Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Biological control of grey mould in strawberry fruits by halophilic bacteria

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Keywords
antifungal enzymes, biocontrol, *Botrytis cinerea*, grey mould, halophilic bacteria, postharvest disease, strawberries.

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2008/0241: received 12 February 2008, revised 26 August 2008 and accepted 29 August 2008

doi:10.1111/j.1365-2672.2008.04053.x

Abstract

Aims: Grey mould caused by *Botrytis cinerea* is an economically important disease of strawberries in Tunisia and worldwide. The aim of this study was to select effective halophilic bacteria from hypersaline ecosystems and evaluate the abilities of antifungal bacteria to secrete extracellular hydrolytic enzymes, anti-*Botrytis* metabolites and volatiles.

Methods and Results: Grey mould was reduced in strawberry fruits treated with halophilic antagonists and artificially inoculated with *B. cinerea*. Thirty strains (20·2%) were active against the pathogen and reduced the percentage of fruits infected after 3 days of storage at 20°C, from 50% to 91·66%. The antagonists were characterized by phenotypic tests and 16S rDNA sequencing. They were identified as belonging to one of the species: *Virgibacillus marismortui*, *B. subtilis*, *B. pumilus*, *B. licheniformis*, *Terribacillus halophilus*, *Halomonas elongata*, *Planococcus rifietoensis*, *Staphylococcus equorum* and *Staphylococcus* sp. The effective isolates were tested for antifungal secondary metabolites.

Conclusions: Moderately halophilic bacteria may be useful in biological control against this pathogen during postharvest storage of strawberries.

Significance and Impact of the study: The use of such bacteria may constitute an important alternative to synthetic fungicides. These moderate halophiles can be exploited in commercial production and application of the effective strains under storage and greenhouse conditions.

Introduction

Strawberry is an important small fruit crop grown worldwide, and its production is increasing steadily. Among major factors limiting strawberry yield are susceptibility to a variety of phytopathogenic fungi, bacteria and viruses (Schestibratov and Dolgov 2005). Grey mould caused by *Botrytis cinerea* pers: Fris is one of the most destructive diseases of strawberry in Tunisia and worldwide and can cause yield losses of up to 25% for untreated strawberries. In strawberry, the fungus attacks flowers, setting fruits, mature fruits and leaves (Sutton 1990; Sutton and Peng 1993). Grey mould is also a major cause of postharvest losses during storage, transit and shipment. The postharvest life of the strawberry (*Fragaria ananassa Duch*) is largely limited by *B. cinerea* infection. It is assumed that there are two factors influencing the batch keeping quality: the *Botrytis* inoculum potential and the resistance of the strawberry to infection (Schouten *et al.* 2002).

The most important method of control of *B. cinerea* on strawberries is the application of fungicides during flowering. But fungicides are generally not effective unless they are timed properly and used in conjunction with the cultural practices. However some of the more effective fungicides have not been registered for use in strawberry and others have developed resistance problems (Paulus 1990). Therefore, the reduction of synthetic fungicide applications and elaboration of safe and effective alternative approaches for the control of postharvest disease are necessary (Wilson and Wisniewski 1989).

The search of antagonists for plant protection has intensified in the recent years and several micro-organisms with high activity have been identified. Several biological control agents are effective in reducing decay caused by grey mould on strawberry (Lim *et al.* 1991; Peng and Sutton 1991). However, few of these have been tested under postharvest storage conditions (Burmeister *et al.* 1997; Wszelaki and Mitcham 2000). The use of microbial antagonists is one of the most studied alternatives to fungicides that has lead to the development and release of commercial products such as *Candida oleophila* (Saligkarias *et al.* 2002), *Cryptococcus albidus* (Ippolito *et al.* 2005), *Pseudomonas syringae* (Bull *et al.* 1997) and *Bacillus subtilis* (El-Ghaouth *et al.* 1998; Utkhede *et al.* 2001).

Our previous laboratory studies showed the efficiency of halotolerant *Bacillus* isolates in the control of potato dry rot under storage conditions (Sadfi *et al.* 2001, 2002). We also described the ability of halotolerant and moderately halophilic bacteria isolated from different Tunisian Sebkhas (hypersaline soils) to protect fresh-market tomato fruits from *B. cinerea* (Sadfi-Zouaoui *et al.* 2008). These halophilic micro-organisms produce antibiotics and antifungal metabolites and are able to control phytopathogenic fungi. However, the halotolerant and moderately halophilic bacteria have not been tested yet against grey mould on harvested strawberries.

Moderately halophilic bacteria are a group of halophilic micro-organisms able to grow optimally in media containing a wide range of NaCl concentrations (3–15% NaCl) (Ventosa *et al.* 1998). They constitute a heterogeneous group of micro-organisms and have been studied for their ecology, physiology, biochemistry and genetics (Ventosa *et al.* 1998). However their biotechnological possibilities have not been extensively exploited. Furthermore, halophiles are the most likely source of extracellular hydrolytic enzymes such as amylases, proteases, lipases, DNAses, pullulanases, phosphatases, xylanases and nucleases which have different potential usages in biotechnological and industrial processes such as food industry, feed additive, biomedical sciences and chemical industries (Niehaus *et al.* 1999). Moreover, extremophilic micro-organisms are adapted to survive in ecological niches such as at high temperature, extremes of pH, high salt concentrations and high pressure. These micro-organisms produce unique biocatalysts that function under extreme conditions comparable to those prevailing in various industrial processes (Niehaus *et al.* 1999). The production of hydrolytic enzymes which degrade fungal cell walls, especially chitinases and β-1,3-glucanases, are among key factors involved in the suppression of pathogenic fungi by biocontrol agents (Ordentlich *et al.* 1988).

The objective of our study was to evaluate, under postharvest conditions the effectiveness of halophilic bacteria in reducing strawberry rot caused by *Botrytis cinerea* under storage conditions. The ability of halophilic antagonists to produce diversity of extracellular hydrolytic enzymes, including antifungal ones as well as other antifungal compounds was also investigated.

Materials and methods

Pathogen inoculum

A monosporal isolate of *B. cinerea* (LPV 02) used for laboratory trials has been isolated from infected strawberry fruits with typical symptoms of grey mould rot. The fungal pathogen was cultivated on plates of potato dextrose agar (PDA, Difco) for 10 days at 25°C until sporulation. A monosporal isolate was maintained on PDA at +4°C and was subcultured onto fresh PDA plates every 2 month-intervals.

