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Research Article

Evaluation of *Bacillus amyloliquefaciens* for control of potato blackleg caused by *Pectobacterium carotovorum*

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Abstract

Potato is an important crop in Chiang Mai, Thailand. Blackleg disease is one of the constraints of potato production. The objective of this study was to evaluate the efficacy of antagonistic bacteria to control the causal pathogen. Diseased potato stems were collected in Chai Prakan District, Chiang Mai Province. The symptoms included black stem lesions, vascular discoloration, and foliar yellowing and wilting. The pathogen was identified as the bacterium *Pectobacterium carotovorum* CK3-2 by morphological characteristics and sequencing the 16S rDNA gene. *Bacillus amyloliquefaciens* isolates BB06, BB18, BB23 and NSB4 recovered from the rhizosphere of a number of different plants species, and identified by 16S rDNA sequence, were provided by the Bacteria Plant Disease Laboratory, Faculty of Agriculture, Chiang Mai University. These *B. amyloliquefaciens* isolates produced zones of inhibition of *P. carotovorum* CK3-2 of 6.80, 5.80, 6.60 and 8.20 mm, respectively. Co-inoculation experiments on potato tubers indicated that *B. amyloliquefaciens* BB06 was the most effective treatment in inhibiting invasion of wounds.

Keywords: Potato, *Pectobacterium carotovorum*, *Bacillus amyloliquefaciens*, biological control, blackleg, bacterial soft rot

Introduction

Potato (*Solanum tuberosum*) is considered the fourth most important crop after rice, maize and bean in Chiang Mai Province, Thailand. The cultivation area of potato is around among 2,213 ha with 41,223 tons of production. The period of planting is October to April (Dry season) (Chiang Mai Agriculture, 2015) Blackleg and soft rot diseases are caused by bacteria belonging to *Enterobecteriaceae* family and the genera *Pectobacterium* species (formerly classified as pectinolytic *Erwinia* spp.). *Pectobacterium* spp. cause disease in a wide range of plants, including potato, broccoli, carrot, tomato, cabbage and eggplant. These bacteria cause soft rot of tubers and blackleg of stems on field-grown potato (Czajkowski et al., 2011; Katarina et al., 2014; Sonia et al., 2015). The bacteria produce plant cell-wall degrading enzymes such as cellulase, pectinase, polygalacturonase and protease. The disease always occurs in the field as well as transportation and storage (Lee et al., 2014).

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A number of *Pectobacterium* species and subspecies cause soft rot and blackleg disease on potato, including *Pectobacterium atrosepticum*, *Pectobacterium wasabiae*, and *Pectobacterium carotovorum*, which has been divided into three subspecies including *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium carotovorum* subsp. *odoriferum* (Gardan et al., 2003) and *Pectobacterium carotovorum* subsp. *brasiliensis* (Duarte et al., 2004). *Bacillus* spp., a plant growth promoting rhizobacterium (PGPR) has been associated with suppression of a number of pathogens, and produces endospores which enable it to resist environmental stresses (Jacobsen et al., 2004). The biological mechanisms behind pathogen inhibition by PGPR include habitat occupation, antibiosis, and induction of plant defense systems. The objective of this study was to evaluate the efficacy of *Bacillus amyloliquefaciens* against *Pectobacterium carotovorum*.

Material and methods

Bacterial Isolates

Diseased potato stems were collected in Chai Prakan District, Chiang Mai Province in 2016. The symptoms included black stem lesions, vascular discoloration, and foliar yellowing and wilting. Infected tissue at the leading edge of the infection was excised with a sterile knife, macerated in sterile water, and a loopful of the macerate was streaked on nutrient agar (NA). Pure cultures were cultivated on NA and Eosin methylene blue (EMB) agar for 48 h at room temperature. Isolates of antagonistic rhizosphere bacteria were provided by the Bacterial Plant Disease Laboratory, Faculty of Agriculture, Chiang Mai University and included BB06 (from chilli pepper), BB18 (from tomato), BB23 (from coffee) and NSB4 (from banana).

