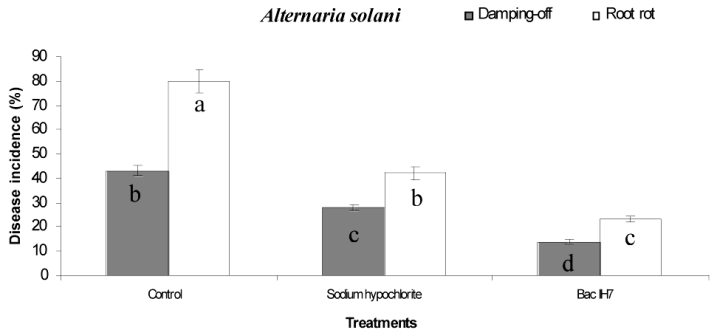


**Fig. 7.** Effect of treatments by distilled water (control), sodium hypochloride, and Bac IH7 (800 AU) on the percentage of damping-off and root rot incidence of tomato (*Lycopersicon esculentum*) plants caused by *Alternaria solani*. Histograms with different letters are significantly different according to the test of Duncan (P=0.05). Values are means of three independent experiments and the standard deviation is reported.

*carnosum* (Keppler *et al*., 1994) are examples of amylase-sensitive bacteriocins. On the other hand, the antimicrobial activity of the culture supernatant of *Lactococcus lactis lactis* MM19, was reported to be reduced following lipase treatment (Millette *et al*., 2007).

Furthermore, Bac IH7 was observed to be stable over a wide range of pH, which is common feature of many bacteriocins (Hammami *et al*., 2009). It was exhibited a pronounced antagonistic activities against various species of Gram-positive and Gram-negative bacteria as well as against fungi strain. The inhibitory spectrum was similar to those shown by cerein 8A (Bizani and Brandelli, 2002), entomocin 9 (Cherif *et al*., 2003) and bacteriocin-like substance produced by *B. licheniformis* P40 (Cladera-Olivera *et al*., 2004), but wide compared with those of coagulin (Hyronimus *et al*., 1998), cerein 7 (Oscariz *et al*., 1999) and thuricin 7 (Cherif *et al*., 2001), bacillocin 490 (Martirani *et al*., 2002) which only inhibited Gram-positive indicator strains.

The present study showed that while Bac IH7 has a bactericidal action on indicator *A. tumefaciens* C58, it exhibited a fungistatic mode of action against *C. tropicalis* R2 CIP203. In fact, some bacteriocins, such as thoeniicin, have previously been reported to be bactericidal on one strain and bacteriostatic on others (Van der Merwe *et al*., 2004).

The application of a Bac IH7 (800 AU $ml^{-1}$) treatment was also observed to improve the germinative energy measured at the half-period of germination. In fact, germinative energy can play an important role in the achievement of quick and uniform seedling emergence and the reduction of damping-off incidences, thus improving the yield.

Bac IH7 (800 AU $ml^{-1}$) solution was also noted to be useful for seed disinfection. Treatment with Bac IH7 (800 AU $ml^{-1}$) also resulted in higher production yields, earlier and better emergence levels, and greater control of both damping-off/root rot and wilt caused by *Alternaria.*

The compelling disinfection properties described above make Bac IH7 a potential strong candidate for application as an alternative substitute to the current inadequate methods employed for the removal of the harmful disinfectants in seed and propagation trays. The results presented in the current study also demonstrate that Bac IH7 (800 AU $ml^{-1}$) represents a class of natural products that may offer an efficient substitute for the currently commercialized corrosive disinfectants. In fact, it can be used to control the most resistant fungal structure (chlamydospores) produced by the species of *Fusarium* (Mavrogianopoulos *et al*., 2000), *Alternaria,* and even *Xanthomonas* and *Pseudomonas*, without causing any damage to the germination ability of crops.

Considering the promising properties of the latter, the determination of the $NH_2$-terminal amino acid sequence and the study of the bacteriocin structural genes as well as its regulatory elements are currently underway in our lab. This would require a further investigation into the structure-function relationship using molecular study and 3-D structure determination.

## ACKNOWLEDGEMENTS

This work was supported by the Ministry of Agriculture and Water Resources and the Ministry of Higher Education and Scientific Research, Tunisia. The authors wish to express their sincere gratitude to Prof. Anouar Smaoui from the Sfax Faculty of Science, Tunisia, for kindly proofreading the manuscript of the present work.

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Received November 29, 2010
Accepted March 2, 2011