A spore suspension was prepared by flooding 10-day-old cultures of *B. cinerea* on PDA dishes with 15 ml of sterile distilled water supplemented with 0·01% Tween80 (w/v) and dislodging the spores with a glass rod. The suspension was filtered through a sterile 30 μm filter to discard conidiophores and fragments of mycelium. Conidial suspension of *B. cinerea* was adjusted to 10^6 spores ml^{-1} by counting with a haemocytometer.

Fruits

Mature and healthy strawberries were uniform in size and colour, free from wounds and rot. Before each test, the fruits were washed with a sodium hypochlorite solution (1% active chlorine), then rinsed in distilled water and left to dry by using sterile filter papers.

Antagonistic bacteria

In a recent study a total of 148 halotolerant to halophilic bacteria were isolated during 2004–2005 from four shallow salt lakes, or Sebkhas, located in different areas of Tunisia (Sadfi-Zouaoui *et al.* 2008). Strains were maintained on Tryptic Soy Agar (TSA, Difco) slants with 7·5%, 10% or 20% NaCl (w/v) at 4°C and subcultured every 2-month intervals. For long-term storage, strains were conserved in 75% glycerol at −80°C.

To evaluate their antagonistic activity against *B. cinerea* on strawberry fruits, all halophilic bacterial strains were incubated on TSA medium supplemented with 7·5%, 10% or 20% NaCl at 30°C for 48 h. After 48 h, the cells were scraped from the agar and diluted in sterilized saline solution (0·1% NaCl). Bacterial suspensions were adjusted to 10^8 colony-forming units (CFU) ml^{-1}. Bacterial concentration was determined by dilution plating on TSA.

In vivo screening of halophilic isolates for anti-*Botrytis* activity

Harvested strawberries were soaked in a bacterial suspension (10^8 CFU ml^{-1}) for 12 s, dried for 1 h and sprayed with a conidial suspension of *B. cinerea* at 10^6 spores ml^{-1} and then placed in plastic tray containers. As positive and negative controls, fruits were either inoculated with the pathogen alone, with the bacterial candidate alone or with distilled water. The fruits were then stored according to Tunisian commercial standard at 20°C, and 95% RH during 3–4 days.

The percentage of disease incidence of grey mould on the strawberry fruits was calculated using the following formula: $(\%) = (A/B) \times 100$, where A is the number of infected strawberry fruits in a bacterial treated tray and B is the number of total fruits per tray. Three independent experiments were performed with 20 fruits per treatment (strain) and each experiment was used as a repetition for the statistical analysis using LSD test. Only the isolates showing disease incidence of less than 50% on strawberry fruits were retained for further identification and analysis.

In vitro screening of halophilic antagonists

The efficiency of halotolerant to moderately halophilic isolates inhibited the growth of *B. cinerea* on strawberry fruits was also verified *in vitro* by applying a dual culture technique (Sadfi-Zouaoui *et al.* 2008). Two mycelial plugs of 5 mm in diameter of *B. cinerea* were placed at each side of the bacterial strip. The distance between the two micro-organisms was 2·5 cm. Plates were then incubated at 25°C for 5 days. The percentage of mycelial growth inhibition was calculated by the formula: Growth inhibition $(GI) = (R_1 - R_2)/R_1 \times 100$, where R_1 represents the distance (measured in millimeter) between the point of implant of the fungal disc and the side of the Petri plate, and R_2 is the distance of fungal growth from the point of inoculation to the colony margin in the direction of the antagonist (Sadfi-Zouaoui *et al.* 2008). All *in vitro* antagonism assays were made in triplicate.

Statistical analysis

Data were analysed according to a factorial design with one factor. Analysis of variance of data collected from the percentage of disease incidence of different bacterial treatments including the control (NTC) and the percentage of growth inhibition of *B. cinerea* on PDA plates and means comparisons by LSD method at the level of 5%, were carried out using STATISTICA program ver. 5.0 (StatSoft France, 1997).

Identification of halophilic bacteria

Phenotypic characterization

The effective biocontrol isolates were phenotypically characterized on the basis of morphological, physiological and chemical analyses. Active strains were identified according to the methods of Grant *et al.* (2001) and Hacene *et al.* (2003), which are based on the following phenotypic features: colony and cell morphology, motility, spore production when present, Gram staining, pigmentation and growth at optimal pH, temperature and salinity. The susceptibility to antimicrobial agents was tested on TSA medium containing 75 g l^{-1} NaCl with antibiotic discs (Difco), by the method of Tindall (1992). The antibiotics tested were: penicillin G (10 units), erythromycin (15 μg), bacitracin (10 units), chloramphenicol (30 μg), neomycin (30 μg), ampicillin (10 μg) and rifampicin (30 μg). Oxidase reaction was performed by impregnating discs with dimethyl-*P*-phenylenediamine (Biomérieux, France). Catalase was determined by adding 10 volumes of H_2O_2 to each strain culture (18 h) on solid TSA medium. Acid production from carbohydrates (D-glucose, sucrose, lactose) was detected on liquid TSB medium supplemented with 7·5% NaCl (w/v). H_2S production was performed according to Clarke (1953). Nitrate reduction was assayed by adding 0·2% (w/v) KNO_3 to the liquid TSB medium. Nitrite was detected as described by Skerman (1967). Urea hydrolysis was detected with Christensen's medium (1946). β-galactosidase and Voges–Proskauer tests were performed by standard procedures (Barritt 1936; Lowe 1962).

16S rRNA gene sequence determination

Chromosomal DNA preparation

The cells were harvested at approximately the late exponential growth phase by centrifugation and resuspended in TE (10 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA pH 8). Cells of Gram-positive bacteria were lysed by adding respectively for 30 min at 37°C; lysozyme (30 mg ml^{-1}); proteinase K (20 mg ml^{-1}) and SDS 10%. Chromosomal DNA was extracted by using the 'Wizard Genomic DNA purification kit' (Promega), according to the manufacturer's protocol. The DNA preparations were visually inspected by 0·8% (w/v) agarose gel electrophoresis, and stored at −20°C.

PCR amplification

The 16S rRNA gene of the isolate was amplified by adding 0·5 μl DNA extract to a thermocycler microtube containing 5 μl 10× *Taq* buffer (Promega), 5 μl 25 mmol l^{-1} $MgCl_2$, 0·5 μl 100 nmol l^{-1} primers, 37·7 μl sterile distilled water and 0·3 μl 5U *Taq* polymerase μl^{-1} (Promega). The universal primers Fd1 (5′-AGAGTTTGATCCTGGCTCAG-3′, positions 8–28) and Rd1 (5′-AAGGAGGTGATCCAGCC-3′,

positions 1547–1531, MWG) (*Escherichia coli* numbering), were used to obtain the PCR product (Winker and Woese 1991). The PCR profile was consisting of initial denaturation at 96°C for 3 min followed by 30 cycles of annealing at 57°C for 30 s, extension at 72°C for 2 min and denaturation at 96°C for 30 s and finally, an extension cycle of 72°C for 7 min.

