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Full Length Research Paper

Isolation and identification of endophytic bacterium W4 against tomato *Botrytis cinerea* and antagonistic activity stability

Cong-Jun Yang[1,2], Xin-Gang Zhang[1,2], Guan-Ying Shi[1,2], Hao-Yu Zhao[1,2], Long Chen[1,2], Ke Tao[1,2]* and Tai-Ping Hou[1,2]*

[1]College of Life Sciences, Sichuan University, Chengdu 610064, China. [2]Key Laboratory of Bio-resources and Eco-environment, Ministry of Education, Sichuan University, Chengdu 610064, China.

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Of the 72 endophytic bacteria isolated from healthy tomato stems and leaves from field-grown plants, the strain W4 gave strongly inhibitory effect on *Botrytis cinerea* Pers, with the inhibition rate 78% in dual culture assay and 100% using fermentation filtrate diluted 20 times. Based on morphological, physiological and biochemical properties, 16S rDNA gene sequences and Biolog system analysis, the isolate W4 was identified and named as *Brevibacillus brevis* W4. The activity determination demonstrated that the antagonistic effect of W4 fermentation filtrate on *B. cinerea* was fairly stable to temperature, pH, ultraviolet light. Only heated at 90°C for 30 min or at 100°C or above for 10 min, the inhibitory effect was significantly reduced. The inhibitory rate remained above 90% at pH 2-11 and above 95% under ultraviolet light radiation for 5-240 min. These stability characteristics of antagonistic activity were conducive to future applications in the field.

Key words: *Botrytis cinerea, Brevibacillus brevis* W4, endophytic bacteria, antagonistic activity.

INTRODUCTION

Tomato grey mould disease caused by *Botrytis cinerea* Pers are one of the most common and widely distributed disease of vegetables throughout the world (Essaid et al., 2002). Control of this disease often depends on frequent fungicide applications (Rosslenbroich and Stuebler, 2000). Unfortunately, *B. cinerea* is a classical 'high-risk' pathogen. Evolution of resistance to different classes of fungicides, such as benzimidazoles, dicarboximides, diethofencarb and sterol biosynthesis inhibitors, has been frequently reported (Chastagner et al., 1990; Elad, 1992; Dianez et al., 2002; Baroffio et al., 2003). More effective and safer biofungicides for the control of *B. cinerea* are required to prevent or delay the development of fungicide resistance or to reduce the risks of chemical pollution (Steel, 1996; Muller et al., 2009).

*Corresponding author. E-mail: houtplab@scu.edu.cn, taoke@scu.edu.cn.

Endophytic bacteria, co-evolved with plants, have been found in virtually every plant studied, where they colonize the internal tissues of their host plant and can form a range of different relationships including symbiotic, mutualistic, commensalistic and trophobiotic (Robert et al., 2008). Endophytic bacteria can not only promote plant growth and act as biocontrol agents, but also produce nature products to control plant diseases (Miller et al., 1998; Beck et al., 2003; Strobel et al., 2004; Guan et al., 2005) . Bacterial endophytes can mediate *de novo* synthesis of novel antimicrobe compounds and antifungal metabolites, which has been accepted as a potential fungicide to restrict the spread of plant diseases (Sturz et al., 2000; Wellington and Marcela, 2004). While a wide range of biologically active compounds have been isolated from endophytic organisms, they still remain a relatively untapped source of novel natural products.

So far, more than 10 species of bacteria have been researched to control tomato *B. cinerea*, mainly including *Bacillus*, *Bacillus subtilis*, *Bacillus polymyxa*, *Bacillus*

lichenformis, *Lactobacillus sp.*, *Bacillus pumilus*, *Xanthomonas maltophilia*, *Pseudomonas sp*., *Pseudomonas fluorescens* and so on (Ge et al., 2007). But these antagonistic bacteria were mostly isolated from soil, and their control effects on grey mould disease were often not obvious or were invalid in the field (Han et al., 2004). The objective of this research attempted to acquire effective endophytic bacteria against *B. cinerea*, and to provide basic data for further research and development of novel fungicide using natural antagonist.