Identification of bacteria

Extraction of the DNA from Gram-negative and Gram-positive bacteria used a modification of the protocol of Cheng & Jiang (2006). Bacterial isolates were cultivated in Lubia-Bertani broth (LB) on a rotary shaker at 30°C and 180 rpm for 18 h. About 2 ml of the cell suspension was centrifuged at 8,000g for 2 min. The pelleted cells were washed with 400 µl of STE Buffer (100 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA, pH 8.0) twice and mixed by a vortex. Then the cells were centrifuged at 8,000g for 2 min. The pellets were resuspended in 200 µl of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), and 100 µl Tris-saturated phenol (pH 8.0) was added and mixed by a vortex. The samples were centrifuged at 13,000g for 5 min. The supernatant containing the DNA (160 µl) was removed into a new sterile 1.5 ml tube. TE buffer (40 µl) was added and mixed with 100 µl of chloroform and centrifuged for 5 min at 13,000g. The supernatant (160 µl) was transferred to a clean 1.5 ml tube. TE buffer (40 µl) was added, mixed well and incubated at 37° C for 10 min. Chloroform (100 µl) was added and the sample was mixed. After centrifugation at 13,000g for 5 min, 150 µl of the supernatant was transferred to a clean 1.5 ml tube. Genomic DNA in TE buffer was visualized in 1% (w/v) agarose gels. DNA concentration was determined by gel electrophoresis.

PCR was performed using the primers 16SF (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16SR (5′-AAGGAGGTGATCCAGCCGCA-3′) amplify a fragment of about 1500 bp (Liu et al., 2009). PCR reactions were performed in a total volume of 50 µl, containing genomic DNA, 10X PCR buffer, 20 µM of each of dNTP, 50 mM $MgCl_2$, 1 U of *Taq* DNA polymerase and 20 µM of each primer. The reaction mixtures were incubated in a Peltier-based Thermal Cycler A100/A200 (LongGene Document Version 1.4). Following an initial denaturation at 94°C for 5 min, the DNA templates were amplified for 35 cycle consisting of 30 s at 94°C, 30 s at 64°C,

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and 2 min at 72°C followed by a final extension at 72°C for 10 min. Amplification products were separated in 1% (w/v) agarose gels, and viewed by gel electrophoresis.

The PCR products were purified and directly sequenced by Fluorescent dye-terminator sequencing ABI Prism^{TM} 3730xl DNA sequencer (Bio Basic Inc). The obtained sequences were edited and assembled using MAGA5 software and then compared with available sequences in the GenBank (http://www.ncbi.nlm.nih.gov/Genbank) using Basic local Alignment Search Tool (BLAST) for nucleotide alignments.

Inhibition assay

The pathogen (3 ml of 10^8 cfu/ml) was transferred to a 250-ml flask containing 97 ml of NA and poured into petri-dishes. After that, the antagonistic bacteria (10^8 cfu/ml) were evaluated by a disc diffusion assay. Paper discs (6 mm diameter) were plated on the agar, followed by incubation at 28°C for 3 d, and measurements of the inhibition zone have been done. The experimental was designed as a CRD with five replications.

Co-inoculation Experiment

Potato tubers were washed with a 1% sodium hypochlorite solution for 5 min and rinsed twice with sterile water. The tubers were dried and then were sprayed with 70% ethanol. The pathogen strain and antagonistic bacteria were diluted in sterile 0.8% NaCl to 10^8 cfu/ml. The tubers were inoculated with 10 µl of the bacterial pathogen suspension and each bacterial antagonist suspension, and incubated at room temperature. The experimental design was a CRD with 4 replications (Esmaeil et al., 2011).