16SrDNA gene sequencing and phylogenetic analysis

PCR products were purified with a QIAquick gel extraction Kit. Direct sequencing of the PCR product was performed by Genome Express (Grenoble, France). The rRNA gene sequences of the strains have been aligned with related bacteria sequences obtained from Genbank (Benson *et al.* 1999), using the SEQUENCE ALIGNER software from the Ribosomal Database Project II (Maidak *et al.* 2001) and the sequence alignment editor BioEdit (Hall 1999) as previously detailed by Sadfi-Zouaoui *et al.* 2008. All the phylogenetic programs are implemented in the Treecon package (Van de Peer and De Wachter 1994).

Detection of antibiotic activity

The selected isolates exhibiting antifungal activity against *B. cinerea* were grown on TSB medium with 7·5%, 10% or 20% NaCl (w/v) at 30°C for 24 h. The bacterial suspension was centrifuged at 10 000 ***g*** for 10 min at 4°C.

Antibiotic assays were performed on 9-cm Petri plates containing 20 ml of PDA medium. A fungal plug (5 mm) cut from the outer edges of a 5-day old culture of *B. cinerea* was placed in the centre of the Petri plate. A well (diameter, 10 mm) was made at 2 cm from the fungal plug using a No. 3 sterile cork borer and filled with 100-μl of the crude antibiotic extract that has been passed through a 0·2-μm Acrodisc syringe filter (Pall Corp., East Hills, USA). For the crude antibiotics extracted from liquid cultures, a control consisted of filter-sterilized distilled water. Plates were kept at 5°C for 2 h to allow for diffusion of the sample, then were inverted and incubated at 25°C for 5 days. The results were reported as a percentage of fungal growth reduction. Three plates with one measurement per plate were used for each sample and the experiment was repeated twice.

Detection of antifungal activity of volatiles

The production of volatile compounds by selected halophilic strains (i.e. *B. subtilis* J9; *Terribacillus halophilus* J31; *B. pumilus* M3-16; *B. marismortui* M3-23; *Halomonas elongata* L80; *Planococcus rifietoensis* M2-26) was assayed by a sealed plate method as described by Fiddman and Rossall (1995). From a 24-h TSB culture of halophilic isolate, 200 μl were spread on TSA medium in a Petri dish. After incubation at 30°C for 24 h, a second Petri dish (containing PDA), was inoculated with a 6-mm plug of the test fungus in the centre of the plate, inverted and placed over the bacterial culture. The two plates were sealed together with a parafilm and further incubated at 25°C. This ensured that both organisms were growing in the same atmosphere with being physically separated. As a control, a Petri dish containing TSA medium without bacteria was placed over the PDA medium inoculated with the fungal pathogen. Fungal growth was measured by following the increase in radial spread of the test fungus for a period of 5 days. Each test was replicated three times.

Detection of extracellular hydrolytic enzymes

Chitinase activity

The selected halotolerant and halophilic isolates with antifungal activity against *B. cinerea* were cultured in a medium containing (per litre) $(NH_4)_2SO_4$, 7 g; K_2HPO_4, 1 g; NaCl, 1 g, $MgSO_4.7H_2O$, 0·1 g; yeast extract, 0·5 g; agar, 15·0 g. The medium was supplemented with 7·5, 10·0 or 15·0% NaCl (w/v) and 0·5% colloidal chitin prepared according to Rodriguez-Kabana *et al.* (1983). Bacteria showing clearing zones after 7 days of incubation at 30°C on colloidal chitin agar were considered as chitinase producers.

Glucanase activity

Halophilic strains were cultured at 30°C for 3 days on a rotary shaker in 15 ml tubes containing 6 ml peptone medium containing laminarin (0·1%) (from *Laminarin digitata*; Sigma) (Lim *et al.* 1991) plus 7·5%, 10%, 15% or 20% NaCl (w/v). The cultures were then centrifuged at 12 000 ***g*** for 20 min at 4°C and the supernatant was used as enzyme solution. The standard assay was performed according to the method of Leelasuphakul *et al.* (2006). The amount of reducing sugar from Laminarin was measured. The standard assay contained 10 μl of the enzyme solution and 90 μl of 5 mg ml^{-1} of laminarin in 0·1 mol l^{-1} sodium acetate buffer pH 5·0. After incubation at 40°C with gentle shaking for 10 min, the reaction was stopped by boiling for 5 min and 0·2 ml of 1% dinitrosalicylate (DNS) and 0·2 ml of sodium acetate buffer were added and boiled for another 5 min, then placed in an ice bath and 0·9 ml distilled H_2O was added. The optical absorbance was measured at 540 nm. The amount of reducing sugars released was calculated from a standard curve prepared with glucose and the glucanase activity was expressed in units (μmole glucose equivalent min^{-1}).

Protease activity

Proteolytic activity was assayed by measuring zones of precipitation of paracasein around bacterial colonies

grown for 48 h at 30°C in agarized saline medium supplemented with 50% (w/v) milk, 10% (w/v) NaCl and 0·5% (w/v) yeast extract (Difco).

Cellulase activity

Cellulolytic activity of isolates was detected by screening for zones of hydrolysis around the colonies growing on TSA medium supplemented with 1% cellulose. After incubation at 30°C for approx. 7 days, the plates were flooded respectively with Congo-red (1 mg ml^{-1}) for 15 min and NaCl 1 mol l^{-1} for 15 min. Cellulolytic activity was taken as evidence by appearance of clear zone around the colonies.

Chitinase quantification and induction

Bacterial cultures were grown in M1 or M4 media prepared by supplementing the Difco tryptone glucose yeast medium with 7·5% or 10% (w/v) NaCl and with either 1 g l^{-1} glucose (M1) or 1 g l^{-1} glucose plus 0·3% (w/v) colloidal chitin (M4). For the induction test by the presence of the pathogen; the autoclaved spores of *B. cinerea* (10^6 spores ml^{-1}) were added to the TGY medium containing 0·1 or 1 g l^{-1} of glucose respectively in media M3 and M2. After 3 days of incubation at 30°C, 180 rev min^{-1}, the cell-free supernatant (enzyme sample) was used for the enzyme assay. The chitinase activity was determined according to the method of Gomez Ramirez *et al.* (2003). Enzyme samples (one millilitre) were added to 1 ml of colloidal chitin suspension (10%) in 0·2 mol l^{-1} sodium phosphate buffer (pH 7·0). The mixture was incubated for 1 h at 50°C. The reaction was terminated by adding 1 ml of 1% NaOH followed by boiling for 5 min and the absorption was measured at 535 nm. The product was determined by 3, 5 dinitrosalicylic acid assay (DNS) as detailed by the study of Gomez Ramirez *et al.* (2003). A calibration curve was plotted using *N*-acetylglucosamine (NAG; Sigma). In this case, the chitinase activity was defined as the amount of enzyme required to produce one μmol of NAG per hour (Roja Avelizapa *et al.* 1999).

