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SHORT NOTES

Selection of halophilic bacteria for biological control of tomato gray mould caused by *Botrytis cinerea*

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Summary. In Morocco, tomato gray mould caused by *Botrytis cinerea* Pers: Fr. is a serious threat for postharvest storage of tomatoes. Fifteen halophilic bacteria were evaluated for their antagonistic activity against *B. cinerea*: 11 Gram positive strains assigned to the genera *Bacillus* (9), *Jeotgalibacillus* (1) and *Planococcus* (1) and four Gram negative strains assigned to the genera *Salinivibrio* (1), *Vibrio* (2) and *Photobacterium* (1). In *in vitro* screening, 12 antifungal isolates secreted diffusible compounds, hydrolytic enzymes or volatile compounds. *In vivo* screening of the isolates, *Bacillus safensis* CCMM B582 and *Bacillus oceanisediminis* CCMM B584 showed permanent antagonistic activity on tomato fruits, with 100% inhibition of *B. cinerea* after 7 days. These two strains may offer potential for biological control of tomato gray mould.

Key words: halophilic bacteria, antifungal compounds, *Lycopersicon esculentum*.

Introduction

Botrytis cinerea Pers: Fr. is an important pathogen that causes gray mould on tomato, apple, strawberry and a range of other economically important crops (Jarvis, 1977; Williamson *et al*., 2007; Sharma *et al*., 2009). In Morocco, significant economic losses due to this pathogen occur on tomato. Tomatoes grown in plastic greenhouses cover about 9000 ha with 50% of exported tomato production (Hanafi and Schnitzler, 2004).

Gray mould may be controlled by chemical and non-chemical methods. However, frequent application of fungicides has resulted in the selection of pathogen resistant strains (Chung *et al*., 2009). Elad *et al*. (1992) showed that *B. cinerea* developed resistance to specific fungicides (benzimidazoles, dicarboximides and the sterol biosynthesis inhibitors) within a relatively short time. Similarly, Hmouni *et al*. (2003) reported a high level of resistance of *B. cinerea* to benzimidazoles, dicarboximides and dithiocarbamates in Moroccan tomato greenhouses. Moreover, fungicides potentially cause soil pollution and may have detrimental effects on humans (Martínez-Romero *et al*., 2008).

Biological control offers an alternative to the use of synthetic fungicides and has become a well established principle over the last few decades (Cook and Baker, 1983). Various bacteria (e.g. *Bacillus, Pseudomonas*, and *Enterobacter*), fungi (e.g. *Pythium*, and *Trichoderma*) and actinomycetes (e.g. *Streptomyces)* have shown good biocontrol potential (Lange *et al*., 1993; Emmert and Handelsman, 1999; Yang *et al*., 2006; Benchabane *et al*., 2008).

For the control of *B. cinerea*, many fungi, bacteria and actinomycetes have been used (Elad *et al*., 1994; Loqman *et al*., 2009; Mónaco *et al*., 2009). Sadfi-Zouaoui *et al*. (2007, 2008) showed that moderately

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halophilic bacteria from Tunisia (e.g. *Bacillus* spp., *Halomonas* spp., *Planococcus* spp., *Salinicoccus* spp., *Halobacillus* spp. and *Marinococcus* spp.) inhibited *B. cinerea*, and could be considered as potential biocontrol candidates.

The aim of the present study was to select effective antifungal bacteria from Moroccan ecosystems and evaluate their abilities to control the tomato gray mould pathogen.

Materials and methods

Bacterial strains and growth conditions

Fifteen bacterial strains were selected from a range of halophilic isolates obtained in 2005 and 2006 from two hypersaline environments, located in Larache at 35°10'N, 006°06'W and 35°11'N, 006°07'W (North Morocco) (Tables 1 and 2). We have previously reported on them (Berrada *et al.*, 2012), and all the bacterial isolates have been deposited in the Moroccan Coordinated Collections of Microorganisms. Isolates were grown at 30°C and stored at 4°C on Tryptic Soy Agar medium (TSA, Difco, Detroit, USA). For long-term storage, strains were preserved in 20% glycerol at -80°C.

Preparation of pathogen inoculum

Botrytis cinerea CCMM-M185 causing typical symptoms of gray mould was grown on plates of potato dextrose agar (PDA, Difco) for 7 to 10 days at 25°C until sporulation. A monospore isolate was maintained on PDA at 4°C and was subcultured onto fresh PDA plates every week. Fungal spores from the PDA plates were used to test for *in vitro* antagonism.