MATERIALS AND METHODS

Isolation of endophytic bacteria from tomato leaves and stems

Ten tomato plants of two months old were randomly sampled. One leaf or 0.5 cm long stem was excised from middle of each plant. These samples (about 3 to 5 g) were washed under running tap water, surface-sterilized by dipping successively into 70% ethanol for 1 min, 100% sodium hypochlorite for 3 min, and finally rinsed three times in sterilized distilled water. After surface disinfection, the leaves or stems tissues were homogenated with two volumes of 0. 85% NaCl solution in mortar together with quartz sand, then 100 l homogenate and its four serial decimal diluents were separately spread on the surface of nutrient agar medium (NA; 3 g beef extract, 10 g peptone, 5 g NaCl, and 17 g agar in 1 liter distilled water. pH 7.0 to 7.2) (containing 100 g/ml Carbendazim) in Petri dishes. Plates were incubated in darkness at 30°C for 7 day. The colonies representing different morphologies were picked at random and purified by restreaking on the same medium plates for 2- 3 times. Pure bacteria were subcultured on NA slopes and stored at 4°C.

Screening of endophytic bacteria against *B. cinerea in vitro*

The *in vitro* activity of isolated endophytic bacteria against *B. cinerea* was determined in a dual culture assay firstly (Cottyn et al., 2000). One mycelial plug (6 mm diameter) was taken from an actively growing culture of *B. cinerea* on potato dextrose agar medium (PDA; 200 g potato, 20 g dextrose, and 17 g agar in 1 L distilled water). Each plug was placed at the center and four bacterial cultures grown at 35°C for 24 h on NA medium were symmetrically placed around the plug with distance of 2.5 cm in plate containing PDA medium. All dual culture plates were incubated at 27°C for 5-7 day and the diameter of fungal colonies was measured to score for inhibition of fungal growth.

Further screening of endophytic bacteria against *B. cinerea* was carried out by using fermentation filtrate. The strains gave strongly inhibitory action (with inhibition rates of above 60%) in dual culture assay were individually incubated in 100 ml Erlenmeyer flask containing 30 ml NA liquid medium at 35°C, 150 rpm for 1 day. Fermentation broth was centrifuged (9391 g, 10 min at 4°C) and the supernatant was filtered with a germ filter (0.22 m diameter). Then the fermentation filtrate was mixed with PDA in a ratio of 1:19 and the mixture was poured into a petri dish. A plug of mycelium of *B. cinerea* (6 mm diameter) was placed directly on the surface centre of the mixed medium and incubated at 27°C for 5 to 7 day. Inhibition effect was evaluated with the formula as follows.

$$P\ (\%) = 100 \times [(C\text{-}d) - (T\text{-}d)] / (C\text{-}d)$$

P: Inhibitory rate, C: Diameter of colony of control, T: Diameter of colony of treatment, d: Diameter of mycelial plug.

Identification of endophytic bacteria

The screened endophytic bacterium with best inhibitory effect on *B. cinerea* was used for identification. By negative staining, electron microscope and Bergey's Manual (Buchanan and Gibbons, 1984), the endophytic bacterium morphology, physiological and biochemical properties were observed. For further identification, 16S rDNA gene sequences and Biolog system analysis were applied.

Genomic DNA of endophytic bacterium was extracted by DNA extraction kit (Tiangen, China). The 16S rDNA gene sequences was amplified by primers (5 -TGTAAAACGACGGCCAGTAGAGTTTGATCCTGGCTCAG-3), (5 -CAGGAAACAGCTATGACAAGGAGGTGATCCAGCCGCA-3). Polymerase chain reaction (PCR) was carried out in a 25 l reaction volume containing 50 ng genomic DNA, 1 x PCR buffer (TaKaRa, China), 2.5 mM $MgCl_2$, 2.5 M of each primer, 50 M of each dNTP and 1.0 U TaqDNA polymerase (TaKaRa, China). PCR conditions were as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, 50°C for 45 s, 72°C for 60 s, with a final extension at 72°C for 10 min. PCR products were electrophoresed on a 1.0% agarose gel and purified with the DNA Agarose Gel Extraction kit (Omega, USA). The purified fragments were sequenced using ABI 3730 genetic analyser (Applied Biosystems). The 16S rDNA gene sequences similarity was identified using the BLAST program from the GenBank data library, and the phylogenetic tree was constructed using MEGA (Version 4.0) by Kimura2-Parameter distance model and neighbour-joining method.