Results

Screening of effective antagonists *in vivo*

Results presented in Fig. 1 indicate that 30 out of 148 tested isolates (*c.* 20·2%) significantly reduced the strawberry grey mould rot incidence comparing with the nontreated

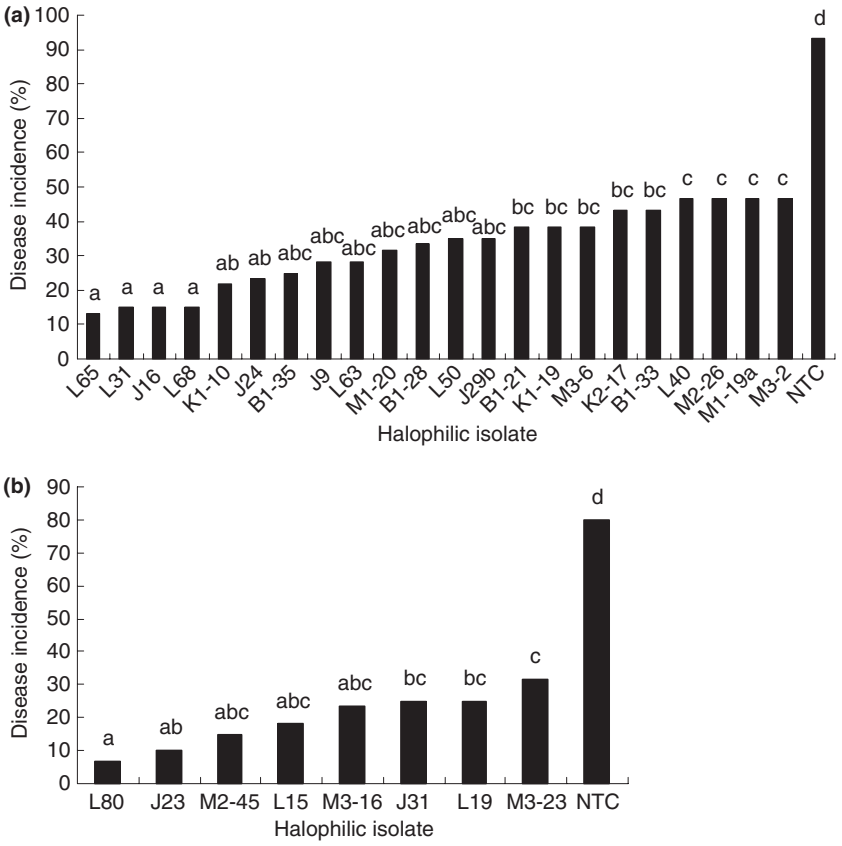

Figure 1 Effect of the most active halotolerant (a) and halophilic (b) bacterial isolates on the disease incidence on strawberries. Strawberries were soaked in a bacterial suspension, dried and sprayed with a conidial suspension of *Botrytis cinerea*. After 3–4 days of storage at 20°C, the number of infected fruits were recorded in both treated and control trays. Twenty fruits per treatment were used and data were recorded from three experiments. Note: bars with the same letter did not differ significantly at $P = 0{\cdot}05$ by LSD test. NTC: nontreated controls (fruits inoculated with *Botrytis*).

control (NTC). The halotolerant to moderately halophilic strains isolated in a medium containing 7·5–10% (w/v) NaCl gave a percentage of grey mould reduction ranging from 50% to 85·71% (Fig. 1a). The most effective strains were L65, L31, J16 and L68 which can reduce grey mould disease on strawberries from 83·93% to 85·71%. The highest reduction was obtained with two moderately halophilic isolates of J23 and L80 which almost completely inhibited the growth of the pathogen and resulted in percentage grey mould reduction by respectively 87·5% and 91·66% (Fig. 1b). The latter strains were isolated in a medium containing 20% NaCl (w/v).

Identification of selected antagonists

The phenotypic characterization was carried out with the 30 effective strains (Table 1). This preliminary identification revealed that 33·4% of the biocontrol isolates belonged to the genus *Bacillus* and 40% to *Staphylococcus*. Isolate L80 was identified as a member of the genus *Halomonas*. Isolates B1-21, K1-19 and M3-23 were identified as belonging to the genus *Virgibacillus,* while J16, L15 and J31 belonged to *Terribacillus* genera.

From the 16S rDNA sequence analysis (Fig. 2), the most halotolerant to moderately halophilic active isolates were identified as belonging to *Virgibacillus marismortui* (B1-21), *Terribacillus halophilus* (J31), *Bacillus subtilis* (B1-33), *B. pumilus* (M3-16, L65, K1-10), *Halomonas elongata* (L80), *Staphylococcus equorum* and *Staphylococcus* spp. The 16S rDNA sequences of these isolates have been deposited in the GenBank database, under the accession numbers EU435360 for B1-21, EU435359 for J31, EU435361 for B1-33, EU435356 for M3-16, EU435357 for L65 and EU435355 for L80. *B. licheniformis* J24 (accession no EF471917), *B. subtilis* J9, M1-20 (accession no EF471915, EF471916), and *Planococcus rifietoensis* M2-26 (accession no EF471920) previously selected on tomato fruits (Sadfi-Zouaoui *et al.* 2008) were among the active isolates on strawberries.

Screening of effective antagonists *in vitro*

The results of the *in vitro* dual culture assay showed that 21 isolates (70%), previously selected, reduced the mycelial growth of *B. cinerea*. Although very effective *in vivo* on strawberries, nine isolates were unable to inhibit *B. cinerea* on solid medium. Among 21 active isolates, 16 gave a percentage inhibition growth ranging from 81% to 99·5% (Table 1). All the active halotolerant bacteria *in vitro*, growing at salt concentration ranging from 5% to 10% belonged to *B. licheniformis* (J24) and *B. subtilis* (M1-20, B1-33) species, while only the halotolerant strain of *B. subtilis* J9 failed to inhibit the pathogen (Table 2). Furthermore, the selected moderately halophilic bacteria growing over a broad range of NaCl (5–15%; 5–20%) effectively inhibited mycelial development of *B. cinerea*. The best inhibitory activity was obtained by the strain L80 of *Halomonas elongata*. However, *Virgibacillus marismortui* strains, *Terribacillus halophilus* strains, *Staphylococcus equorum* (B1-35) and *Staphylococcus* sp. (J23) were ineffective *in vitro*.

