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ORIGINAL PAPER

Control of postharvest soft rot caused by *Erwinia carotovora* of vegetables by a strain of *Bacillus amyloliquefaciens* and its potential modes of action

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Abstract *Erwinia carotovora* subsp. *carotovora* (*Ecc*), the causal agent of bacterial soft rot, is one of the destructive pathogens of postharvest vegetables. In this study, a bacterial isolate (BGP20) from the vegetable farm soil showed strong antagonistic activity against *Ecc* in vitro, and its twofold cell-free culture filtrate showed excellent biocontrol effect in controlling the postharvest bacterial soft rot of potatoes at 25 °C. The anti-*Ecc* metabolites produced by the isolate BGP20 had a high resistance to high temperature, UV-light and protease K. Based on the colonial morphology, cellular morphology, sporulation, and partial nucleotide sequences of 16S rRNA and *gyrB* gene, the isolate BGP20 was identified as *Bacillus amyloliquefaciens* subsp. *plantarum*. Further in vivo assays showed that the BGP20 cell culture was more effective in controlling the postharvest bacterial soft rot of green peppers and Chinese cabbages than its twofold cell-free culture filtrate. In contrast, the biocontrol effect and safety of the BGP20 cell culture were very poor on potatoes. In the wounds of potatoes treated with both the antagonist BGP20 and the pathogen *Ecc*, the viable count of *Ecc* was 31,746 times that of BGP20 at 48 h of incubation at 25 °C. But in the wounds of green peppers, the viable count of BGP20 increased 182.3 times within 48 h, and that of *Ecc* increased only 51.3 %. In addition, the treatment with both BGP20 and *Ecc* induced higher activity of phenylalanine ammonia-lyase (PAL) than others in potatoes. But the same treatment did not induce an increase of PAL activity in green peppers. In conclusion, the present study demonstrated that the isolate BGP20 is a promising candidate in biological control of postharvest bacterial soft rot of vegetables, but its main mode of action is different among various vegetables.

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Introduction

Vegetables are highly perishable products, especially during the postharvest including storage, transport, retail and at consumer sites (Sharma et al. 2009). Phytopathogens are major causes of postharvest decays of vegetables (Liao 2009; Sharma et al. 2009). Bacterial soft rot, caused by *Erwinia carotovora* subsp. *carotovora* (*Ecc*), is one of the destructive diseases of postharvest vegetables worldwide, especially potatoes (*Solanum tuberosum*), green peppers (*Capsicum annuum*) and Chinese cabbages (*Brassica campestris* subsp. *pekinensis*) (Kikumoto 2000; Pérombelon 2002; Liao 2009; Bhat et al. 2010). Potatoes, green peppers and Chinese cabbages are important component parts in Chinese diet (Du et al. 2007; Fan et al. 2008; Jansky et al. 2009). Currently, the control of postharvest bacterial soft rot depends mainly upon the use of bactericides, such as hypochlorite, formaldehyde solution, and antibiotics (Bhat et al. 2010). Increasing public concern for food safety and environment has resulted in the cancellation of many chemical bactericides and antibiotics in developed and some developing countries. Therefore, many researches have been focused on the development of alternative methods of controlling postharvest diseases. Biological control has been proposed as one of the most promising alternatives for reducing the decay loss of

postharvest vegetables and fruits (Droby et al. 2009; Sharma et al. 2009). Among various biological approaches, microbial antagonists are quite promising for controlling postharvest diseases of vegetables and fruits, especially *Bacillus* species and yeasts (Sharma et al. 2009; Abraham et al. 2010; Osman et al. 2011).

Bacillus spp. is considered as potential biocontrol agents due to their broad-spectrum antagonistic activity against fungi and bacteria, high spore production ability, and resistance to adverse environments (Romero et al. 2007; Ongena and Jacques 2008). Some strains of *Bacillus* spp. have been successfully applied to controlling postharvest pathogens on many vegetables and fruits, such as *Ecc* on potatoes and bell peppers, *Botrytis cinerea* and *Monilinia fructicola* on peaches, *Colletotrichum gloeosporioides* on papayas (Dong et al. 2004; Liao 2009; Arrebola et al. 2010; Osman et al. 2011; Casals et al. 2012). But, a series of questions needed to be resolved before these antagonists were commercialized, especially understanding their mechanisms of action to pathogens.

Accumulated evidences showed that bacterial antagonists exerted their biocontrol ability against pathogens through several modes of action. The main mode of action of *Bacilus* spp. as biocontrol agents is believed to inhibit the growth of pathogens by synthesizing antimicrobial peptides (Finking and Marahiel 2004; Ongena and Jacques 2008). The other important mode of action is believed to be competition for nutrient and space (Demoz and Korsten 2006; Sharma et al. 2009; Osman et al. 2011). Induced systemic resistance (ISR) in plants by *Bacilus* spp. is also an important antagonistic mechanism, which could increase activity of pathogenesis related (PR) proteins, such as phenylalanine ammonia-lyase (PAL) (Ongena et al. 2005; Ongena and Jacques 2008). Additional modes of action, such as the production of lytic enzymes and the interference of quorum-sensing signal, were also proposed (Dong et al. 2004; Sharma et al. 2009). In fact, biological control is a kind of complicated interaction among host, pathogen and biocontrol agent (Droby et al. 2009).

The objectives of this study were: (a) to identify effective bacterial antagonists against *Ecc*; (b) to evaluate its efficacy in controlling postharvest bacterial soft rot of potatoes, green peppers and Chinese cabbages, respectively; (c) to investigate its possible modes of action.

