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RESEARCH ARTICLE

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Isolation and characterization of *Bacillus subtilis* EB-28, an endophytic bacterium strain displaying biocontrol activity against *Botrytis cinerea* Pers.

Abstract The fungal pathogen *Botrytis cinerea* Pers. causes severe rotting on tomato fruits during storage and shelf life. As a biological control agent, endophytic bacterium was regarded as an effective alternative to chemical control. Out of 238 endophytic bacterial isolates, three strains (EB-15, EB-28, and EB-122) isolated from *Lycopersicum esculentum* Mill., *Speranskia tuberculata* (Bge.) Baill, and *Dictamnus dasycarpus* Turcz. respectively were found to be strongly antagonistic to the pathogen *in vitro* and were selected for further *in vivo* tests. One endophytic bacterium strain, encoded EB-28, was selected from the three *in vivo* tested isolates. The inhibitive rate of EB-28 reached 71.1% *in vitro* and 52.4% *in vivo*. EB-28 was identified as *Bacillus subtilis* according to its morphological, physiological, and biochemical characteristics and 16S rDNA sequence analysis.

Keywords endophytic bacterium, *Bacillus subtilis*, *Botrytis cinerea* Pers., biological control

1 Introduction

The pathogen *Botrytis cinerea* Pers. causes serious losses in more than 200 crop species worldwide (Williamson et al., 2007). Currently, the use of synthetic fungicides is the primary method to control post-harvest fungal decay (Chen et al., 2008). However, there is a continuing need for more effective and safer fungicides, especially those with novel modes of action and resistance-breaking properties, and natural products have a key role to play in the search for such compounds. Scientists have focused on biological methods to protect crops from invasion and infection by the pathogen (Liu et al., 2007). The management of pathogens by the use of antagonistic microorganisms or their secondary metabolites is now considered to be a feasible disease control technology (Han et al., 2005).

B. cinerea is the most destructive on mature or senescent tissues of dicotyledonous hosts, but it usually penetrates into plant tissue at an earlier stage in crop development and remains quiescent for a considerable period before rapidly rotting tissues under a conducive environment, and the host physiology changes. Therefore, serious damage is caused following the harvest of apparently healthy crops and the subsequent transport to distant markets where the losses become evident (Williamson et al., 2007). Endophytes, living for most of their life cycles inside healthy plant tissues, are mutualistic to their hosts. In particular, endophytic bacteria are thought to interact closely with their host plants and can therefore potentially be used as biological control agents in sustainable crop production (Sturz et al., 2000). A variety of endophytes have been reported to have antagonistic activities toward bacterial and fungal pathogens (Lodewyckx et al., 2002; Sessitsch et al., 2004). At least seven products have been approved for use on food and non-food plants in greenhouses, under plastic tunnels, or in the fields in different countries. They have achieved niche markets in situations where heavy use of conventional fungicides is restricted because of accumulating residues or because of the restriction imposed by importing countries (Elad and Stewart, 2004).

The aim of the present study was to isolate endophytic bacteria from *Lycopersicum esculentum* Mill. (tomato), *Speranskiae tuberculatae* (tuberculation speranskia herb), and *Dictamnus dasycarpus* Turcz. Densefruit Pittany Koot-bark, which showed a strong inhibition on the

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growth of *B. cinerea*. A potential endophytic antagonist, *Bacillus subtilis*, denoted as EB-28, effective against the growth of *B. cinerea*, was identified in healthy stems of *L. esculentum* by *in vitro* and *in vivo* screening techniques and was characterized by morphology, physiological tests, and 16S rDNA sequence analysis.

2 Materials and methods

2.1 Isolation of endophytic bacteria

Healthy stems from *L. esculentum* and *S. tuberculata*, and stem bark from *D. dasycarpus* were collected from Baoding, Hebei, in April 2007. The endophytic bacteria were isolated according to the method described by Liu et al. (2001). Three segments were randomly cut from each stem or bark. They were first washed in running water and then had their surface sterilized. The segments were washed again using sterile distilled water in autoclaved Petri dishes and then sectioned to 0.5 cm length using a small knife and placed on potato dextrose agar (PDA: potato, 200 g; dextrose, 20 g; agar, 15 g; distilled water, 1 L). After incubation for 1–2 days at 30°C in the dark, bacteria appeared on the plates and were individually isolated as single colonies on PDA.