Results and Discussion

The pathogen isolate CK3-2 of blackleg disease was isolated from a potato field in Chai Prakan District, Chiang Mai, Thailand. Typical symptoms of the disease were observed including black stem lesions, vascular discoloration, and foliar yellowing and wilting (Figure 1A). The isolate CK3-2 showed motile with peritrichous flagella (Figure 1B). The isolate produced white to creamy colonies on NA (Figure 1C) and emerald green colonies on EMB which developed after 2 d (Figure 1D). Therefore, the isolate was preliminarily identified as belonging to the genus *Pectobacterium* (Golkhandan et al., 2013). The phylogenetic tree was based on a 1500 bp alignment sequence of 16S rDNA of the isolate from the potato blackleg sample. The pathogen was identified as *Pectobacterium carotovorum* and clustered closely with the type strain of *P. carotovorum* DSM30168 (GenBank NR_041971.1) and *P. carotovorum* CFBF2046 (GenBank NR_118227.1). The database showed 99% sequence similarity with the type strain (Figure 2). Fujimoto et al. (2017) studied potato blackleg disease in Japan and identified the causal pathogen as *P. carotovorum* subsp. *brasiliense* by 16S rDNA sequences. Similar results were obtained by Duarte et al. (2004). All strains from blackleg and stem rot disease samples yielded a specific amplified (322 bp) in a PCR with primers Br1f and L1r for *P. carotovorum* subsp. *brasiliense.*

The antagonistic bacterial isolates BB06, BB18, BB23 and NSB4 were identified as *Bacillus amyloliquefaciens* by 16S rDNA sequences. They were grouped with *B. amyloliquefaciens* BCRC11601 (GenBank NR_116022.1), *B. amyloliquefaciens* MPA1034 (GenBank NR_117946.1), *B. amyloliquefaciens* NBRC15535 (GenBank NR_112685.1) and *B. amyloliquefaciens* (GenBank NR_118227.1) (Figure 3). The disc diffusion assay found that isolates NSB4 and BB06 produced equivalent zones of inhibition that were significantly greater than BB10. BB23 produced an intermediated zone of inhibition (Figure 4).

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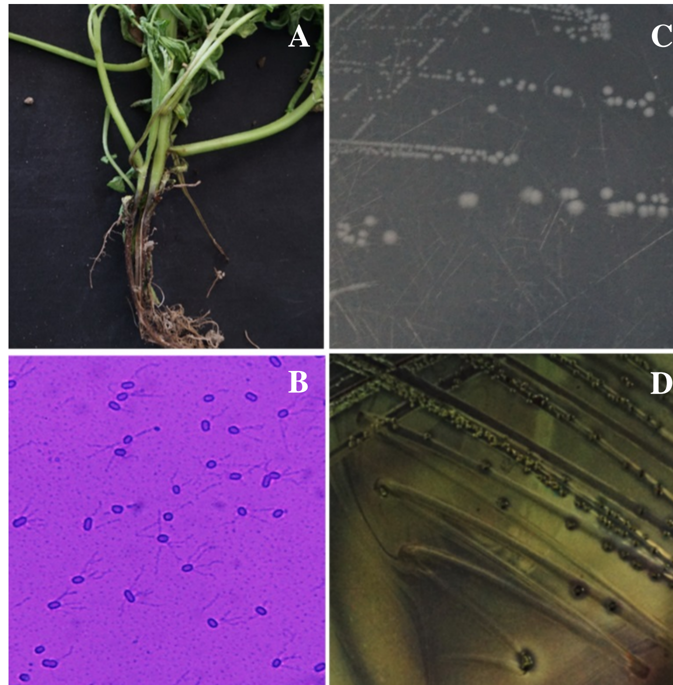

Figure 1. Blackleg symptoms and morphology of *Pectobacterium carotovorum* CK3-2: (A) blackleg symptom on potato stem, (B) peritrichous flagella, (C) the colony on NA, (D) the colony on EMB agar.