Materials and methods

Isolation of antagonistic bacteria

Potential antagonistic bacteria were isolated from the vegetable farm soil where green peppers and Chinese cabbages were grown for many years. The sample was collected randomly from the topsoil (0–10 cm), and mixed thoroughly. A portion of 10 g was suspended in 100 mL of sterile water, and incubated in a shaker at 200 rotations per minute (rpm) for 4 h. Serial dilutions of 100 μL were plated on 2YT (tryptone at 17 g/L, NaCI at 5 g/L, Yeast extract at 10 g/L, pH 7.0) agar plates, respectively. Then the plates were incubated for 36 h at 28 °C. Individual colonies were isolated from each plate. Their pure cultures were performed by sub-culturing from single colony on plates. Subsequently, the isolates were stored at −80 °C in 25 % sterile glycerol for further studies.

Preparation of antagonistic inoculums

Isolates were cultivated in 2YT medium without agar for 36 h at 200 rpm at 28 °C, respectively. The cell-free supernatant was concentrated twofold according to the following method. The cell culture was centrifuged at 15,000×*g* for 30 min. The supernatant was filtered by a 0.22 μm vacuum filter, and then concentrated by lyophilization. The twofold cell-free culture filtrate was stored at −20 °C for further studies.

Preparation of the pathogen *Ecc*

The pathogen *Ecc* with rifampicin resistance (Rif^r) was donated by Dr. Jiaqin Fan (Nanjing Agricultural University, China). *Ecc* was cultured in 2YT medium without agar for 12 h at 200 rpm at 28 °C.

Potatoes, green peppers and Chinese cabbages

In this study, fresh potatoes, green peppers and Chinese cabbages were bought from a super vegetable wholesale market in Nanjing, Jiangsu Province, China. Healthy vegetables of uniform size and colour, and without mechanical damage were selected for this study.

In vitro screening of antagonists

All isolates were preliminarily screened by in vitro antagonist–pathogen interaction on 2YT agar plates. 2 mL of *Ecc* culture (OD_{600} = 1.0) was added to 100 mL of liquid 2YT agar (<40 °C), and then the homogeneous mixture was promptly spread on plates. Holes (4 mm in diameter) were generated on these agar plates by a sterile puncher. The 100 μL culture of each isolate was added into a hole, respectively. The plates were incubated for 24 h at 28 °C. Their antagonistic activities were evaluated by the diameters of clear inhibition zones, and the hole inoculated with sterile water was used as blank control. The same experiment was repeated three times (Hu et al. 2010).

In vivo screening of antagonists

The surface of potatoes was disinfected with 70 % alcohol for 1 min, rinsed with sterile water, and air-dried at room temperature. Then an artificial wound (1.5 cm in depth and 2.5 mm in diameter) was made along the equatorial zone of each potato by a sterile scalpel (Zhang et al. 2011). Each wound was filled with either 100 μL of the antagonist cell culture (10^8 CFU/mL) or its twofold cell-free culture filtrate. Sterile water was used as blank control. In addition, 100 μL aliquot of chloramphenicol (300 μg/mL) was used to treat artificial wounds as an antibiotics control. After 2 h, 20 μL of *Ecc* culture (10^8 CFU/mL) was inoculated into each wound. Each treatment included ten potatoes. The wound-inoculated potatoes were stored in plastic crispers at 25 °C. After 72 h, rotten lesion of each wound was excised from the potato, and weighed. The same experiment was repeated three times.

Identification of the antagonist BGP20

First, taxonomic identification of the isolate BGP20 was carried out according to colonial morphology, cellular morphology and sporulation. After BGP20 was cultured for 36 h on 2YT agar plates at 28 °C, the colonial morphology was observed. The cellular morphology of BGP20 was viewed under the light microscope (YS100, NIKON Inc., Japan) by crystal violet staining method, and its sporulation was viewed by Schaeffer–Fulton staining method (Zhao and He 2002).

Then molecular identification was performed based on the partial nucleotide sequences of 16S rRAN and *gyrB* gene. Genomic DNA was extracted by DNA extraction kit (Tiangen Inc., Beijing, China). The partial DNA fragment of 16S rRNA gene was amplified by PCR using a forward primer fd2 (5′-AGAGTTTGATCATGGCTCAG-3′) and a reverse primer rp1 (5′-ACGGTTACCTTGTTACGACTT-3′) (Weisburg et al. 1991). Then PCR products were directly sequenced (Invitrogen Inc., Shanghai, China). The partial DNA fragment of *gyrB* gene was amplified by using a forward primer UP-1 (5′-GAAGTCATCATGACCGTTCTGCAYGCNGGNGG NAARTTYGA-3′) and a reverse primer UP-2r (5′-AG CAGGGTACGGATGTGCGAGCCRTCNACRTCNGCR TCNGTCAT-3′) (Yamamoto and Harayama 1995). Then PCR products were directly sequenced by using a pair primers UP-1S (5′-GAAGTCATCATGACCGTTCTGCA-3′) and UP-2Sr (5′-AGCAGGGTACGGATGTGCGA GCC-3′) (Yamamoto and Harayama 1995).

Multiple sequence alignments were performed based on sequencing partial nucleotide sequences of 16S rRNA and *gyrB* gene by using ClustalX 1.83 software respectively (Thompson et al. 1997). Two phylogenetic trees were constructed by the neighbour-joining method of MEGA 5.05 software (Tamura et al. 2011). *Pseudomonas fluorescens* SBW25 (NC_012660.1) was used as an outgroup.