2.2 Pathogen inoculum

A strain of *B. cinerea* (MYB01) used for laboratory trials had been isolated from infected tomato fruits with the typical symptom of gray mold in Baoding, China, and identified by its morphological characteristics. The fungal pathogen was cultivated on PDA plates for 10 days at 25°C until sporulation. A monospore isolate was maintained on PDA at 4°C and was subcultured onto fresh PDA plates at one-month intervals. Fresh, 5-mm-diameter mycelia plugs that were cut from the edge of a *B. cinerea* colony that was incubated for 5 days at 25°C were used as inoculum.

2.3 Tomato leaves and cucumber seedling cotyledons

Detached tomato leaves and cucumber seedling cotyledons were used for bioassay. The fourth leaves of the tomato plants (*Lycopersicum esculentum* cv. Hezuo908) and the eight-day-old cucumber seedling cotyledons (*Cucumis sativus* L. cv. Chuangchunmici) were uniform in size and free from wounds and lesions. Thereafter, the leaves were surface-sterilized by soaking in 2% aqueous sodium hypochlorite for 3 min. They were then thoroughly rinsed with sterile distilled water, dried by using sterile filter papers, and finally put into Petri dishes that contained 0.5% water agar medium (Jiao et al., 2009).

2.4 *In vitro* antagonism experiments

The inhibitory effects of endophytic strains on the growth of *B. cinerea* were tested in Petri dishes containing the PDA medium. A 5-mm mycelium disc cut from a 5-day-old culture of *B. cinerea* was deposited in the center of the plates, and bacteria from 2-day-old cultures were streaked across both sides of the mycelium disc approximately 2.5 cm away from the disc center. The plates were then incubated at 25°C for 4 days. The inhibition on fungal growth was evaluated by the reduction percentage of mycelium expansion compared to control plates without bacteria following the formula of Whipps (1987): $(R1–R2)/R1\times100$, where $R1$ is the farthest radial distance (measured in millimeters) grown by *B. cinerea* after 4 days of incubation in the direction of the antagonist (a control value), and $R2$ is the distance of fungal growth from the point of inoculation to the colony margin in the direction of the antagonist. All *in vitro* antagonism assays were made in triplicate (Fig. 1).

2.5 *In vivo* antagonism experiments

For each experiment, fresh cultures of the pathogen and the bacterial antagonists were used. To evaluate their

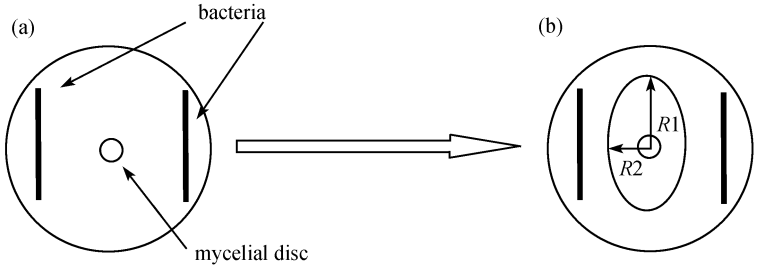

Fig. 1 Description of the *in vitro* antagonism assay

Note: (a) One mycelial plug of *B. cinerea* was placed at the center of the plate, and the bacterial isolates were streaked across each side of the mycelial\ plug. The distance between the two microorganisms was 2.5 cm. Plates were then incubated at 25°C for 4 days. (b) The percentage growth inhibition of the fungus was calculated by the formula of Whipps (1987): $(R1–R2)/R1\times100$, where $R1$ is the farthest radial distance (measured in millimeters) grown by *B. cinerea* after 4 days of incubation in the direction of the antagonist (a control value), and $R2$ is the distance of fungal growth from the point of inoculation to the colony margin in the direction of the antagonist.