Detection of antibiotic activity

The bacterial cell-free extracts of the species *B. licheniformis* (J24) and *B. pumilus* (M3-16) were bioactive against *B. cinerea* as inhibition zones were detected around the wells. These antifungal substances resulted respectively in 41·96% and 42·15% reduction in fungal growth after 5 days of incubation. The cell-free filtrates of all other active isolates were unable to inhibit the growth of *B. cinerea in vitro*.

Detection of antifungal activity of volatiles

The results of experiments of the effect of halophilic isolates volatiles on the growth of the pathogen are presented in Table 3. These experiments showed variable fungal growth according to the tested bacterial antagonist compared to the control. On PDA, six halophilic isolates out of seven showed inhibitory effect on *Botrytis* growth (Table 3).

The highest volatile activities were obtained by the strains, M3-16 of *B. pumilus*, M3-23 of *B. marismortui* and L80 of *H. elongata* which seem to be effective in producing volatiles against the pathogen. These isolates resulted in more than 49·4% reduction in fungal growth after 120 h of incubation.

Extracellular hydrolytic activities

All *B. cinerea*-inhibiting halophilic strains produced chitinase and β-1,3-glucanase except *H. elongata* L80, which did not form clearing zones on colloidal chitin agar (Table 4). On average, *Bacillus* spp. produced higher amounts of antifungal enzymes when compared to other halophilic bacterial species. Four tested isolates (*T. halophilus* J31, *T. halophilus* J16, *B. subtilis* B1-33, and *B. pumilus* M3-16) produced protease, and only three isolates (*B. subtilis* B1-33, *B. pumilus* M3-16, and *B. pumilus* M3-2) tested positive for cellulase activity.

Chitinase quantification and induction

The production of chitinase by halophilic antagonists was significantly influenced by the carbon source incorporated into the medium and the presence or

Table 1 Phenotypic characterization of active isolates

Characteristics	B1-28	B1-33	B1-35	M2-45	M3-2	M3-6	M3-16	M3-23	B1-21
Colonial morphology	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Irregular	Circular
Cell shape	Coccoid	Rod	Coccoid	Coccoid	Rod	Rod	Rod	Rod	Rod
Spore production	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+
Pigmentation	Cream	Cream	Cream	Cream	Cream	Cream	Cream	Cream	Cream
Anaerobic growth	–	–	–	–	–	–	–	–	–
Gram stain	+	+	+	+	+	+	+	+	+
Optimal NaCl for growth (%)	10	7·5	10	10	7·5	7·5	10	10	10
Optimal temperature for growth (°C)	37	30	37	30	30	30	30	30	30
Optimal pH for growth	6–7	7·5	6–7	6–7	7	7	7	6	8
Sensitivity to antibiotics									
Erythromycin	R	S	R	R	R	S	R	S	S
Bacitracin	S	S	S	S	S	S	R	R	R
Neomycin	R	R	S	R	S	R	R	S	R
Chloramphenicol	S	S	S	S	S	S	S	S	S
Ampicillin	S	S	S	R	S	S	R	R	S
Penicillin	S	S	S	R	S	R	R	R	R
Rifampicin	S	S	S	S	S	S	S	S	S
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	–	–	–	–	–	+	+	+	+
H_2S production	–	–	–	+	–	–	–	–	–
Nitrate reduction	+	++	+	+	++	++	++	+	+
Vogues-Proskauer test	–	–	–	–	+	+	+	–	–
Gas formation	–	–	–	+	+	+	+	+	–
Acid production from									
Sucrose	–	–	–	–	–	–	–	–	–
Lactose	–	–	–	–	–	+	+	–	–
D-Glucose	–	–	–	–	–	+	+	–	–
Hydrolysis of									
Esculin	–	+	+	+	+	+	+	+	+
Starch	–	+	–	–	–	+	+	–	–
Casein	–	+	–	–	–	–	+	–	–
Gelatin	–	–	+	+	+	–	+	+	+
Tween80	–	–	–	–	–	–	+	–	–
Urease	+	–	–	–	+	–	+	+	–
β-galactosidase	+	+	+	+	+	+	+	+	+

Characteristics	L65	L80	L63	L68	J23	J31	J16	J29b
Colonial morphology	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Cell shape	Rod	Rod	Coccoid	Coccoid	Coccoid	Rod	Rod	Coccoid
Spore production	+	–	+	+	+	+	+	+
Motility	+	+	+	+	+	–	–	+
Pigmentation	Cream	Cream	Cream	Cream	Cream	None	White	Cream
Anaerobic growth	–	–	–	–	–	–	–	–
Gram stain	+	–	+	+	+	+	+	+
Optimal NaCl for growth (%)	7·5	10	10	10	10	10	10	10
Optimal temperature for growth (°C)	30	37	37	37	30	30	30	37
Optimal pH for growth	7	6–7	6–7	6–7	6–7	7·5	6–7	6–7
Sensitivity to antibiotics								
Erythromycin	S	R	R	R	R	S	S	R
Bacitracin	S	S	S	R	S	R	S	S
Neomycin	S	R	S	S	R	R	R	R
Chloramphenicol	S	S	S	S	S	S	S	S
Ampicillin	S	R	S	S	R	R	R	S
Penicillin	R	R	S	S	R	R	S	S
Rifampicin	S	R	S	S	S	R	S	S

Table 1 *(Continued)*

Characteristics	L65	L80	L63	L68	J23	J31	J16	J29b
Catalase	+	+	+	+	+	+	+	+
Oxidase	+	+	+	–	–	+	+	+
H_2S production	–	–	–	–	–	–	–	–
Nitrate reduction	++	+	++	++	+	++	++	++
Vogues–Proskauer test	+	nd	–	–	–	nd	nd	–
Gas formation	+	–	–	–	+	+	+	–
Acid production from								
Sucrose	–	–	–	–	–	–	–	–
Lactose	–	–	–	–	–	–	–	+
D-Glucose	–	–	–	–	–	–	–	+
Hydrolysis of								
Esculin	+	+	+	+	+	+	+	+
Starch	–	–	–	–	–	+	+	+
Casein	–	–	–	–	–	+	+	–
Gelatin	+	–	+	+	–	–	–	–
Tween80	–	–	–	–	–	–	–	–
Urease	–	+	–	–	+	–	–	+
β-galactosidase	+	+	+	+	+	–	+	+