Biocontrol effects and safety of the antagonist BGP20 on potatoes, green peppers and Chinese cabbages

In this experiment, the surface of vegetables was disinfected with 70 % alcohol for 1 min, rinsed with sterile water, and air-dried at room temperature. This experiment included five treatments: artificial wounds of vegetables were inoculated with only sterile water as blank control, the pathogen *Ecc* or the antagonist BGP20, respectively; artificial wounds of vegetables were pretreated with either BGP20 or its twofold cell-free culture filtrate, and then inoculated with *Ecc* after 2 h.

The experiment on potatoes was performed as described previously in "In vivo screening of antagonists" section.

An artificial wound (4.0 cm in length and 1 mm in depth) was generated in the surface of each green pepper by a sterile scalpel. Inoculations were performed as described previously in "In vivo screening of antagonists" section. The wound-inoculated green peppers were stored in plastic crispers at 25 °C. After 48 h, lesion areas were investigated.

Fleshy leaves of Chinese cabbage were cut into 10 cm lengths. An artificial wound (2.5 cm in length and 1 mm in depth) was generated in the surface of each segment by a sterile scalpel. Inoculations were performed as described previously in "In vivo screening of antagonists" section. The wound-inoculated leaves were stored in plastic crispers at 25 °C. After 30 h, lesion areas were investigated.

Stability analysis of anti-*Ecc* metabolites

To determine effects of high temperature, protease K and UV-light on the stability of the anti-*Ecc* metabolites produced by the antagonist BGP20, aliquots of the twofold cell-free culture filtrate of BGP20 were treated as the following methods (Martin-Visscher et al. 2008). Heat stability was assessed by incubating aliquots of the sample for 1 h at 80 °C and for 20 min at 121 °C, respectively. Aliquots of the sample were treated with protease K (50 μg/mL, pH 7.5) for 6 h at 30 °C. Aliquots of the sample were irradiated with UV-light (254 nm, 100 μw/cm^2) for 2 h. Antagonistic activities of the treated samples against *Ecc* were assessed on 2YT agar plates as described previously in "In vivo screening of antagonists" section. Sterile water was used as blank control. The same experiment was repeated three times.

Population dynamics of the antagonist BGP20 and the pathogen *Ecc* in wounds of potatoes and green peppers

Potatoes and green peppers were treated as described previously in "In vivo screening of antagonists" and "Biocontrol effects and safety of the antagonist BGP20 on potatoes, green peppers and Chinese cabbages" section, respectively. The population dynamics of the antagonist BGP20 and the pathogen *Ecc* were investigated after 0, 6, 24, 48, 72 and 96 h in the wounds of potatoes, and after 0, 6, 24 and 48 h in the wounds of green peppers, respectively. The entire wound was excised from the potatoes and the green peppers by a sterile scalpel, respectively (Nally et al. 2012). The sample was homogenized in a sterile porcelain mortar with 2 mL of sterile water. Diluted homogenates were plated on 2YT agar plates for BGP20 or on 2YT agar plates supplemented with 100 μg/mL Rif for *Ecc*. The number of bacterial colonies on each plate was counted after incubating for 24 h at 28 °C, respectively (Zhao et al. 2012). Population densities of BGP20 and *Ecc* were expressed as $\log_{10}$ CFU per wound (Nally et al. 2012). Five potatoes or green peppers were randomly sampled at each sampling time. Each diluted homogenate was plated on three plates, respectively. The same experiment was repeated three times.

Induction of PAL and dynamic analysis

Potatoes and green peppers were treated as described previously in "In vivo screening of antagonists" and "Biocontrol effects and safety of the antagonist BGP20 on potatoes, green peppers and Chinese cabbages" section, respectively. Fresh samples were excised from the connecting part of the lesion and the healthy tissue after 0, 24, 48, 72 and 96 h in the treated potatoes, and after 0, 6, 24 and 48 h in the treated green peppers, respectively. The samples were cut into pieces (5 × 5 mm), frozen quickly by liquid nitrogen, and then stored at −80 °C for further studies.

A sample of 5 g was homogenized in 10 mL of ice-cold sodium borate buffer (50 mmol/L, pH 8.8) with 0.5 g polyvinylpyrrolidone (PVP) and 5 mmol/L β-mercaptoethanol, and then centrifuged for 40 min at 16,000×*g* at 4 °C. The supernatant was used directly for the enzyme assay. PAL activity was determined as described previously with some modifications (Assis et al. 2001). In brief, the reaction mixture was composed of 1 mL of the supernatant, 2 mL of sodium borate buffer (50 mmol/L, pH 8.8) and 1 mL of L-phenylalanine (20 mmol/L), and incubated for 90 min at 30 °C. Then the enzymatic reaction was ceased by adding 1 mL HCI (1 mol/L). PAL activity was determined based on the production of cinnamate, and measured by the absorbance change at 290 nm. Isopyknic sterile water replaced the supernatant as blank control. PAL enzyme activity was defined as nmol cinnamic acid per h per mg of protein. Every treatment included three repetitions. The same experiment was repeated twice.

Statistical analysis

Results were means of three independent experiments ± standard deviation (SD). Tukey's test was used to identify statistically significant differences ($P < 0.05$). All statistical analyses were performed by using SPSS 13.0 software (SPP Inc., Chicago, USA).

Results

Isolation and in vitro screening of antagonists

Among 267 isolates from the vegetable farm soil, only three isolates (BGP14, BGP20and BCL9) generated clear and obvious antagonistic zones (diameter >10 mm) on 2YT agar plates with *Ecc*. The diameters of their antagonistic zones were 11.5 ± 0.33 mm (BGP14), 12.7 ± 0.22 mm (BGP20) and 12.2 ± 0.56 mm (BCL9), respectively. There was not significant difference among BGP14, BGP20 and BCL9 ($P < 0.05$).