antagonistic activities against *B. cinerea* on the detached tomato leaves, all bacterial strains selected from *in vitro* tests were grown for 48 h on Luria-Bertani (LB) medium at 30°C, shaken at 150 $r \cdot min^{-1}$. The cells were collected by centrifugation at 4000 $r \cdot min^{-1}$ and re-suspended with sterilized saline solution (1% NaCl). Bacterial suspension was adjusted to 1×10^8 colony-forming units (CFU)$\cdot mL^{-1}$. The bacterial concentration was determined by dilution plating on LB plate. After the bacterial suspension was sprayed on the surface of the detached tomato leaves, the leaves were allowed to air dry in a sterile cabinet for up to 60 min, followed by inoculation with a mycelium plug at the center of each leaf. The control treatments included bacterial candidate alone (non-*Botrytis* inoculated leaves, positive control) and distilled water (*Botrytis*-inoculated leaves, untreated control). The leaves were then stored in a growth chamber with 16 h of light and 8 h of darkness at 24°C for 48 h. The percentage of disease reduction of the gray mold on the tomato leaves was calculated using the following formula:

$$\text{Reduction rate } (\%) = (A - B)/A \times 100,$$

where A is the lesion diameter recorded in untreated control, and B is the lesion diameter in the infected tomato fruit treated with the antagonists (Sadfi et al., 2008). For each treatment, twelve tomato leaves were assayed (three leaves as one replicate), and the experiment was repeated at least three times.

2.6 Identification of antagonistic bacteria strain EB-28

The antagonistic bacteria strain EB-28 was phenotypically characterized on the basis of morphology, physiological tests, and chemical analysis, and identified according to the methods of Bergey's Manual of Determinative Bacteriology (1994) and Hacene et al. (2004), which were based on the following phenotypic features: colony and cell morphology, motility, spore production, Gram staining, pigmentation, and growth at different NaCl concentrations of 2%, 5%, 7%, and 10% (w/v). Catalase was determined by adding 10 volumes of H_2O_2 to cell culture (18 h) on solid LB medium. H_2S production was performed according to Clarke (1953). Nitrate reduction was assayed by adding 0.2% (w/v) KNO_3 to the liquid LB medium. Voges-Proskauer tests were performed by standard procedures (Barritt, 1936).

16S rRNA gene sequence determination was conducted by extracting chromosomal DNA from a single colony for PCR procedure according to Xiao et al. (2000). 16S rDNA from EB-28 genomic DNA was amplified with the primers 63F 5′-CAGGCCTAACACATGCAAGTC-3′ and 1494R 5′-GGTTACCTTGTTACGACTT-3′. The PCR amplification condition was as follows: denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 40 s each, at 56°C for 40 s, and at 72°C for 1.5 min, with another extension at 72°C for 10 min (Guo et al., 2007). The PCR products were purified using a UNIQ-10 gel extraction kit (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.) and identified by horizontal electrophoresis on 1.2% agarose gel, and then sequenced with an ABI 3100-Avant Genetic Analyzer (Applied Biosystems). The DNA sequence homology searches were performed using the online BLAST search engine in GenBank (available at: http://www.ncbi.nlm.nih.gov).

2.7 Statistical analyses

The results of the experiments were analyzed with DPS (Data Processing System 3.01) software. The data presented were the mean values of the three replications. The data were subjected to variance analysis, and one-way ANOVA or *t*-test was used to estimate the significance of differences between mean values ($P < 0.05$).

3 Results

3.1 Screening of effective antagonists *in vitro*

The results of the *in vitro* screening revealed that seven out of 238 endophytic bacterial isolates (EB-7, EB-11, EB-15, EB-19, EB-28, EB-112, and EB-122) significantly reduced the mycelial growth of *B. cinerea* by forming an inhibition zone (Table 1, Fig. 2(c)). Isolates EB-15, EB-28, and EB-122, which are the representative isolates from *S. tuberculata*, *L. esculentum*, and *D. dasycarpus*, were selected from the *in vivo* test.