Characteristics	K1-10	K1-19	K2-17	M1-19a	L15	L19	L31
Colonial morphology	Circular	Irregular	Circular	Circular	Circular	Circular	Circular
Cell shape	Rod	Rod	Coccoid	Rod	Rod	Coccoid	Coccoid
Spore production	+	+	+	+	+	+	+
Motility	+	–	+	–	–	+	+
Pigmentation	Cream	Cream	Cream	Cream	White	Cream	Cream
Anaerobic growth	–	–	–	–	–	–	–
Gram stain	+	+	+	+	+	+	+
Optimal NaCl for growth (%)	7·5	10	10	7·5	10	10	10
Optimal temperature for growth (°C)	30	30	30	30	30	30	37
Optimal pH for growth	7	8	6–7	7	6–7	6–7	6–7
Sensitivity to antibiotics							
Erythromycin	S	S	S	R	R	S	R
Bacitracin	S	R	S	R	S	S	R
Neomycin	R	R	S	R	R	S	R
Chloramphenicol	R	R	S	S	S	S	S
Ampicillin	S	S	S	S	R	S	S
Penicillin	S	R	S	R	R	S	S
Rifampicin	S	S	S	S	R	S	S
Catalase	+	+	+	+	+	+	+
Oxidase	+	+	–	+	+	+	+
H_2S production	–	–	–	–	–	–	–
Nitrate reduction	++	+	+	++	++	+	+
Vogues–Proskauer test	+	–	–	–	nd	+	–
Gas formation	±	–	+	+	+	+	–
Sucrose	–	–	–	–	–	–	–
Lactose	+	–	–	–	+	–	–
D-Glucose	+	–	–	–	+	–	–
Hydrolysis of							
Esculin:	–	–	–	–	–	–	–
Starch	+	+	–	+	–	+	+
Casein	–	–	+	–	+	–	+
Gelatin	–	–	+	–	–	–	–
Tween80	–	+	+	+	–	+	–
Urease	–	–	+	–	–	nd	–
β-galactosidase	–	–	–	+	–	–	–

(+) Positive reaction; (–) negative reaction; (R) resistant to tested antibiotic; (S) sensitive to tested antibiotic; (++) reduction of nitrate to nitrite; (±) unclear reaction; and (nd) not determined.

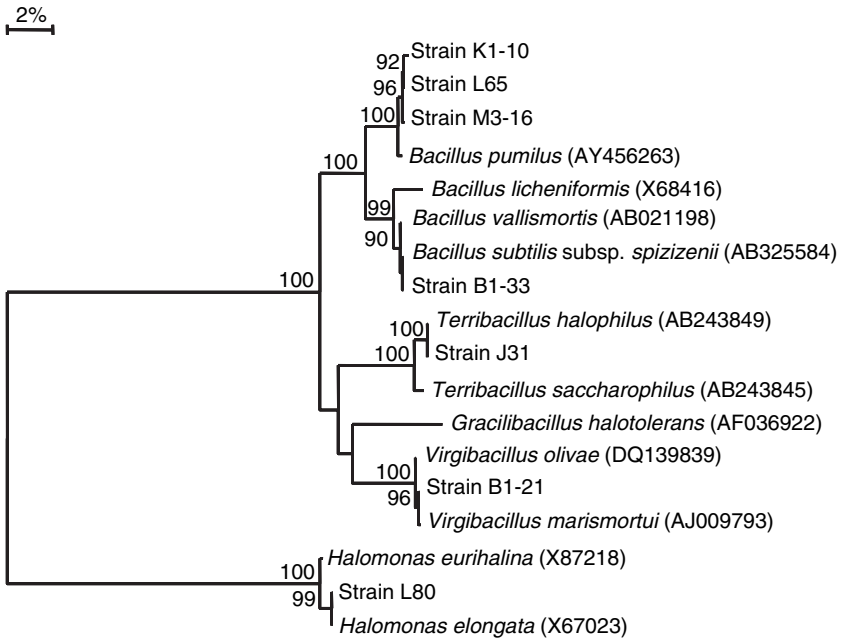

Figure 2 Neighbour-joining tree based on 16S rDNA (1368 bases) sequences showing the phylogenetic relationship between strains K1-10, L65, M3-16, B1-33, J31, B1-21, L80 and other related species of *Bacillus* and *Halomonas*. Bootstrap values (expressed as percentages of 100 replications) greater than 70% are given at the nodes.

absence of the pathogen (Fig. 3). Chitinase production was significantly higher in medium (M2) containing glucose (1g l^{-1}) as a sole carbon source and the pathogen (10^6 conidia ml^{-1}). The chitinolytic activity in the latter medium ranged from 55·21 U ml^{-1} for M3-23 to 295·8 U ml^{-1} for M2-26. Lower chitinase amounts, were detected in media containing glucose (M1) or colloidal chitin (M4) as a sole carbon source (Fig. 3). In fact, the chitinolytic activities were not higher than 30·19 U ml^{-1} and 67·71 U ml^{-1} respectively in M1 and M4 media.

Finally, for media (M3) which contains glucose at 0·1 g l^{-1}, chitinase activities ranged from 30·66 U ml^{-1} for M3-23 to 1·39 U ml^{-1} for J31. The results showed that chitinase activities were highly dependent with media composition (source of carbon) and presence or absence of the pathogen and the origin of the bacterial strain.

Discussion

Recent studies undertaken by Sadfi *et al.* (2001, 2002), showed the successful biocontrol of halotolerant bacteria in the inhibition of postharvest disease of potatoes induced by *Fusarium sambucinum* under storage conditions. *Bacillus cereus* and *B. thuringiensis* were found to reduce the disease incidence from 50% to 80% (Sadfi *et al.* 2002). Control of grey mould using halophilic antagonists has been successful for pre- and postharvest treatments of tomato crop in Tunisia (Sadfi-Zouaoui *et al.* 2007, 2008). The isolates halophilic strains from shallow salt lakes (Sadfi-Zouaoui *et al.* 2008) were tested on harvested strawberries for their effectiveness against *B. cinerea*. 20·2% of the selected strains satisfactory controlled the pathogen. These bacteria are halotolerant to moderate halophiles, as they grew in media containing 0·5–20% NaCl (w/v) (Ventosa *et al.* 1998). The moderate halophiles of *Bacillus subtilis* (M1-20, J9), *B. licheniformis* (J24), *Staphylococcus equorum* (L40, L50) and *Planococcus rifietoensis* (M2-26) had inhibited the mycelial growth of grey mould on wounded tomatoes (Sadfi-Zouaoui *et al.* 2008), and were active against the development of the disease on strawberries. Moreover, *Virgibacillus marismortui*, *B. pumilus*, *Terribacillus halophilus*, *Halomonas elongata*, *Staphylococcus equorum* and *Staphylococcus* sp., identified in this study by phenotypic tests and 16S rDNA sequencing, showed a good inhibitory effect against *B. cinerea* on harvested strawberry fruits. These bacteria produced extracellular hydrolytic antifungal enzymes, which may contribute to the suppression of grey mould rot on strawberry fruits.