In vitro screening of antagonists

A loopful of bacteria from a 2-day-old culture was streaked across the centre of a TSA (Difco, Detroit, USA) plate. Two 5-mm discs were cut from a 7-day-old culture of *B. cinerea* CCMM-M185 and placed at a distance of 25 mm on each side of the bacterial streak. Control plates containing fungus were prepared without bacteria. The plates were then incubated at 25°C for 7 days. Percentage inhibition of pathogen growth was calculated by the formula: (r1 - r2)/r1×100, where r1 (a control value) represents the radial growth of the fungus in the control, and r2 represents the radial growth of the fungus in the plates inoculated with the test bacteria (Chaurasia *et al.*, 2005).

Antagonism due to volatile compounds was evaluated by preparing a bacterial culture on Tryptic Soy Broth TSB medium (Difco) plates. After incubation for 48 h, the lid of each Petri dish was replaced by a plate containing 5mm agar discs of the pathogen. The two plates were sealed with parafilm. Controls were prepared without bacteria in the bottom plate. Petri dishes were incubated at 25°C, and observations were recorded after 7 days. The percentage growth inhibition of the pathogen was calculated using the same formula: (r1 - r2)/r1×100. All *in vitro* antagonism assays were made in triplicate.

Detection of extracellular hydrolytic activities of the bacterial antagonists

Proteolytic activity of each isolate was screened qualitatively as described by Sadfi-Zouaoui *et al.* (2008). The extracellular chitinase or glucanase activities were detected on an agar medium containing 0.5% chitin from crab shell (Sigma C 9752, Sigma Chemical Co., Saint Louis, MO, USA) or 0.1% laminarin from *Laminaria digitata* (Sigma L9634) as the sole carbon source. This medium contained per litre of water: 7 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 1 g NaCl, 0.1 g $MgSO_4.7H_2O$, 0.5 g yeast extract, 15 g agar plus 5% NaCl (w/v).

Preparation of tomato fruits

Intact red tomatoes (*Lycopersicon esculentum* Mill.) uniform in size and colour, were obtained from the market. The fruits were surface-sterilized by soaking in 2% aqueous sodium hypochlorite for 5 min (Sadfi-Zouaoui *et al.*, 2008). They were thoroughly rinsed, dried using sterile filter papers, and then wounded by removing two plugs at the equator of each fruit, 3 mm in diam. and 3 mm in depth, from the surface, using a sterile scalpel.

In vivo screening of antagonists for antifungal activity against *B. cinerea* on tomato fruits

Fresh cultures of the pathogen and the bacterial antagonists were used for each experiment. To evaluate their antagonistic activity, twelve bacterial strains were grown for 48 h in TSB (Difco) and ad-

Table 1. *In vitro* screening of bacterial isolates for their antifungal activities against *Botrytis cinerea*

Antagonist[a]	% inhibition of *Botrytis cinerea*	
	Diffusion	Volatile
Bacillus oceanisediminis B584[b]	100	87.5 ±2.5
Bacillus gibsonii B578	100	49.5 ±6.7
Bacillus safensis B582	100	41.7 ±8.5
Bacillus aquimaris B656	96.7 ± 5.8	0.0
Bacillus aquimaris B597	95.6 ±7.6	0.0
Bacillus niabensis B579	95.8 ±7.2	0.0
Bacillus oceanisediminis B655	93.0 ±6.1	0.0
Bacillus thioparans B649	55.3 ±2.8	0.0
Bacillus oceanisediminis B645	26.7±3.8	0.0
Planococcus rifietoensis B654	95.6 ±7.7	0.0
Jeotgalibacillus salarius B657	36.8 ±1.6	0.0
Photobacterium sp. B665	100	61.6 ±4.5
Salinivibrio costicola B659	100	76.3 ±10.1
Vibrio fluvialis B664	100	0.0
Vibrio sp. B663	100	0.0

[a] Antagonists were chosen as representatives of strains from a recently reported collection of halophilic bacteria from Morocco (Berrada *et al.*, 2012).
[b] B-numbers refer to the Moroccan Coordinated Collection of Micro-organisms (CCMM) accession numbers of strains. Antifungal activity was measured by diffusion after 7 days (Dif.) / Volatile antifungal activity was measured after 7 days (Vol.). Values are means ± SE (Standard Error) of three replicates.

Table 2. Extracellular activities of 15 bacterial isolates from Morocco.

