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Biocontrol Efficacy of *Bacillus subtilis* BCB3-19 against Tomato Gray Mold

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Abstract

The soil bacterium, BCB3-19, isolated from rhizosphere of tomato and identified as *Bacillus subtilis* was evaluated for biocontrol activities against *Botrytis cinerea*, a phytopathogenic fungus causing gray mold rot of tomatoes after harvest. *In vitro* assay by co-culture of BCB3-19 and *B. cinerea* on agar plates showed that the bacterium effectively inhibited radial growth of the fungus at 4° C and 23°C. *In vivo* evaluation on tomato fruits showed that the bacterium effectively suppressed the development of gray mold at 4°C and 23°C. The population dynamic study showed that the bacterium successfully colonized tomato fruits. Population of the bacterium was continually increasing at 23°C but was static at 4°C. The bacterium did not exhibit hemolytic and lecithinase activities. These findings support the potential use of *Bacillus subtilis* BCB3-19 for biological control of postharvest tomatoes against *B. cinerea*.

Keywords: *Bacillus subtilis*, *Botrytis cinerea*, Biocontrol, tomato

1. Introduction

Biological control, by means of using antagonistic microorganisms in fighting plant pathogens, is now widely recognized as a safer and more sustainable alternative to the chemical based strategy. During the past decades, various groups of microorganisms including bacteria, yeasts and filamentous fungi have been screened for antagonistic activities against certain plant pathogens and numerous effective antagonists have been reported. Among them, *Bacillus subtilis* is one of the most promising antagonists due to its ability to produce various antimicrobial compounds which contribute to its biocontrol capability. Those compounds include predominantly peptides that are either of ribosomal origin or are formed non-ribosomally [1]. Several strains of *B. subtilis* with suppressive effect against plant pathogens have been identified and are expected to be used as potential biocontrol agents [2-4].

Beside its prominent antagonistic activities, safety is an additional property that support the usefulness of *B. subtilis,* since it is ubiquitous in food supplies [5] and is generally recognized as safe (GRAS) [6]. Moreover, some strains of *B. subtilis* have been characterized as potential probiotics for human use [7-8]. These features lead to the belief that the bacterium is suitable for use in the protection of food crops. In this study, a strain of *B. subtilis* was isolated from tomato

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growing soil and was evaluated *in vitro* and *in vivo* for its biocontrol capabilities against tomato gray mold, caused by *Botrytis cinerea.* As additional safety criteria, the hemolytic and lecithinase activities of the bacterium were also determined.

2. Materials and Methods

2.1 Microorganisms

Three isolates of *B. cinerea* (N01-01, N2-01 and N03-01) virulent to tomato were obtained from the plant pathogen collection of the Applied Biology Program, Phranakhon Si Ayutthaya Rajabhat University (Thailand). Fresh cultures were prepared by transferring of conidia suspension of each strain from deep freeze culture to new potato dextrose agar (PDA) plates. The working cultures were monthly sub-cultured and stored on PDA slant at 4ºC.

The antagonistic bacterium, coded as BCB3-19, was isolated from tomato rhizosphere soil taken from a farm in Nakhon Pathom, Thailand. The bacterium was maintained on PDA slant at 4ºC

2.2 *In vitro* efficacy bioassay

The *in vitro* efficacy of BCB3-19 was evaluated against *B. cinerea* based on plate confrontation technique. First, bioassay plates each containing 20 ml of PDA were prepared. Secondly, a mycelia plug of *B. cinerea* was taken from periphery of active growing culture using a sterile cork borer (6 mm Ø) and aseptically placed on to a PDA plate, approximately 25 mm from the center of the plate. After that, a colony plug of BCB3-19 (6 mm Ø) was taken from a 24 hour old swab culture and placed on one side of the plate, approximately 45 mm distant from the fungal plug. PDA plates singly inoculated with *B. cinerea* were prepared as controls. All the bioassay plates were incubated at 4 or 23ºC and routinely checked till the radial growth of *B. cinerea* in control plates reached 45 mm, the inhibition zone in between the bacterial and fungal colonies was recorded (see Figure 1). The *in vitro* efficacy was calculated according to following equation:-