Table 1 Antagonistic effect of endophytic bacteria strains against *B. cinerea*

endophytic bacteria strain	original plant	inhibition zone /mm	inhibitory rate/%
EB-7	*S. tuberculata*	10	60.00
EB-11	*S. tuberculata*	10	62.50
EB-15	*S. tuberculata*	11	70.27
EB-19	*S. tuberculata*	9	58.06
EB-28	*L. esculentum*	12	71.05
EB-112	*D. dasycarpus*	9	60.61
EB-122	*D. dasycarpus*	10	69.44

3.2 Effective antagonist tests *in vivo*

Isolates EB-15, EB-28, and EB-122, which are the representative isolates from *S. tuberculata, L. esculentum*, and *D. dasycarpus* were tested on cucumber seedling cotyledons and detached tomato leaves. The most effective was the EB-28 that was isolated from the tomato stem, with a percentage of gray mold reduction of 44.8% (cucumber cotyledon) and 52.4% (tomato leaves) (Fig. 2 and Fig. 3).

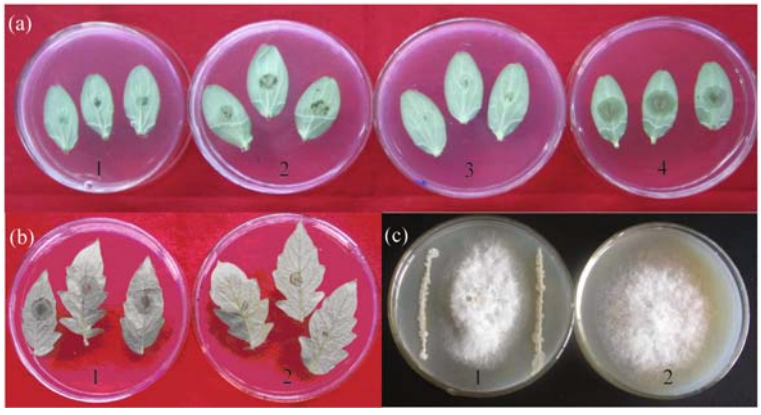

Fig. 2 *In vivo* and *in vitro* test of endophytic antagonistic bacteria to *Botrytis cinerea*

Note: (a) Antagonistic effect of endophytic bacterium isolates EB-122, EB-15, and EB-28 (a1, a2, and a3) on cucumber seedling cotyledons against tomato gray mold (a4 was the untreated control). (b) Antagonistic effect of endophytic bacterial isolates EB-28 (b2) on detached tomato leaves against gray mold (b1 was the untreated control). (c) Dual cultures of pathogen-antagonists showing (c1) a strong inhibition of radial growth of *B. cinerea* (c2 was the untreated control).

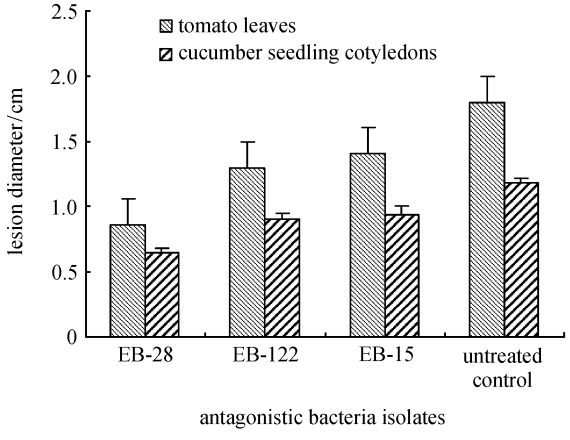

Fig. 3 Inhibitive effect of three effective endophytic bacterium isolates against *B. cinerea* on cucumber cotyledons and tomato leaves

Table 2 Phenotypic characterization of EB-28

characteristic	EB-28	characteristic	EB-28
colonial morphology	circular	catalase	+
cell shape	rod	H_2S production	+
spore production	+	nitrate reduction	+
motility	+	vogues-Proskauer test	+
anerobic growth	–	growth temperature	
gram stain	+	4°C	–
growth in presence of:		10°C	–
2% NaCl	+	20°C	+
5% NaCl	–	30°C	++
7% NaCl	–	40°C	+
10% NaCl	–	55°C	–

3.3 Characterization and identification of EB-28

The optimum growth occurred in medium containing 2% salt (w/v; Table 2). The EB-28 isolates were Gram-positive, motile endospore-forming rods. The spores were spherical or sometimes ellipsoidal and were located at subterminal positions. The spores survived heating at 80°C for at least 10 min. Colonies of EB-28 were 1–2 mm in diameter after being cultivated for 48 h on LB medium, and circular, smooth, and cream-pigmented (Fig. 4). EB-28 was identified as the member of the genera *Bacillus* from its morphological, biochemical, and physiological characteristics (Table 2).