Antagonist[a]	Enzyme pattern[c]		
	Protease	Chitinase	Glucanase
Bacillus safensis B582[b]	+	+	+
Bacillus oceanisediminis B584	-	+	+
Bacillus gibsonii B578	-	+	+
Bacillus aquimaris B656	-	+	+
Planococcus rifietoensis B654	-	+	+
Vibrio sp. B663	-	+	+
Salinivibrio costicola B659	+	+	-
Photobacterium sp. B665	-	+	-
Vibrio fluvialis B664	-	+	-
Bacillus aquimaris B597	-	-	-
Bacillus niabensis B579	-	-	-
Bacillus oceanisediminis B655 and B645	-	-	-
Bacillus thioparans B649	-	-	-
Jeotgalibacillus salarius B657	-	-	-

[a] See Table 1.
[b] See Table 1.
[c] Symbols: +, positive growth; -, no growth.

justed to 10^8 colony forming units (CFU) per mL. Conidial suspensions of *B. cinerea* in Potato Dextrose Broth (Difco) were adjusted to 10^4 spores mL^{-1}. Twenty microlitres of the bacterial suspensions were inoculated into the wounded fruits. After 60 min drying in a laminar flow cabinet, the tomatoes were each inoculated with 20 μL of a conidial suspension of the pathogen. As positive and negative controls, fruits were either inoculated with the pathogen alone or with distilled water.

The fruits were then stored at 20°C for 7 days in autoclaved glass jars with hermetic covers. The percentage of disease reduction of gray mould on the tomato fruits was calculated using the following formula: (%) = (A-B)/A×100, where A is the lesion diameter recorded in tomato fruit inoculated with the pathogen alone and B is the lesion diameter in infected tomato fruit treated with the antagonist. All *in vivo* antagonism assays were made in triplicate.

Results and discussion

In vitro screening of bacterial antagonists and detection of extracellular hydrolytic activities

In vitro screening for diffusible compounds (Table 1) revealed that among the 15 tested bacteria, 12 had clear antagonistic activity towards *B. cinerea*. The isolates *Bacillus oceanisediminis* B584, *Bacillus gibsonii* B578, *Bacillus safensis* B582, *Photobacterium* sp. B665,

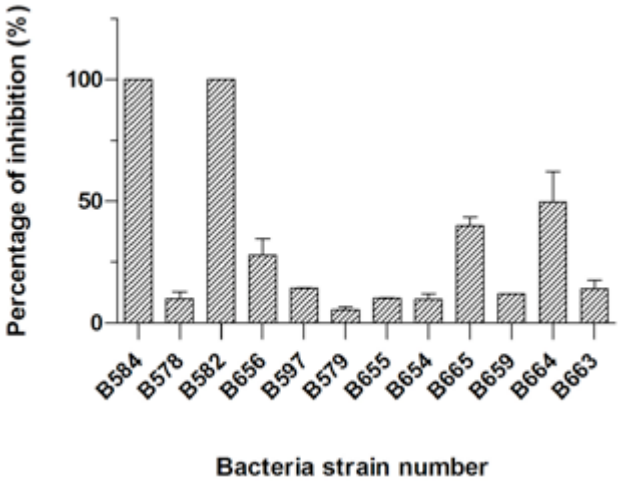

Figure 1. *In vivo* screening of bacterial antagonists against *Botrytis cinerea.* The percentage of inhibition is given after 7 days. The error bars represent the mean + SE of three replicates.

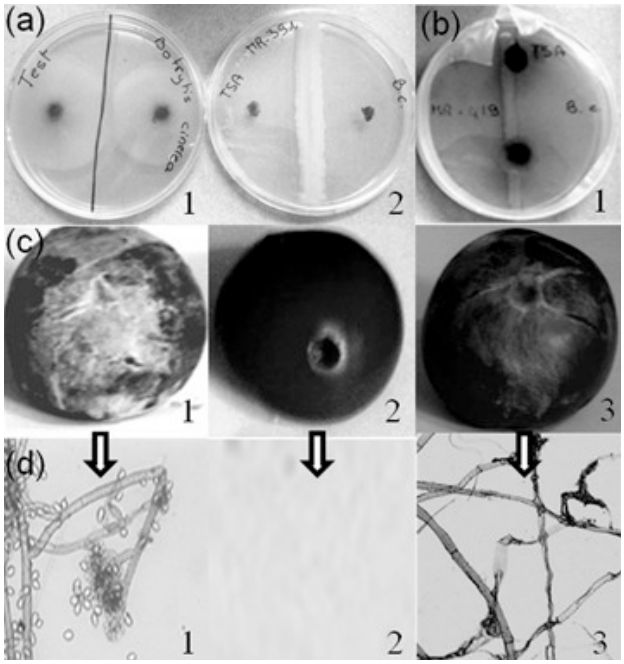

Figure 2. Screening *in vitro* and *in vivo* of bacterial antagonists against *Botrytis cinerea.* (a and b) test *in vitro* : (a1) control, (a2) antagonism due to diffusible compounds of *Bacillus safensis* B582 and (b1) antagonism due to volatile compounds of *Bacillus oceanisediminis* B584. (c) Test *in vivo*: (c1) control, (c2) Total inhibition by *Bacillus safensis* B582 and (c3) Partial inhibition by *Vibrio* sp. B663. (d) Microscopic observation (Magnification: 10 × 100 Observation): (d1) Normal mycelia of *B. cinerea*, (d2) No visible mycelia *Bacillus safensis* B582. (d3) Abnormal mycelia of *B. cinerea* inhibited by the antagonistic bacterial isolate *Vibrio* sp. B663.