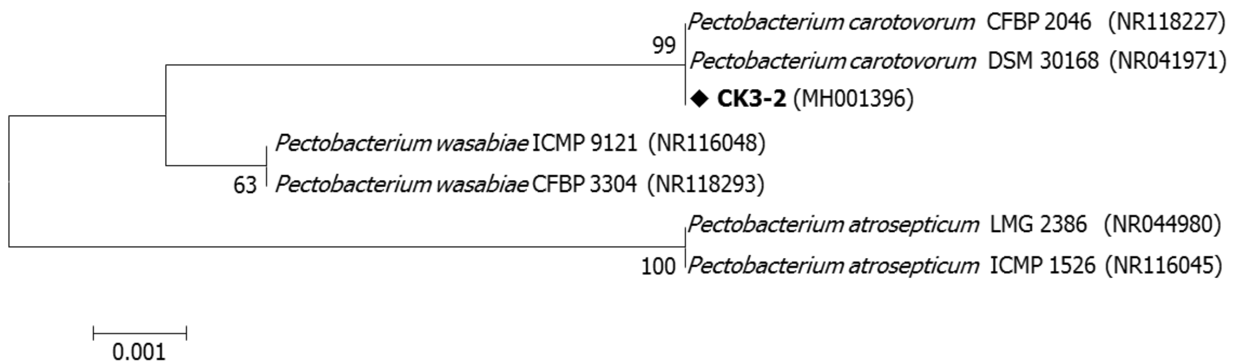

Figure 2. Phylogenetic tree based on comparative 16S rDNA sequence analysis of isolate CK3-2.

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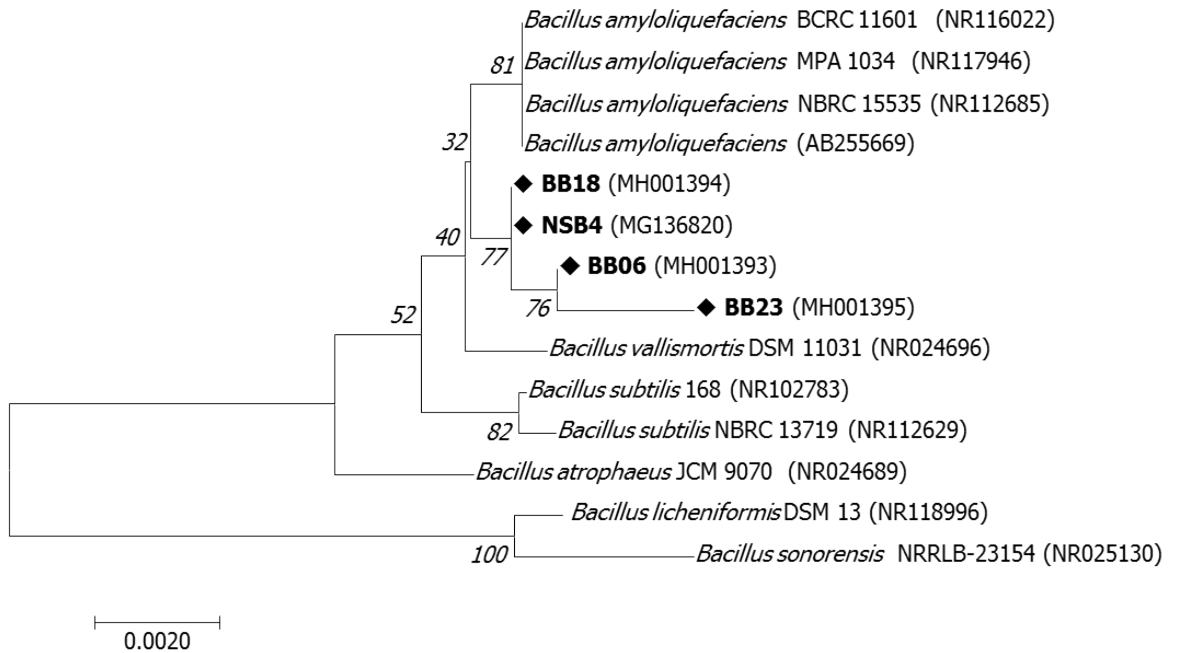

Figure 3. Phylogenetic tree based on comparative 16S rDNA sequence analysis of isolates BB06, BB18, BB23 and NSB4.