Further in vivo screening of antagonists on potatoes

The treatments of three antagonists (BGP14, BGP20 and BCL9) and their twofold cell-free culture filtrates significantly reduced the decay caused by *Ecc* on potatoes compared to the treatment of *Ecc* control ($P < 0.05$) (Fig. 1). Furthermore, the biocontrol effect of the twofold cell-free culture filtrate significantly excelled that of the corresponding antagonist cell culture ($P < 0.05$). After 72 h at 25 °C, the best biocontrol effect was achieved with the twofold cell-free culture filtrate of BGP20. The rotten biomass of this treatment was only 0.90 ± 0.45 g per wound, and there was not significant difference between it and the treatment of 300 μg/mL chloramphenicol (0.79 ± 0.42 g/wound) (Fig. 1).

Identification of the antagonist BGP20

The colonial morphology of the antagonist BGP20 was cream colour, observably convex, rough, and circular with undulate margins (Fig. 2a). It was sporiparous bacilli (Fig. 2b, c). Based on these results, BGP20 was preliminarily identified as *Bacillus* species. To clarify the classification of BGP20, the molecular identification was carried

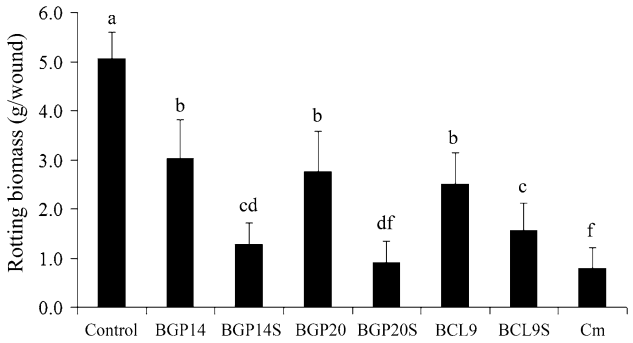

Fig. 1 *In vivo* screening of potential antagonists on potatoes. Control: sterile water; BGP14, BGP20 and BCL9: potential antagonists; BGP14S, BGP20S and BCL9S: the twofold cell-free culture filtrates of BGP14, BGP20 and BCL9, respectively; Cm: 300 μg/mL chloramphenicol. Values in each band with *different letters* mean significant difference at $P < 0.05$ level by Tukey's test. *Vertical bars* represent standard errors of the means

out based on the partial nucleotide sequences of 16S rRNA and *gyrB* gene.

The expected sequences of 16S rRNA (1,431 bp) and *gyrB* gene (1,181 bp) were successfully amplified from BGP20. These sequences were deposited at GenBank database under accession numbers JQ734535 (16S rRNA) and JQ734538 (*gyrB*). Based on the sequences, two phylogenetic trees were constructed by the neighbour-joining method (Fig. 3), in which BGP20 was clustered with the isolates of *Bacillus amyloliquefaciens*. BLASTn analysis showed that the 16S rRNA and *gyrB* gene sequences of BGP20 shared 100 % identity with those of *Bacillus amyloliquefaciens* subsp. *plantarum* CAU B946 from the rice rhizosphere, respectively. Based on these results, BGP20 was classified as *B. amyloliquefaciens* subsp. *plantarum*.

Biocontrol effects and safety of the antagonist BGP20 on potatoes, green peppers and Chinese cabbages

The cell culture of the antagonist BGP20 not only showed poor biocontrol effect in controlling the bacterial soft rot of potatoes, but also caused the decay of potatoes (Fig. 4a, d). But its twofold cell-free culture filtrate could provide excellent protection on potatoes (Fig. 4a, d). Both the cell culture of BGP20 and its twofold cell-free culture filtrate could effectually control the bacterial soft rot on green peppers and Chinese cabbages, especially its cell culture (Fig. 4b, c, e and f). Moreover, BGP20 did not cause obvious decay in the wounds of green peppers and Chinese cabbages (Fig. 4b, c, e and f). These results showed that the biocontrol effect of BGP20 against *Ecc* was different on various vegetables, and it might cause harm to some vegetables.

Stability analysis of antagonistic substances

When the cell-free culture filtrate of the antagonist BGP20 was heated for 1 h at 80 °C, its antagonistic activity was not impaired compared to the untreated sample (Fig. 5). However, when it was treated for 20 min at 121 °C, the diameter of the antagonistic zone was significantly decreased from 17.9 ± 0.6 mm to 15.4 ± 1.3 mm ($P < 0.05$) (Fig. 5). In addition, the antagonistic activity of the cell-free culture filtrate was not impaired when it was treated with protease K and UV-light respectively (Fig. 5).

Population dynamics of the antagonist BGP20 and the pathogen *Ecc*

This experiment showed that both the antagonist BGP20 and the pathogen *Ecc* could colonize the wounds of potatoes and green peppers when they were individually inoculated, and rapidly proliferated, especially the pathogen *Ecc* (Fig. 6). In the wounds of potatoes treated with both BGP20 and *Ecc*, BGP20 showed poor competitive position compared to *Ecc*, and the viable count of *Ecc* was 31,746 times that of BGP20 at 48 h of incubation (Fig. 6a). In contrast, during interacting between BGP20 and *Ecc* in the wounds of green peppers, the viable count of BGP20