Salinivibrio costicola B659, *Vibrio* sp. B663, and *Vibrio fluvialis* B664 gave 100% inhibition of *B. cinerea* after 7 days of incubation.

Among the 15 bacteria tested, two showed protease activity, nine chitinase activity and six glucanase activity (Table 2). *Bacillus* strains B656, B582, B578, B584, *Planococcus rifietoensis* B654 and *Vibrio* sp. B663 were able to degrade colloidal chitin and β-1, 3-glucan. Only one isolate (*Bacillus safensis* B582) secreted protease, chitinase and glucanase.

Bacillus species may exhibit their antagonistic effects against fungal pathogens by degradation of cell walls of pathogenic fungi via the production of extracellular lytic enzymes. Several *Bacillus* species produce chitinases, glucanases and proteases e.g., *B. circulans* (Rombouts *et al.*, 1976), *B. subtilus* (Manjula and Podile, 2005) and *B. pumilis* (Essghaier *et al.*, 2009). As far as we know, this is the first report of the inhibitory effects of *Bacillus gibsonii, Bacillus oceanisediminis, Bacillus safensis* and *Bacillus aquimaris* against *B. cinerea*.

Sadfi-Zouaoui *et al.* (2008) previously reported that *Planococcus rifietoensis* inhibited *B. cinerea*. Our strain *Planococcus rifietoensis* B654 was able to degrade colloidal chitin and β-1,3-glucan and gave clear diffusible antifungal activity *in vitro* against *B. cinerea.*

Strains *Photobacterium* B665, *Salinivibrio costicola* B659 and *Vibrio fluvialis* B664 were able to degrade colloidal chitin. This is the first report of the inhibitory effects of the genera *Vibrio, Salinivibrio* and *Photobacterium* on *B. cinerea*.

Strains B655, B579 and B597, which were not able to degrade colloidal chitin, protein and β-1, 3-glucan, showed diffusible antifungal activity *in vitro* against *B. cinerea.* In this case, anti-fungal antibiotic action is supposed. Sadfi *et al.* (2002a) showed that bacterial antagonists assume their antagonistic effects also by the production of antifungal antibiotics (Katz and Demain, 1977).

The results of the *in vitro* dual culture screening for volatile compounds revealed that among the 15 tested bacteria, five gave partial antagonistic activity against *B. cinerea: Bacillus oceanisediminis* B584, *Bacillus gibsonii* B578, *Bacillus safensis* B582, *Photobacterium*

sp. B665 and *Salinivibrio costicola* B659. It has been reported that volatile compounds may inhibit fungal spore germination, germ tube growth and hyphal morphology (Arrebola *et al.*, 2010).

In vivo screening of bacterial antagonists for antifungal activity

Among 12 bacteria tested, two (*Bacillus oceanisediminis* B584 and *Bacillus safensis* B582) showed antagonistic activity against *B. cinerea* on tomato fruits (Figures 1 and 2), with 100% inhibition after 7 days. Strains B665, B663, B664, B597 and B654 showed partial inhibition and caused abnormal mycelial growth of *B. cinerea* (Figures 2c3, 2d3).

Our results confirm the observations of Mari *et al.* (1996) and Sadfi-Zouaoui *et al.* (2008), that *Bacillus* is particularly effective against *B. cinerea* on tomatoes.

An earlier studies (Zhao and Kong, 2003; Sadfi-Zouaoui *et al.*, 2008) showed that *Bacillus* isolates from salty soils had biocontrol activity against *B. cinerea*. Sadfi *et al.* (2002b) showed that *Bacillus* isolates from salty soils were able to protect potato tubers from *Fusarium* rot under traditional storage for 6 months and cold storage for 8 months. *Bacillus cereus, B. subtilis, B. mycoides* and others *Bacillus* spp. have been used successfully against fungal pathogens belonging to the genera *Rhizoctonia, Sclerotinia, Fusarium, Gaeumanomyces, Nectria, Pythium,* and *Phytophthora* (Cook and Baker, 1983; McKnight, 1993).

Conclusions

In this report, halophilic bacteria isolated from saline environments were tested for their antagonistic potential against *B. cinerea*, the main causal agent of gray mould on tomato in Morocco. Two bacterial strains offer potential as biological agents to control this tomato postharvest pathogen: *Bacillus safensis* B582 and *Bacillus oceanisediminis* B584 were able to secrete extracellular hydrolytic enzymes, and exhibited diffusible and volatile antifungal activity against *B. cinerea*.

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