$$\% \text{ inhibition} = \frac{r_o - r}{r_o} \times 100$$

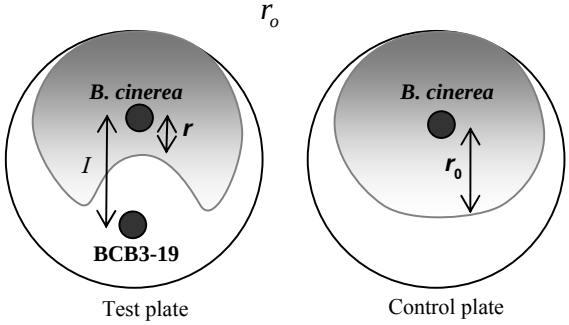

Figure 1 The placement of *B. cinerea* and BCB3-19 in bioassay plates (*in vitro*) and the measurement of inhibition zone, where:-
r_0 = the radius of *B. cinerea* colony (about 45 mm)
r = the radius of *B. cinerea* inhibited by BCB3-19
I = the distance between *B. cinerea* and BCB3-19 plugs, approximately 45 mm

2.3 *In vivo* biocontrol of tomato gray mold

Early ripening tomatoes (*Lycopercicon esculentum* Mill. cv. Tho), obtained from a local grower, were surface sterilized by immersion in a sodium hypochlorite solution (0.016 mol·L^{-1}) for 3 min, washed three time with sterile distilled water and allowed to dry in a constant air flow cabinet. A uniform 6 mm wide and 3 mm deep wound was made at the equator of each fruit using a sterile cork borer. Wounded fruits were left in a laminar flow cabinet till the wounds were dry. An aliquot (50 µL of 10^5 cfu·mL^{-1} spore suspension) of *B. cinerea* was injected into each wound. Thirty minutes later, 50 µL of 0 (as control), 10^6, 10^7 and 10^8 cfu·mL^{-1} cell suspensions of the test bacteria was separately inoculated into each wound. Each 6 fruits with the same treatment were arranged in a plastic box. The boxes were tightly closed before subjected to incubation at 4 and 23ºC in a moistened chamber. The diameter of decay lesion on each fruit was measured after 5 days storage. The experiment was repeated twice.

2.4 Population dynamics of BCB3-19 on tomato

Tomato fruits (≥ 42 fruits) were surface sterilized and wounded as described previously in 2.3. The wounds were inoculated with 50 µL of suspension of BCB3-19 (10^8cfu·mL^{-1}). The fruits were arranged, based on complete randomized block design, in moistened plastic boxes and incubated at 4 and 23ºC. Three fruits were sampled and removed at daily intervals starting at 1 h after incubation (day 0). The wounded area was removed with a 9 mm cork borer, ground and serially diluted in 0.05 M phosphate buffer (pH 6.8), and spread on PDA plates. The colonies were counted after incubation at 23ºC for 24 h.

2.5 Characterizations of BCB3-19

Morphologic, cultural and biochemical features of BCB3-19 were characterized according to the methods of Bergey's Manual of Determinative Bacteriology [9] and Wang *et al.* [10]. In addition, hemolysis and lecithinase activities were detected by cross streaking the bacteria on 5% sheep blood agar and *B. cereus* selective agar (containing egg yolk and polymyxin B) respectively, and incubated for 24–48 h at 37ºC [8].

Molecular identification was conducted based on the analysis of 16S rRNA gene. Genomic DNA from BCB3-19 was prepared by using "Genomic DNA mini kit (Blood/culture cell)" (Geneaid Biotech Ltd., Taiwan). The 16S rDNA gene fragments were amplified by PCR using the universal primers 20F (5'-GAG TTT GAT CCT GGC TCA G-3') and 1500R (5'- GTT ACC TTG TTA CGA CTT-3') [11]. PCR reaction and DNA sequencing were performed as previously described [12]. DNA sequence homology search were performed using BLAST search engine in GenBank databases (available online at: http://www.ncbi.nlm.nih.gov).

3. Results and Discussion

Throughout this study, the bacterial strain BCB3-19 isolated from tomato rhizoshere was characterized and evaluated for its potential use as biocontrol agent. The scope of this study covers the *in vitro* and *in vivo* efficacy test, population dynamics experiment and the morphologic, biochemical, cultural and molecular characterizations of the bacterium.