Previous study showed the efficacy of hot water, biocontrol with *Pichia guilliermondii* yeast and controlled atmosphere in controlling grey mould on strawberries (Wszelaki and Mitcham 2000). Here, we first report on the inhibitory effects of moderately halophilic bacteria belonging to diverse genera of *Planococcus*, *Virgibacillus*, *Terribacillus* and *Halomonas* on the suppression of *B. cinerea* growth on strawberries. Their potential as biocontrol agents for grey mould is interesting and further investigation is needed to verify the effectiveness of these antagonists under long-term storage conditions and their ability to persist on strawberry plant for a long time.

Table 2 Identity of the selected halophilic bacterial isolates, their range of tolerance to NaCl, and their effect on the mycelial growth of *B. cinerea in vitro*

Growth range at salt concentrations (% NaCl)	Strain	Identified as	*GI (%)
5–10	J9	*Bacillus subtilis*	0a
	B1-33	*Bacillus subtilis*	13·82b
	J24	*Bacillus licheniformis*	81f
	M1-20	*Bacillus subtilis*	94hi
5–15	B1-21	*Virgibacillus marismortui*	0a
	K1-19	*Virgibacillus marismortui*	0a
	M3-23	*Virgibacillus marismortui*	0a
	K1-10	*Bacillus pumilus*	65·05d
	M2-26	*Planococcus rifietoensis*	74·5e
	M3-16	*Bacillus pumilus*	90·5gh
	M1-19a	*Bacillus pumilus*	91·5gh
	M3-2	*Bacillus pumilus*	95hij
	M3-6	*Bacillus pumilus*	98ij
	L65	*Bacillus pumilus*	98·5ij
	L80	*Halomonas elongata*	99·5i
5–20	J16	*Terribacillus halophilus*	0a
	B1-35	*Staphylococcus equorum*	0a
	L15	*Terribacillus halophilus*	0a
	J31	*Terribacillus halophilus*	0a
	J23	*Staphylococcus* sp.	0a
	L63	*Staphylococcus equorum*	51·35c
	L68	*Staphylococcus equorum*	79·5f
	J29b	*Staphylococcus* sp.	88·80g
	M2-45	*Staphylococcus* sp.	97ij
	L31	*Staphylococcus equorum*	97·35ij
	L50	*Staphylococcus* sp.	97·5ij
	K2-17	*Staphylococcus* sp.	97·5ij
	L19	*Staphylococcus* sp.	99j
	L40	*Staphylococcus* sp.	99j
	B1–28	*Staphylococcus* sp.	99j
	Control		0a

*Growth inhibition (GI) = $(R_1-R_2)/R_1 \times 100$, where R_1 represents the distance (measured in millimeter) between the point of implant of the fungal disk and the side of the Petri plate, and R_2 is the distance of fungal growth from the point of inoculation to the colony margin in the direction of the antagonist (Sadfi-Zouaoui *et al.* 2008). Halotolerant to moderately halophilic strains were identified by phenotypic tests and 16S rDNA sequencing.

Means followed by a common letter are not significantly ($P = 0·05$) different according to LSD test.

In controlling fungal plant pathogens, a variety of mechanisms contribute to the biocontrol activity of microbes (Kim and Kim 1994). Cell-wall-degrading enzymes such as β-1,3-glucanases, cellulases, proteases and chitinases are involved in the antagonistic activity of some biological control agents against phytopathogenic fungi (Chérif *et al.* 1992). The antifungal enzymes from halophilic bacteria have not been investigated (Sadfi-Zouaoui *et al.* 2008). Suppression of *B. cinerea* growth

Table 3 Effects of volatiles of halophilic bacteria on radial growth of *B. cinerea*

Treatment	Mean radial growth of fungal colony (mm)
Control	64·83 ± 4·53d
J31	62·33 ± 2·51d
J9	52·50 ± 3·77cd
M2-26	39·17 ± 3·54bc
J24	27·17 ± 5·53ab
L80	33·00 ± 3·90b
M3-23	26·67 ± 12ab
M3-16	9·50 ± 1·32a

Data are the average of three replications ± the standard error of the means. Means followed by a common letter are not significantly ($P = 0·05$) different according to LSD test.

in vitro by the selected moderately halophilic isolates and formation of inhibition zones were presumably due to the metabolites being released from bacteria into the culture medium. Although ineffective *in vitro* on PDA medium, *V. marismortui* (B1-21, M3-23, K1-19) and *Terribacillus halophilus* (J16, J31) isolates effectively inhibited grey mould on strawberry fruits. This confirms the idea that bacterial isolates unable to form inhibition zones on solid medium are not necessarily incapable of inhibiting disease development *in vivo*. These isolates may assume their antagonistic effect by mainly producing cell-bound antifungal compounds (Edwards 1993; Walker *et al.* 1998) or

Table 4 Extracellular hydrolytic activity of antifungal halophilic strains

	Characteristics			
Strains	Cellulase	Protease	Chitinase	β-1, 3-glucanase
Terribacillus halophilus L15	–	–	+	+
Terribacillus halophilus J31	–	+	+	+
Terribacillus halophilus J16	–	+	+	+
B. subtilis B1-33	+	+	+	+
B. pumilus M3-16	+	+	+	+
B. pumilus M3-2	+	–	+	+
B. pumilus K1-10	–	–	+	+
B. pumilus L65	–	–	+	+
B. pumilus M3-6	–	–	+	+
B. pumilus M1-19a	–	–	+	+
B. marismortui M3-23	–	–	+	+
B. marismortui B1-21	–	–	+	+
B. marismortui K1-19	–	–	+	+
Halomonas elongata L80	–	–	ND	+

Symbols: (+) strain produces the enzymes; (–) strain does not produce the enzyme; ND - none detected.