Biolog system using GP2 microplate (Biolog Inc, Hayward, CA. USA) was adopted to further confirm the identification result. The isolate was prepared according to Biolog instructions. After incubation (at 37°C for 16 to 24 h), the optical density was read using a Biolog Microplate reader in conjunction with the MicroLog (Version 4.20.04). A correct identification was attained when the similarity index (SIM) and genetic distance (DIS) values were >0.500 and <5.00, respectively.

Antagonistic activity stability of fermentation filtrate

The antagonistic activity stability of the fermentation filtrate produced by the screened endophytic bacterium W4, including temperature, pH, ultraviolet (UV) light stability, all were determined according to the method described by Wu et al. (2006). The fermentation filtrate was heated at various temperatures (50 to 121°C) for 10 or 30 min, respectively. Then the activity of the treated fermentation filtrate against *B. cinerea* was determined.

The pH of fermentation filtrate was adjusted to different values (pH 2.0-12.0) by 1 M HCl or 1 M NaOH solution, and maintained at room temperature for 1 h. After that, the pH was adjusted to the initial value (pH 8.0) for antagonistic activity test. After separately irradiated for various time (5 to 240 min) under UV light with wavelength 254 nm, power of 15 W, distance of 30 cm, the antagonistic activity of fermentation filtrate was determinated.

RESULTS AND DISCUSSIONS

Isolation of endophytic bacteria from tomato stems and leaves

A total of 72 endophytic bacteria were isolated, of which 45 strains isolated from stems and 27 strains from leaves. The isolation efficiency of bacteria from stems was higher than from leaves. The key of endophyte isolation is the efficacy of surface-sterilization, epiphytic

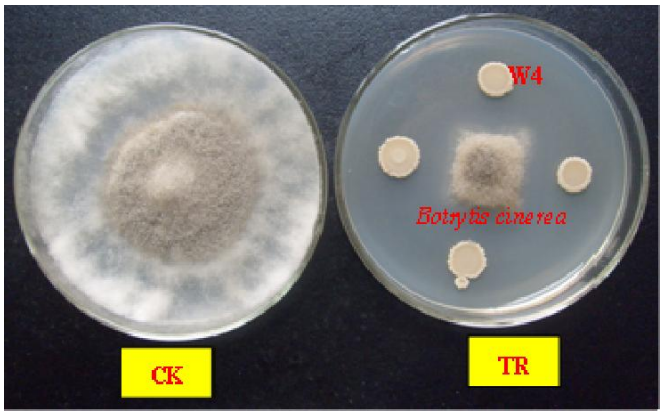

Figure 1. Antagonistic effect of the isolate W4 on *Botrytis cinerea* in dual culture assay.

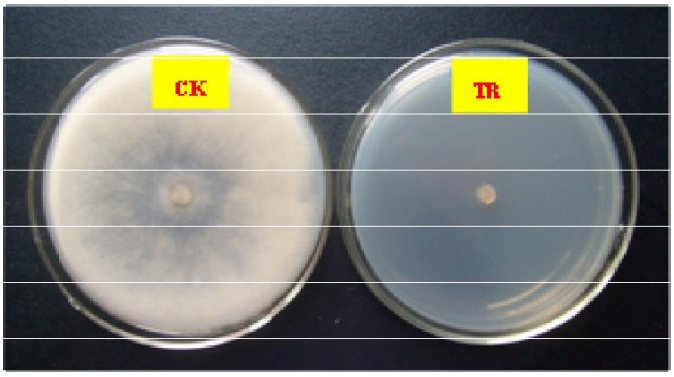

Figure 2. Antagonistic activity of W4 fermentation filtrate against *Botrytis cinerea*.CK: 1 ml sterilized distilled water was mixed with 19 ml PDA medium. TR: 1 ml fermentation filtrate of the isolate W4 was mixed with 19 mL PDA medium, the inhibitory rate was 100%.

microorganisms described by several authors must be removed or killed (Monaco et al., 1999) . Available information indicates that the routine surface disinfection techniques commonly are used before isolation of the endophytes (Larran et al., 2001). To ensure the bacteria were isolated from plant inner tissues, the sterilized distilled water which had been used to wash the sterilized tomato leaves or stems was cultured on NA plates. If there were no colonies formed within 2-3 d, it was considered disinfected thoroughly, and the isolated bacteria were proven endophytic bacteria.