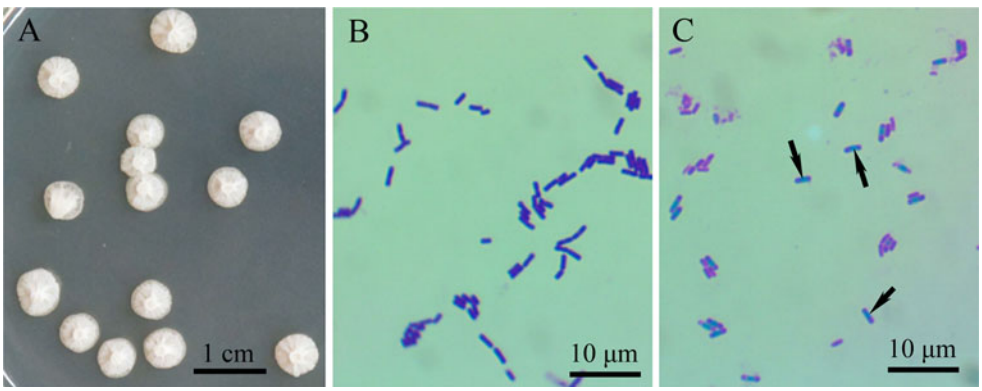

Fig. 2 The colonial morphology (**a**), cellular morphology (**b**) and sporulation (**c**) of the antagonist BGP20. *Arrows* point out spores of BGP20

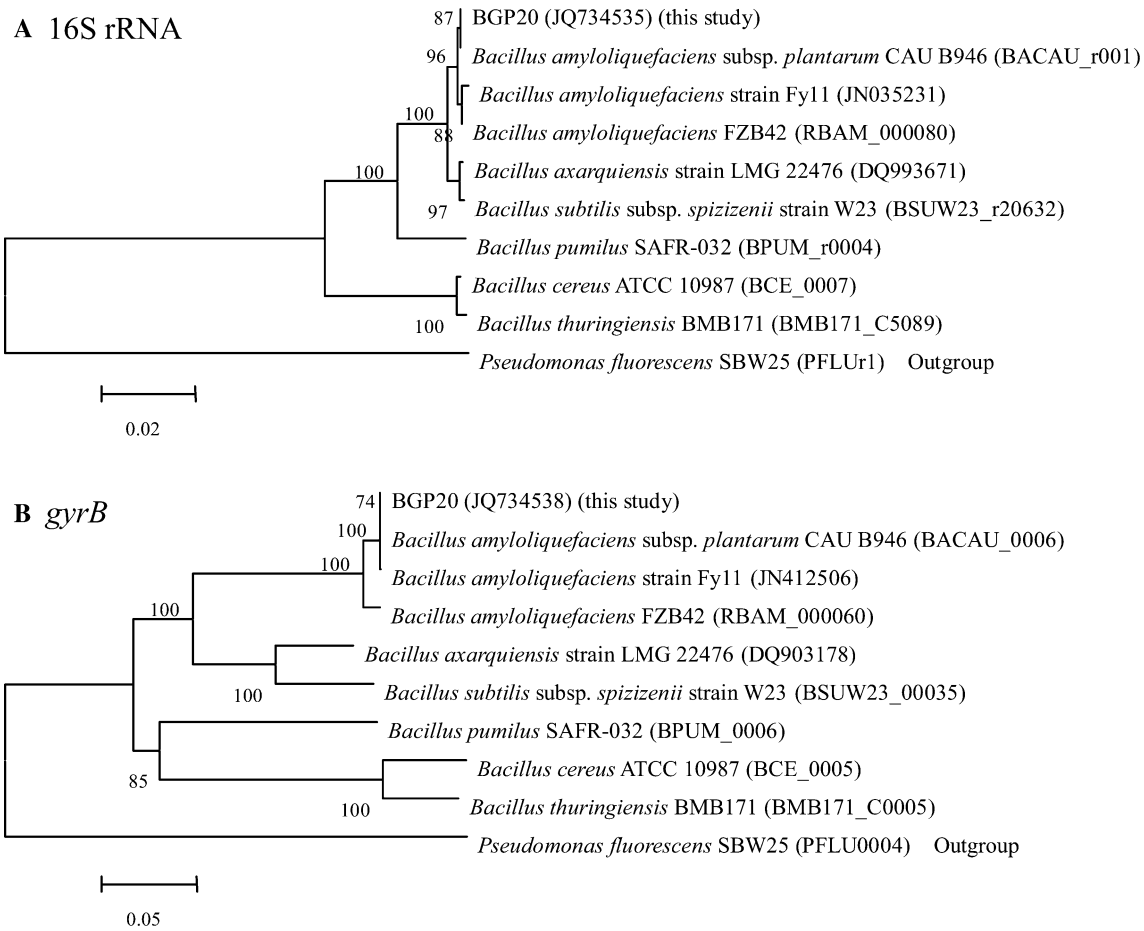

Fig. 3 Two phylogenetic trees of the antagonist BGP20 were constructed by the neighbour-joining method based on the partial nucleotide sequences of 16S rRNA and *gyrB* gene from bacteria related to *Bacillus* species. Numbers at the branches denote the bootstrap percentages for 1,000 replicates. JQ734535 and JQ734538 in *parentheses* indicate the accession numbers deposited in the GenBank database in this study, and the accession numbers for reference sequences are shown in *parenthesis*. The sequences of 16S rRNA and *gyrB* gene from *Pseudomonas fluorescens* SBW25 were used as outgroups. The *scales* indicate the evolutionary distance of the nucleotide substitutions per site

increased 182.3 times within 48 h, and that of *Ecc* increased only 51.3 % (Fig. 6b). In addition, the twofold cell-free culture filtrate of BGP20 could sharply reduce the population of *Ecc* within 6 h after potatoes and green peppers were inoculated with *Ecc*, but subsequently the population of *Ecc* rapidly increased, especially on potatoes (Fig. 6).