By the 16S rDNA sequence analysis, EB-28 showed 99.1% similarity to *B. subtilis* EF488979. Therefore, it was identified as *B. subtilis* (Fig. 4).

4 Discussion

The aim of this research was to isolate endophytic bacteria with an ability to control or at least reduce the pernicious effects of the pathogen *Botrytis cinerea* Pers. The *in vitro* pre-screening test of dual culture allowed us to select three endophytic bacterium strains. Among the three tested trains, *B. subtilis* EB-28 showed potential as a biocontrol agent for gray mold *in vivo* and may be used in biologically controlling the phytopathogenic fungus *B. cinerea*. Further investigation is needed to verify the effectiveness of EB-28 in field and long-term storage conditions.

It is shown previously that *Bacillus* species was the predominant bacterium of all the isolated endophytic bacteria. When Moundt and Hinckle (1976) isolated the endophytic bacteria from the surface-sterilized ovules and

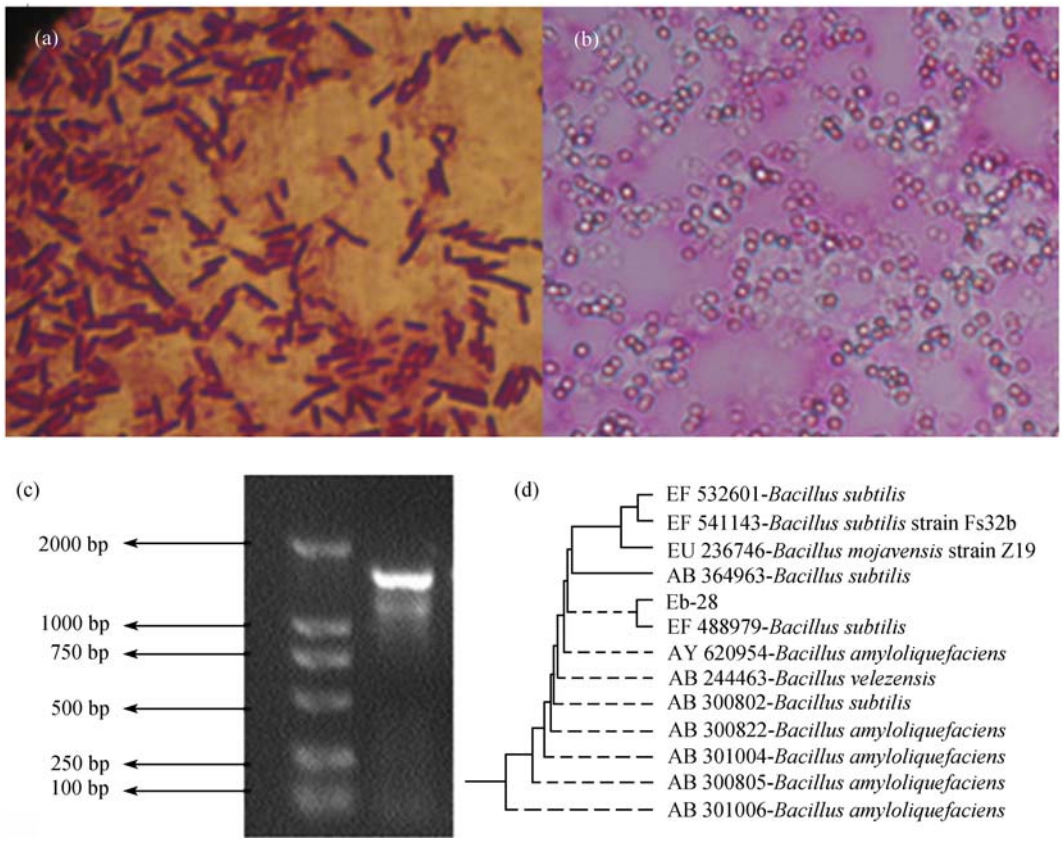

Fig. 4 Identification characteristic of EB-28

Note: (a) and (b) Morphology and endospores of EB-28. (c) Electrophoresis of 16S rDNA of EB-28. (d) Neighbor-joining tree based on 16S rDNA (1468 bases) sequences showing the phylogenetic relationship between EB-28 and other related species of the *Bacillus* sp. Bootstrap values (expressed as percentages of 100 replications) > 70% are given at the nodes.

seeds of 27 plant species, the *Bacillus species* occurred more frequently than any other species of single bacterial genus, accounting for almost one-third of all bacteria isolated. In the work of Walker et al. (1998), 72 of the 92 isolates obtained from the spermospheres of peas and dwarf French beans were found to be *Bacillus* species. Recent studies undertaken by Sadifi et al. (2001, 2002), showed the successful biocontrol of the *Bacillus* species in the inhibition of the post-harvest disease of potatoes induced by *Fusarium sambucinum* under storage conditions. In our research, more than half of the isolated endophytic bacterium strains were identified as *Bacillus* species according to their morphological characteristics (data not shown). This special microbial flora should be exploited more extensively in the biological control of phytopathogenic fungi.