Screening of antagonistic bacteria against *B. cinerea*

In dual culture assay, of 72 entophytic bacteria strains, 49 strains could inhibit *B. cinerea* in varying degrees. The inhibitory rates of 28 strains of 49 were less than 50%, 13 Strains between 50 to 60%, 8 strains more than 60%. Among them the isolate W4 isolated from tomato stem gave the strongest antagonistic effect on *B. cinerea* with the inhibition rate of 78% (Figure 1).

Further screening for antagonistic bacteria were carried out using fermentation filtrate among the 8 strains which had inhibition rates of above 60%. The experimental results showed that the strain W4 had the best activity against *B. cinerea*, with the inhibition rate of 100% (Figure 2).

Our experimental results showed that the antagonistic activity determination using fermentation filtrate was more sensitive than in dual culture assay. The possible reason was that antagonistic substances produced by endophytic bacteria could be exposed to the tested *B. cinerea* more fully. However, the activity of endophytic bacteria against pathogen *in vitro* and their control effect on the disease caused by the same pathogen are not necessarily positive correlation (David, 1998). Further screening of antifungi is necessary *in viv*o.

Identification of antagonistic isolate W4

The isolate W4 was short rod with 2 to 3 end flagella, colony smooth, pale yellow, opaque, thicker, non-pigment, aerobic growth, gram-positive. Catalase test and gelatin hydrolysis were both positive, but starch hydrolysis and V.P-reaction were both negative. Through the phylogenetic tree constructed by 16S rDNA gene sequences, the antagonistic isolate W4 (1543 bp) blonged to *Brevibacillus* (Figure 3). Based on the carbon substrate oxidation patterns in Biolog system analysis (Figure 4), the isolate W4 was identified as *Brevibacillus brevis* with the PROB, SIM, and DIS values of 99%, 1.000 and 0.00, respectively.

According to the morphology, cultural characteristics, physiological and biochemical properties, 16S rDNA gene sequences phylogenetic tree and Biolog system analysis, the endophytic bacterium strain W4 was identified and named as *B. brevis* W4. Although, the 16S rDNA gene sequences analysis has became the golden index for bacteria identification (Ma et al., 2008; Ojo et al., 2008), and the Biolog system analysis also has been recognized by U.S. authorities, including the FAD, NASA, TIGR, NIAID and so on (William, 1991), as a means of identification, 16S rDNA gene sequences and Biolog system have their own advantages and shortage. Several authors have recommended that Biolog system analysis and 16S rDNA gene sequences should be simultaneously used to achieve a correct taxonomy of some strains (Paolis and Lippi, 2008).

Antagonistic activity stability of fermentation filtrate from isolate W4

The antagonistic activity of W4 fermentation filtrate had stronger thermal stability (Figure 5). Compared with the