3.1 The *in vitro* efficacy against *B. cinerea*

Of the 3 isolates of *B. cinerea*, all were inhibited by BCB3-19 (Figure 3A). The rates of inhibition were around 53-56 % either at 4°C or 23°C (Table 1).

Table 1 *In vitro* efficacies of the strain BCB3-19 against the three strains of *B. cinerea* at two different temperatures.

Isolates of *B. cinerea*	% inhibition (n = 3)	
	23 °C	4 °C
N01-01	56.7±4.16	54.3±2.08
N02-01	55.0±2.65	53.6±1.53
N03-01	56.0±2.00	54.7±2.08

3.2 *In vivo* biocontrol of tomato gray mold

The *in vivo* assay on tomato fruits showed that cell concentration and storage temperature influenced the effectiveness of BCB3-19 (Table 2). The bacterium found to be more effective at higher temperature and higher concentration and was found to exhibit highest activity at 10^8 cfu·mL^{-1}. However in general, BCB3-19 at concentrations of 10^7-10^8 cfu·mL^{-1} was highly effective in the control of tomato gray mold either at 4°C or 23°C. It is important to note that the strain BCB3-19 did not damage tomato fruits, at least when examined directly under a stereo microscope (Figure 3F).

Table 2 *In vivo* efficacies of the strain BCB3-19 against the three strains of *B. cinerea* at two different temperatures.

BCB3-19 (cfu·mL^{-1})	*B. cinerea*	% inhibition (n = 12)	
		23°C	4°C
10^6	N01-01	100^{b}*	56.7±3.56^{c}
	N02-01	86.0±2.10^{a}	42.3±2.88^{b}
	N03-01	89.0±4.43^{a}	35.7±3.67^{a}
10^7	N01-01	100^{b}	86.7±11.71^{d}
	N02-01	100^{b}	90.3±8.90^{d}
	N03-01	100^{b}	92.3±8.80^{de}
10^8	N01-01	100^{b}	100^{e}
	N02-01	100^{b}	100^{e}
	N03-01	100^{b}	100^{e}

* Data of the same column marked with different letters are significantly different (Fisher's LSD, $P<0.05$)

3.3 Population dynamics

As a critical criterion for selection, suitable biocontrol agents should efficiently colonize and survive on targeted crops for long period of time, especially under the conditions the crops are stored. Consider Figure 2, the strain BCB3-19 could successfully colonize tomato fruit, but failed to grow at lower temperature (4ºC). This suggest that strain BCB3-19 can successfully survive on tomato fruit and still display its biocontrol function, hence tomato gray mold was found to be suppressed at this temperature. At the higher temperature (23ºC), the bacterium was found to grow continually. This should be the reason why the *in vivo* activity of BCB3-19 was found to be aggressive at this temperature.

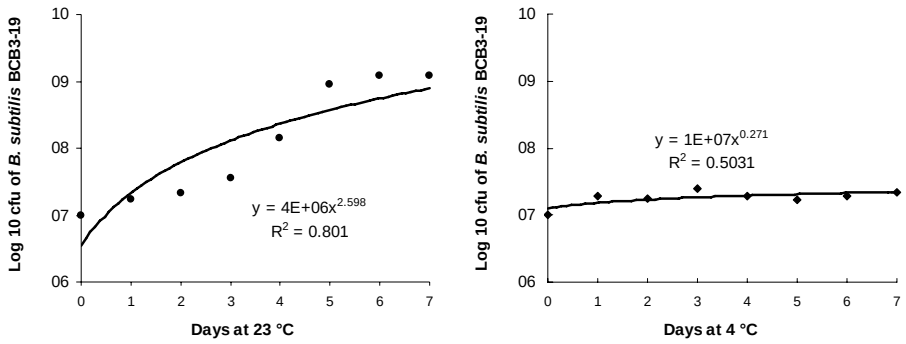

Figure 2 Population dynamics of BCB3-19 in wounds of tomato fruits stored at 4 and 23°C. The data are from one trial, with each point representing the mean of the number of colony counts from three fruits.