The low rate of polymorphism in the rDNA transcription unit allowed the characterization of the rDNA of each species using only a few specimens and made this DNA useful for interspecific comparisons. In addition, the different coding regions of the rDNA repeats usually showed distinct evolution rates. As a result, this DNA could provide information about almost any systematic level (Hillis and Dixon, 1991). The comparison of 16S rDNA sequences was one of the most powerful tools for the classification of microorganisms (Wu et al., 2006). In our study, seven endophytic bacterium isolates from *L. esculentum* Mill., *S. tuberculatae*, and *D. dasycarpus* effectively inhibited the mycelial growth of *B. cinerea*, and one strain encoded EB-28 significantly inhibited the lesion extension of gray mold on the cucumber seedling cotyledons and detached tomato leaves. The characterization of EB-28 was performed by conventional methods using morphology, physiological tests, and 16S rDNA sequence analysis. It was identified as *B. subtilis*. Further efforts are needed to exploit *B. subtilis* EB-28 and other endophytic bacterium isolates for commercial production and application under storage and greenhouse conditions.

The *Lycopersicum esculentum* Mill., *Speranskiae tuberculatae* (Bge.) Baill, and *Dictamnus dasycarpus* Turcz. used to isolate endophytic bacteria in the present work were selected from dozens of plant species during pre-screening experiments (unpublished data). The tomato (*L. esculentum*) is an important host plant of *B. cinerea*. Endophytic bacterium strains isolated from tomatoes are considered to have the potential to be used as a biocontrol agent against tomato gray mold in field and post-harvest conditions. *S. tuberculatae* and *D. dasycarpus* are two

Chinese traditional medicinal herbs. *S. tuberculatae* is useful in treating all types of acne when used in conjunction with *Herba Menthae*, *Radix Gentiana*, and other herbs of the same family (Li et al., 2000). *Dictamnus* (*Dictamnus dasycarpus*; baixianpi) is one of the most commonly used Chinese herb for the treatment of eczema (Du et al., 2005). *S. tuberculatae* and *D. dasycarpus* have been found to have antifungal activity against some plant pathogens, such as *Rhizoctonia solani*, *Alternaria longipes*, and *Curvulavia lunata* (Du et al., 2005; Huang et al., 2007) and are regarded as potential biocontrol agents to plant diseases. In this study, four endophytic bacterium isolates from *S. tuberculatae* and two isolates from *D. dasycarpus* showed significant antagonistic effects against *B. cinerea in vitro*, while the inhibitive effects of these two plants against *B. cinerea* were still unclear. An interesting question is whether combined biological control agents made up of endophytic bacteria and plant extracts would have a stronger biocontrol effect against tomato gray mold or other plant diseases.

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