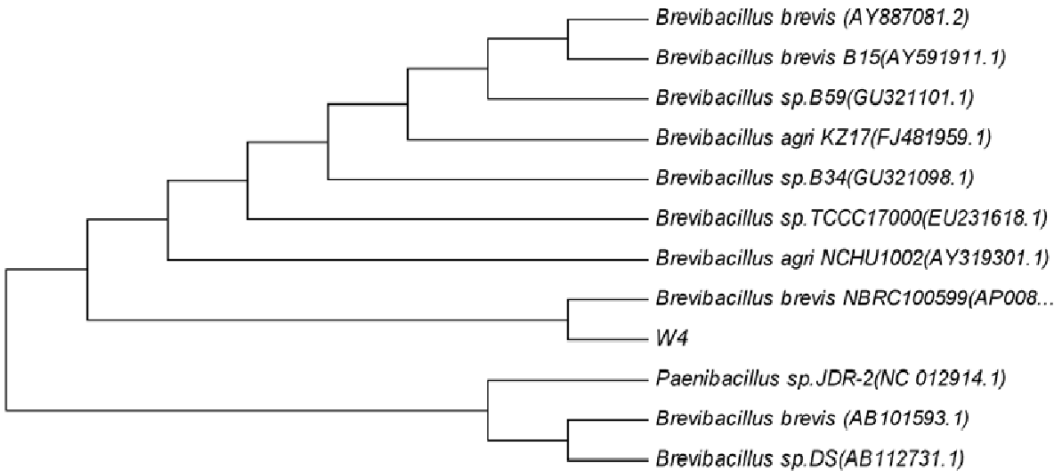

Figure 3. Phylogenetic relationships of the 16S rDNA gene from the isolate W4 against *Botrytis cinerea* and near neighbor sequences. In the phylogenetic tree, the antagonistic endophytic bacterium W4 isolated from tomato stems were clusterd with *Brevibacillus*.

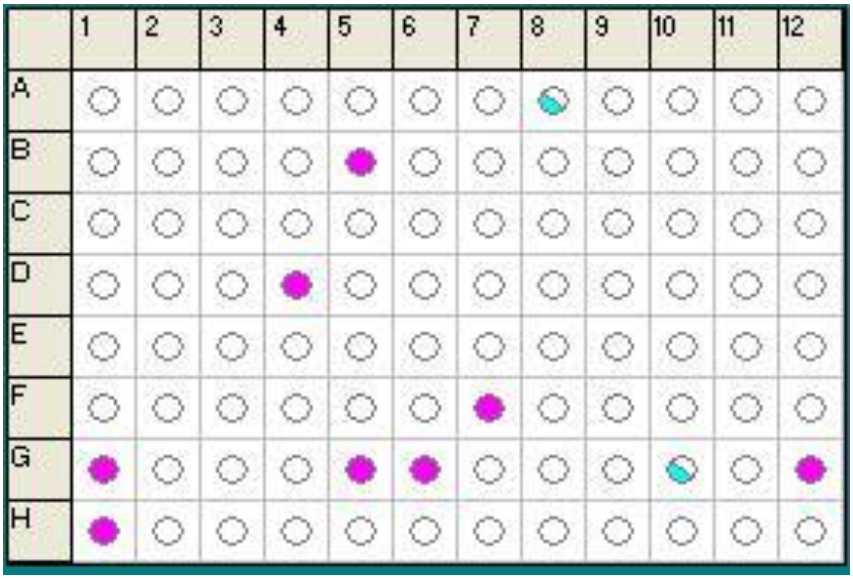

Figure 4. Metabolic fingerprint of the endophytic bacteria W4 in Biolog GP2 Microplate. According to the metabolic fingerprinting, the strain W4 was identified as *Brevibacillus brevis* after 16-24 h incubation with probability (PROB), similarity index (SIM) and genetic distance (DIS) of 99%, 1.000 and 0.00, respectively. available carbon not available carbon partly available carbon.

control, the antagonistic effect on *B. cinerea* had no change at 70°C or below for 10 to 30 min. Only heated at 90°C for 30 min or at 100°C or above for 10 min, the inhibitory activity was significantly reduced. The results also indicated that the antagonistic activity of W4 fermentation filtrate was stable to acid and alkali (Figure 6). The inhibitory rate remained above 90% at pH 2-11, and only at pH 12, the inhibition rate decreased to 81.7%.

The antagonistic activity was very stable to UV light (Figure 7), and the inhibition rates sustained above 95% under UV light radiation for 5 to 240 min. Overall, the antagonistic activity of the fermentation filtrate from the isolate W4 against *B. cinerea* was fairly stable to temperature, pH, UV light. These characteristics of antagonistic activity were conducive to future application in the field.