PAL activity

As shown in Fig. 7a, when potatoes were treated with sterile water as blank control, the antagonist BGP20 or the pathogen *Ecc* respectively, their PAL activities changed in a small scale. However, in the potatoes treated with both BGP20 and *Ecc*, the PAL activity significantly increased compared to the control ($P < 0.05$), and its level was 4.9 times that of the control at 48 h of incubation. Then the PAL activity declined sharply to 10.3 % of the maximum value at 72 h of incubation. In addition, in the potatoes treated with both the twofold cell-free culture filtrate of BGP20 and *Ecc*, the PAL activity increased 74.4 % compared to that of the control at 24 h of incubation, and then declined rapidly.

In the green peppers treated with only BGP20, the PAL activity increased 117.5 % within 6 h of incubation, and then declined sharply (Fig. 7b). The PAL activities of other treatments declined slowly within 24 h of incubation, and then increased slowly (Fig. 7b). In addition, our study found that peroxidase (POD) and polyphenol oxidase (PPO) activities in postharvest potatoes and green peppers had not obvious relationships with the biocontrol effects and the population dynamics of BGP20 (data not shown).

Discussion

In this study, a promising antagonist strain BGP20, isolated from the vegetable farm soil, was identified as *Bacillus*

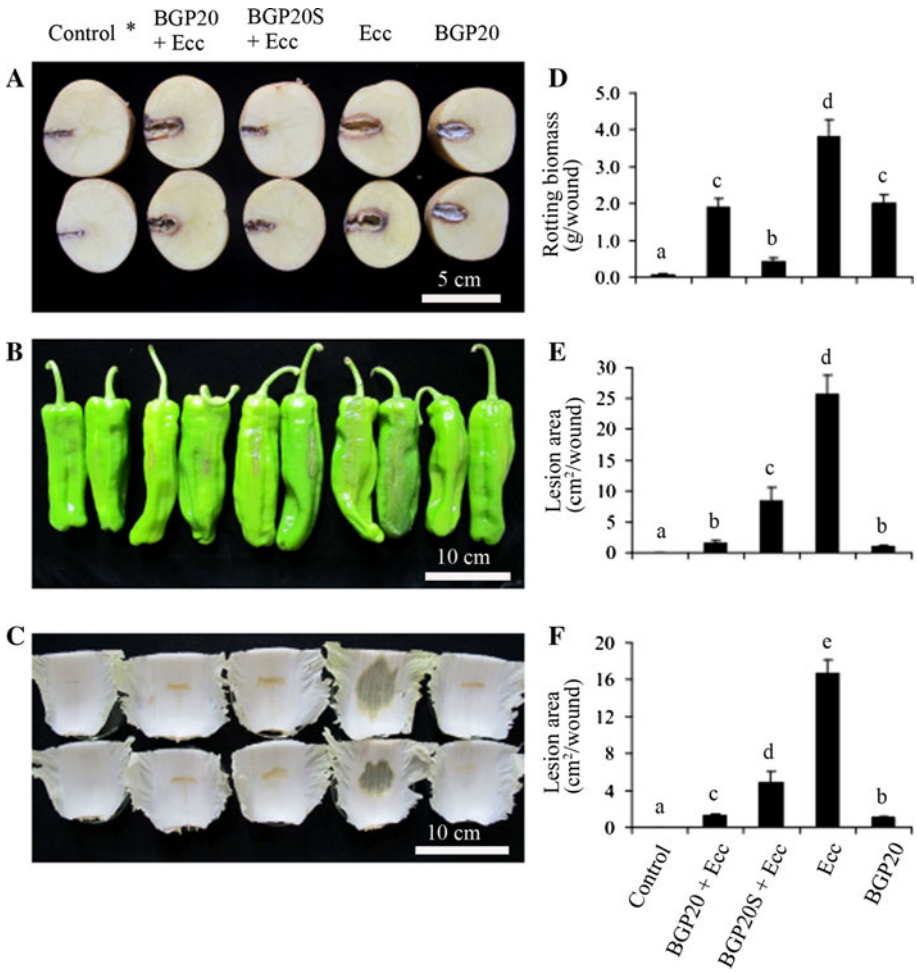

Fig. 4 Biocontrol of bacterial soft rot caused by *Erwinia carotovora* subsp. *carotovora* by use of the antagonist BGP20. **a** and **d**, potatoes; **b** and **e**, green peppers; **c** and **f**, Chinese cabbages. Control: sterile water; BGP20 + *Ecc*: the cell culture of BGP20 was applied preventatively 2 h before inoculation with the pathogen *Ecc*; BGP20S + *Ecc*: the twofold cell-free culture filtrate of BGP20 was applied preventatively 2 h before inoculation with *Ecc*; *Ecc*: inoculation with only *Ecc*; BGP20: inoculation with only the cell culture of BGP20. Values in each band with *different letters* mean significant difference at $P < 0.05$ level by Tukey's test. *Vertical bars* represent standard errors of the means. *Five treatments were always arrayed in the same order in six secondary figures

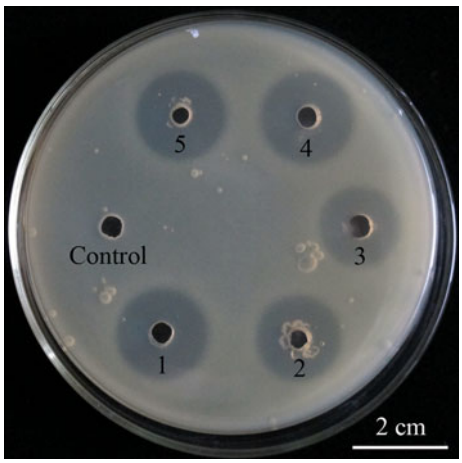

Fig. 5 Stability analysis of the antagonistic substances produced by the antagonist BGP20. Control: sterile water; *1* the twofold cell-free culture filtrate; *2* the twofold cell-free culture filtrate heated for 1 h at 80 °C; *3* the twofold cell-free culture filtrate heated for 20 min at 121 °C; *4* the twofold cell-free culture filtrate treated with protease K; *5* the twofold cell-free culture filtrate irradiated with UV-light

amyloliquefaciens subsp. *plantarum* based on its morphology, sporulation, and partial nucleotide sequences of 16S rRNA and *gyrB* gene. Its biocontrol effect and potential modes of action against the pathogen *Ecc* were investigated on postharvest vegetables, which was the first step in developing it as a biocontrol agent (Droby et al. 2009).