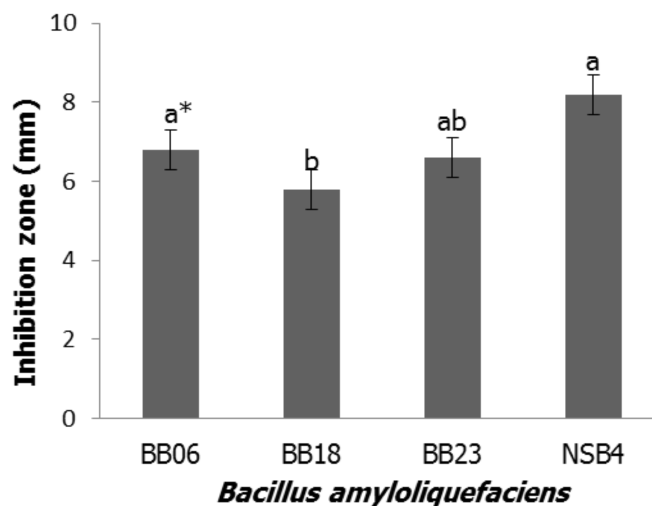

Figure 4. Inhibition zone of *Bacillus amyloliquefaciens* BB06, BB18, BB23 and NSB4 against *Pectobacterium carotovorum* CK3-2 by a disc diffusion assay at 28°C after incubation for 3 d on NA. *The values within the table with different superscripts are significantly different by LSD (p=0.05).

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The antagonism of the four isolates of *B. amyloliquefaciens* was tested in the co-inoculation experiment on potato tuber. The results showed that the direct inoculation of *P. carotovorum* CK3-2 gave tissue maceration after 72 h of incubation. As shown in Figure 5 and Table 1, the co-inoculation of the pathogen isolate CK3-2 and the antagonistic isolates BB06, BB18, and NSB4 led to significant reductions in tissue maceration area, measuring 1.73, 9.30, and 11.72 mm^2, respectively, when compared to the pathogen alone (23.56 mm^2). Co-inoculation with isolate BB23 did not significantly reduce the maceration area. *Bacillus amyloliquefaciens* BB06 was the most effective show in inhibiting invasion of wounds and reducing tissue maceration. Similar results were obtained by Esmaeil et al. (2011). The inoculation of *P. carotovorum* subsp. *carotovorum* EMPCC caused extensive tissue maceration in 48 h, while the ability of EMPCC to macerate potato tubers tissue was reduced when co-inoculated with by *Bacillus* sp. DMA133. Yin et al. (2010) reported that *B. amyloliquefaciens* showed biocontrol efficacy against *P. carotovorum* subsp. *carotovorum* on carrot. *Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus megaterium*, *Bacillus cereus*, and *Bacillus pumilus* showed good activity by zones of inhibition against *P. carotovorum* subsp. *carotovorum* (Issazadeh et al., 2012). Furthermore, Hoda & Kamal (2016) found that antagonistic isolate *Bacillus subtilis* Bs3 restricted the growth of *Pectobacterium atrosepticum*, a causal agent of blackleg disease of potato. Isolate BB06 will be tested for disease control efficacy under field conditions by mixing the tuber with a formulation of the bacterium before planting or by spraying.

Table 1. Effectiveness of antagonistic bacteria in reducing disease on potato 72 h after inoculation.

Treatment	**Diameter of wound (mm^2)***
Negative control (0.8% NaCl)	0.00^{c}**
Positive control (pathogen (CK3-2) only)	23.56^{a}
Co-inoculation of CK3-2 and BB06	1.73^{c}
Co-inoculation of CK3-2 and BB18	9.30^{b}
Co-inoculation of CK3-2 and BB23	19.00^{a}
Co-inoculation of CK3-2 and NSB4	11.72^{b}
LSD (p=0.05)	6.08
CV (%)	30.84

*The average was calculated using data from four replications.
**The values within the table with different superscripts are significantly different by LSD (p=0.05).

Conclusion

The causal agent of potato blackleg disease in Chai Prakan District, Chiang Mai Province was identified as the bacterium *Pectobacterium carotovorum* CK3-2 by morphological characteristics and sequencing of the 16S rDNA. The symptoms included black stem lesions, vascular discoloration, and foliar yellowing and wilting. The pathogen was tested with *Bacillus amyloliquefaciens* BB06, BB18, BB23 and NSB4 by a disc diffusion assay. The results indicated that, isolates NSB4 and BB06 showed the greatest inhibition zones of *P. carotovorum* CK3-2. The co-inoculation experiment on potato tubers found that *B. amyloliquefaciens* BB06 was the most effective in inhibiting the invasion of wounds by *P. carotovorum* CK3-2.

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Figure 5. Attenuation of the maceration capacity of *Pectobacterium carotovorum* CK3-2 by *Bacillus amyloliquefaciens* BB06, BB18, BB23 and NSB4 at 72 h after incubation.

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