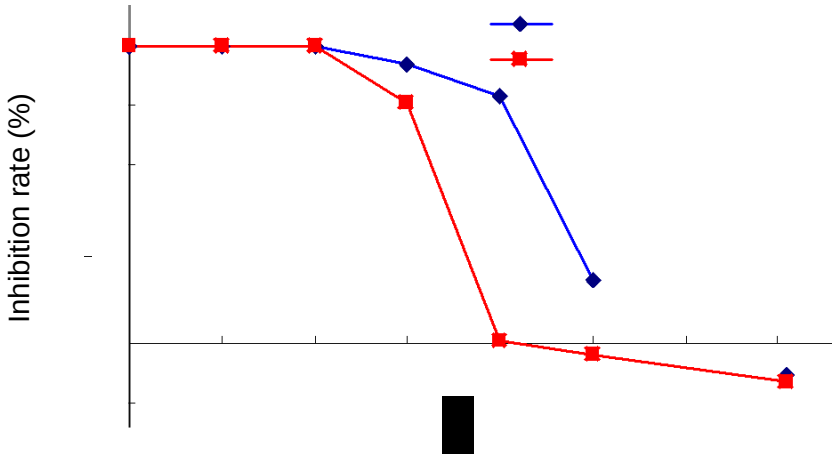

Figure 5. Thermal stability of W4 fermentation filtrate against *B. cinerea*.

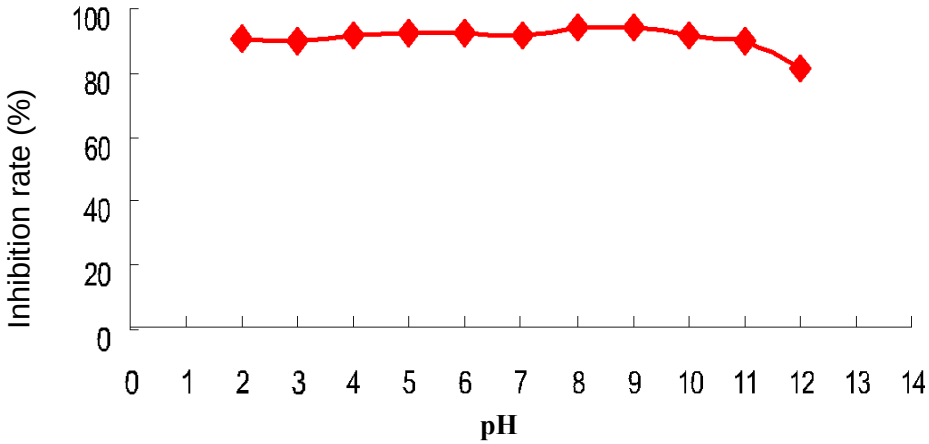

Figure 6. Acid and alkali stability of W4 fermentation filtrate against *Botrytis cinerea.*

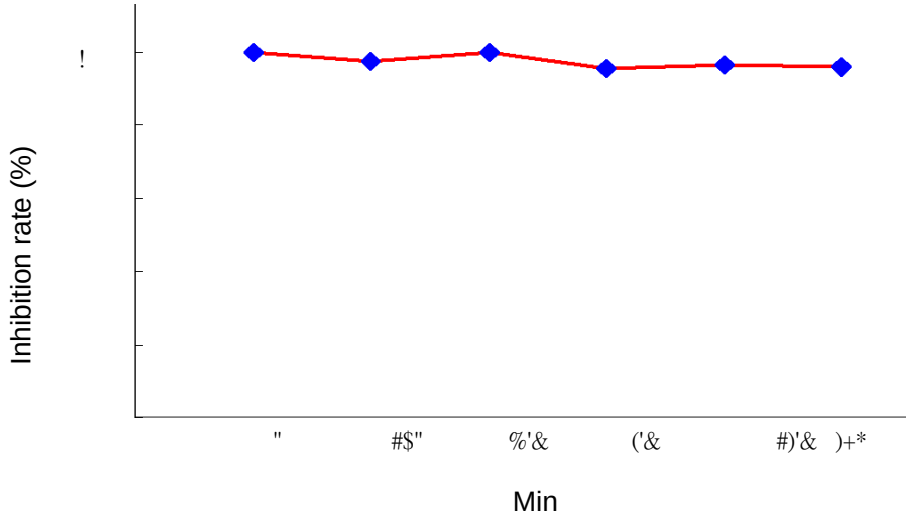

Figure 7. Ultraviolet light radiation stability of W4 fermentation filtrate against *B. cinerea*.

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