In vitro antagonism assays indicated that three isolates (BGP14, BGP20 and BCL9) showed strong antagonistic activity against *Ecc* in this study. However, their outstanding antagonistic ability in culture medium did not necessarily imply that they could effectively control bacterial soft rot caused by *Ecc* on postharvest vegetables (Nally et al. 2012). Several reports had demonstrated that only one or few strains could effectively control the disease among lots of bacterial isolates which showed obvious inhibition zones against the corresponding pathogen (De Costa et al. 2008; Hu et al. 2010). In this study, in vivo screening on potatoes demonstrated that only the isolate BGP20 showed excellent biocontrol effect against *Ecc*.

This study indicated that the biocontrol effect of the antagonist BGP20 cell culture was very poor in controlling bacteria soft rot on potatoes, but it could exert perfect biocontrol effects on green peppers and Chinese cabbages. Abraham et al. (2010) also found that the biocontrol effect of the antagonist *Bacillus* sp. strain B3 in controlling green

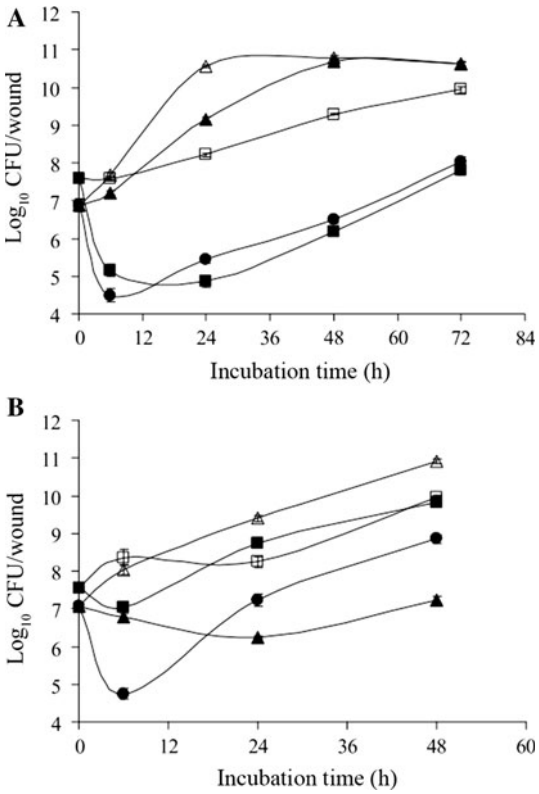

Fig. 6 Population dynamics of the antagonist BGP20 and the pathogen *Erwinia carotovora* subsp. *carotovora* in the wounds of potatoes (**a**) and green peppers (**b**). (*filled square*) Population dynamics of BGP20 in the wounds of potatoes and green peppers treated preventatively by BGP20 2 h before inoculation with *Ecc*; (*filled triangle*) Population dynamics of *Ecc* in the wounds of potatoes and green peppers treated preventatively by BGP20 2 h before inoculation with *Ecc*; (*filled circle*) Population dynamics of *Ecc* in the wounds of potatoes and green peppers treated preventatively by the BGP20 twofold cell-free culture filtrate 2 h before inoculation with *Ecc*; (*open square*) Population dynamics of BGP20 in the wounds of potatoes and green peppers inoculated with only BGP20; (*open triangle*) Population dynamics of *Ecc* in the wounds of potatoes and green peppers inoculated with only *Ecc*. Each point represents the mean of colony forming units (CFUs) from five potatoes or green peppers. *Vertical bars* represent standard errors of the means

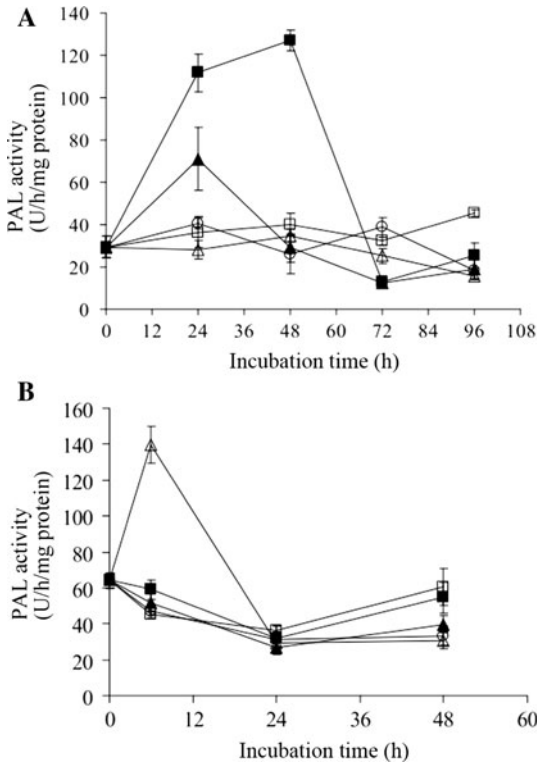

Fig. 7 Induction of phenylalanine ammonia-lyase (PAL) activity in potatoes (**a**) and green peppers (**b**). (*open circle*) sterile water as blank control; (*filled square*) potatoes and green peppers treated preventatively by the antagonist BGP20 2 h before inoculation with the pathogen *Ecc*; (*filled triangle*) potatoes and green peppers treated preventatively by the BGP20 twofold cell-free culture filtrate 2 h before inoculation with *Ecc*; (*open square*) potatoes and green peppers inoculated with only *Ecc*; (*open triangle*) potatoes and green peppers inoculated with only BGP20. PAL activity was determined using a direct spectrophotometric method. *Vertical bars* represent standard errors of the means