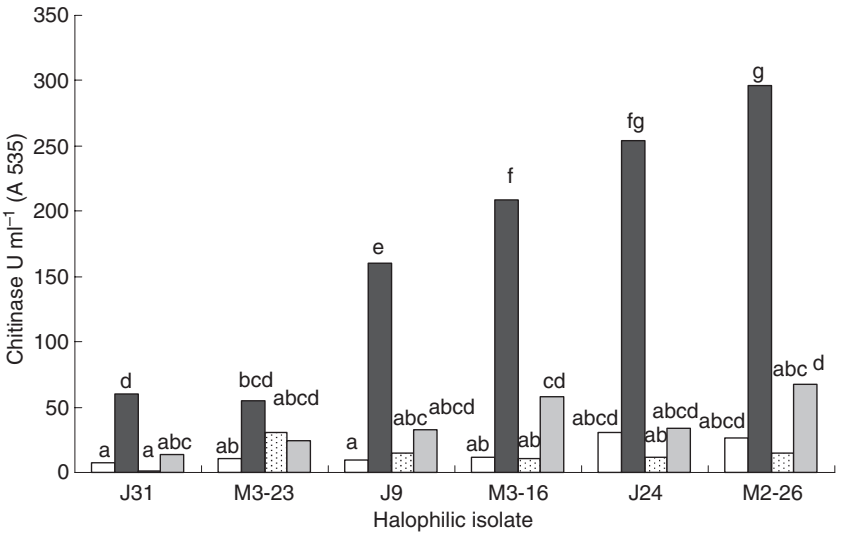

Figure 3 Effect of different growth media on chitinase activity in *T. halophilus* J31, *V. marismortui* M3-23, *B. subtilis* J9, *B. pumilus* M3-16, *B. licheniformis* J24, and *P. rifitioensis* M2-26. For each strain, differentially shaded bars represent (from left to right) chitinase production in M1, M2, M3 and M4 media (also see Materials and methods) after 5 days of incubation at 37°C. Chitinase activity is expressed in units per ml of cell-free supernatant. Bars with the same letter did not differ significantly at $P = 0{\cdot}05$ by LSD test.

indirectly by inducing plant resistance mechanisms and/or by nutrient competition (Kehlenbeck *et al.* 1994; Muninbazi and Bullerman 1998).

This study has shown the production of antifungal enzymes from the halophilic isolates as all isolates possess chitinase and glucanase activities. The chitinase production was highly induced by the presence of autoclaved spores of *B. cinerea in vitro* and it was dependent on the presence of glucose or colloidal-chitin as a sole carbon source in the growth medium. This suggests that medium composition (glucose content) affected induction of chitinase enzyme. These data correlate with results of Nielsen and Sorensen (1997), who suggested the influence of medium composition (glucose content) on the cell wall degrading enzymes produced by *B. pumilus* strains. Furthermore, Leelasuphakul *et al.* (2006) demonstrated that β-1, 3-glucanase produced by *B. subtilis* was induced by the addition of chitin into the growth medium. Although, the number of reports dealing with microbial chitinases is increasing, the mechanisms of degradation and utilization of the insoluble chitin substrate by micro-organisms, including the induction mechanisms remain still unclear (Watanabe *et al.* 1990).

In this study, *B. subtilis*, *B. licheniformis*, *B. pumilus* and *V. marismortui* were shown to secrete chitinolytic enzymes thus supporting earlier reports of chitinase production in several potential biocontrol *Bacillus* species, including *B. circulans* (Watanabe *et al.* 1990), *B. licheniformis* (Trachuck *et al.* 1996), *B. cereus* (Pleban *et al.* 1997) and *B. thuringiensis* (Chigaleichik 1976). 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 (Lorito *et al.* 1994). However, to our knowledge, no data about quantitative chitinase production from *Planococcus rifietoensis* has been published. It was interesting to note very high amounts of chitinases (295·8 U ml^{-1}) produced by this moderately halophilic species determined by the release of reducing sugars from colloidal chitin. This study further confirms the potential of halophilic bacteria to produce important hydrolytic enzymes.

In this study, some *Bacillus* species were found to produce volatile compounds on PDA plates. Whether, these antifungal volatiles contribute to grey mould suppression on strawberries remains to be demonstrated. Nevertheless several studies revealed the importance of antifungal volatiles in the biocontrol of different plant diseases (Dennis and Webster 1971). The effect of volatile compounds has received only limited attention in comparison to the antagonism because of diffusible substances (Chaurasia *et al.* 2004). Only two halotolerant strains of *B. pumilus* and *B. licheniformis* developed antibiotic activity on solid medium, while the rest of isolates were unable to inhibit the growth of *B. cinerea* by the cell-filtrates. Similarly to works of Sadfi *et al.* (2001), the ineffectiveness of culture supernatants of halotolerant *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. Several hydrolytic enzymes are produced by *Bacillus* species especially β-1, 3-glucanase and protease which play a role in the decomposition of fungal cell-walls (Lim *et al.* 1991) and may be important determinants in the antagonistic process (Chérif and Benhamou 1990).

Bacillus and *Staphylococcus* were the most abundant species in the collection established from different hypersaline ecosystems in Tunisia. These results are in

agreement with works of Hacene *et al.* (2003) and Sadfi-Zouaoui *et al.* (2008). *Staphylococcus* spp. are potential pathogens for human and animals and must be omitted in our biocontrol screening because of their serious public health risk.

This study demonstrates that moderate halophiles suppressed or at least reduced the grey mould disease caused by *B. cinerea* on strawberry fruits under commercial standard conditions. The use of such bacteria may constitute an important alternative to synthetic fungicides and may be useful for disease management programmes. The biological control potential of halophilic bacteria may be correlated with their ability to produce several extracellular antifungal hydrolytic enzymes (chitinase, β-1, 3-glucanase, cellulase and protease). Current studies are undertaken to correlate between the concentration of salt in the medium and the production of bioactive compounds (enzymes and antibiotics). Our results showed that for majority of tested strains cultivation under optimal salt concentration yielded highest level of hydrolytic activities (B. Essghaier, H. Jijakli, A. Bondabons, N. Sadfi-Zouaoui and M. Bejji, unpublished data), which is in agreement with results of Xu *et al.* (2003).

Moreover, Greenway and Osmond (1972) have proved that enzymes from halophilic bacteria require high salt concentration during assay for optimal activity. Further studies are currently in progress to select the best producers of hydrolytic enzymes from the novel effective isolates and investigations should be directed towards the characterization and purification of the corresponding encoding genes. In addition to test the efficiency of the antagonistic bacteria in reducing disease on strawberry plant under greenhouse and field conditions.

Acknowledgements

This work was supported by funds from CMCU (Comité Mixte de Coopération Franco-Tunisien no 06S/0901), and by the Ministry of Scientific Research and Competency Development (MRSDTC, Tunisia) 206, 207.

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