mold on navel oranges was 72.4 %, but it had not obvious biocontrol effects on Valencia oranges and lemons. These results indicated that vegetables and fruits played an important role in the course of interacting between antagonists and pathogens in vivo.

Bacillus amyloliquefaciens has been considered as a safe microorganism to human being, and has "Generally Recognized As Safe" status, GRAS (Food and Drug Administration 1999). Several strains of *B. amyloliquefaciens* have been reported to be very effective for controlling plant diseases (Arrebola et al. 2010; Hu et al. 2010; Osman et al. 2011; Shu-Bin et al. 2012). To our knowledge, the safety of antagonistic bacteria to the target plants had not been specially analyzed in the previous reports. In this study, we found that the antagonist *Bacillus amyloliquefaciens* subsp. *plantarum* strain BGP20 caused severe decay in the wounds of potatoes, but only caused slight discoloration in the wounds of green peppers and Chinese cabbages. In addition, its cell-free culture filtrate could effectively control bacterial soft rot on potatoes, green peppers and Chinese cabbages, and was safe for these vegetables. These results indicated that the application range of the antagonist BGP20 was limited, and its application modes should be adjusted according to the characteristics of vegetables.

The antagonism of *Bacillus* is mainly due to the production of the antagonistic substances by its secondary metabolism pathways (Hu et al. 2010). Several reports had demonstrated that the antagonistic substances produced by *Bacillus amyloliquefaciens* could be resistant to protease K and heat (Hu et al. 2010; Shu-Bin et al. 2012). In this study, we found that the anti-*Ecc* metabolites produced by the antagonist BGP20 had high resistance to high temperature, protease K, and UV-light.

Competition for nutrition and space between the microbial antagonist and the pathogen is considered as another important mode of action on postharvest vegetables and fruits (Sharma et al. 2009; Osman et al. 2011). A successful antagonist should be a more aggressive colonizer in the wounds of postharvest vegetables and fruits compared to the pathogen (Demoz and Korsten. 2006; Sharma et al. 2009). This study demonstrated that the antagonist BGP20 was a more aggressive colonizer in the wounds of green peppers compared to the pathogen *Ecc*. However, BGP20 showed poor competitive position in the wounds of potatoes compared to *Ecc*. These results indicated that the colonization capacity of BGP20 was obviously different on postharvest various vegetables. In addition, the twofold cell-free culture filtrate of BGP20 could rapidly kill 99.5 % of the pathogen *Ecc* within 6 h. Cladera-Olivera et al. (2006) also found that the cell-free culture filtrate of *Bacillus licheniformis* P40 could completely kill the pathogen *Ecc* within 8 h. These results showed that the antagonistic metabolites produced by antagonists could enhance their competitive position through sharply impairing the population of *Ecc*.

Plants have evolved several inducible defence mechanisms against pathogen attacks (Ongena and Jacques 2008). Accumulated evidences showed that some antagonists could induce systemic resistance by activating the PR proteins of postharvest vegetables and fruits, such as PAL, POD and PPO (Ippolito et al. 2000; Yao and Tian 2005; Droby et al. 2009; Liu et al. 2010), and then these vegetables and fruits produced various antagonistic substances with broad-spectrum antibiotic activity, such as active oxygen and phytoalexin (Heil and Bostock 2002). These antibiotic substances might also harm biocontrol microorganisms. In this study, the treatment with both the antagonist BGP20 and the pathogen *Ecc* induced stronger PAL activities in potatoes than the treatment with either BGP20 or *Ecc*, and significantly reduced the population of BGP20 in the wounds of potatoes compared to the treatment with only BGP20. However, when green peppers were treated with both BGP20 and *Ecc*, their PAL activity decreased slightly, the population of BGP20 rapidly increased, and bacterial soft rot was completely prevented. These results indicated that the induced systemic resistance (ISR) in potatoes severely harmed the antagonist BGP20. However, some previous reports showed that the PAL activity induced by microbial antagonists in hosts was positively related with their biocontrol effects and population dynamics (Yao and Tian. 2005; Shu-Bin et al. 2012). The difference between the present study and previous reports might be due to the differences of antagonists, pathogens and hosts. These results indicated that ISR was a double-edged sword for the biocontrol effects of microbial antagonists.

In conclusion, the biocontrol agent *Bacillus amyloliquefaciens* subsp. *plantarum* strain BGP20 could effectively control bacterial soft rot caused by *Ecc* on postharvest green peppers, Chinese cabbages and potatoes by use of its cell culture or cell-free culture filtrate. Our data demonstrated that production of antagonistic substances was the main mode of action of BGP20 on potatoes and green peppers, and competition for nutrient and space was also an important mode of action on green peppers. In addition, to our knowledge, this is the first report that ISR stimulated by both an antagonist and a pathogen harmed the antagonist. Further elucidation of biocontrol mechanisms of the antagonist BGP20 would help to support this strain as a promising biocontrol agent.

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