3.4 Characterizations of BCB3-19

Based on morphologic, biochemical and cultural properties (Table 1, Figure 3D and 3E), BCB3-19 was identified as a member of the genus *Bacillus.* Further molecular identification based on the analysis of partial sequence of 16S rRNA gene (643bp) confirm that BCB3-19 is a strain of *Bacillus subtilis*, since its nucleotide sequence showed 100% homology to *Bacillus subtilis* strain C3004 (GenBank accession: HQ154051.1) (Figure 4).

Although *B. subtilis* is generally recognized as safe, some strains of this species have been reported to exhibit hemolytic activity [13-14] and may be virulent for humans. Thus, in this study, the hemolytic and, in addition, lecithinase activities were conducted to determine the virulence of the strain BCB3-19. The results reveal that the bacterium did not exhibit either hemolytic or lecithinase activities (Table 3 and Figure 3B).

Table 3 Morphologic, biochemical and cultural characterizations of BCB3-19

Features	BCB3-19	EB-28[10]	Features	BCB3-19	EB-28[10]
Cell size (μm)	0.6 X 3.0	ND	Growth at 4ºC	–	–
Mobility	Yes	Yes	10ºC	+	–
Spore formation	Elliptical	Elliptical	30ºC	+	+
Spore position	subterminal	subterminal	40ºC	+	+
Gram's reaction	variable	+	Growth in 2% NaCl	+	+
Nitrate reduction	+	+	5% NaCl	+	–
VP reaction	+	+	7% NaCl	+	–
Catalase	+	+	10% NaCl	–	–
Hemolysis	–	ND	Growth at pH 5.7	+	ND
Lecithinase	–	ND	Anaerobic growth	–	–

ND, Not determined

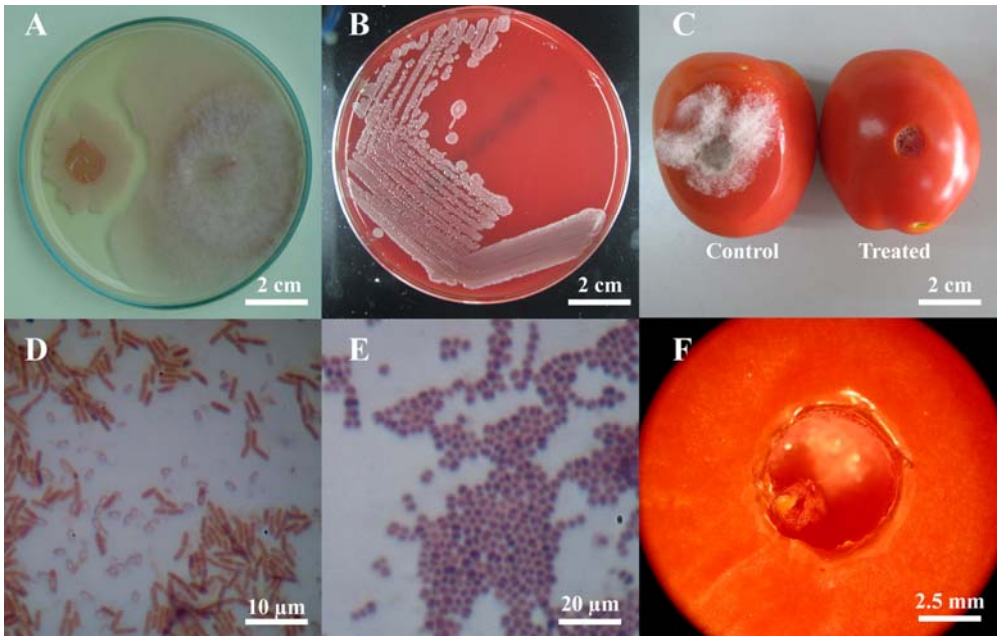

Figure 3 Biocontrol activities and some morpho-physiological features of BCB3-19, (A) *in vitro* activity against *B. cinerea*, (B) hemolytic activity on sheep blood agar, (C) *in vivo* activity against tomato gray mold, (D) vegetative cells, (E) endospores and (F) stereo micrograph of artificial wound site on a tomato fruit, 5 days after being inoculated with BCB3-19.

TTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGT
GGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACC
GGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAA
ACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCC
GCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAG
GCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA
GCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGG
AGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAA
GCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGG
CGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT
TGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTT
CTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGG
GTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGA
GTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTG
GAGGAAC

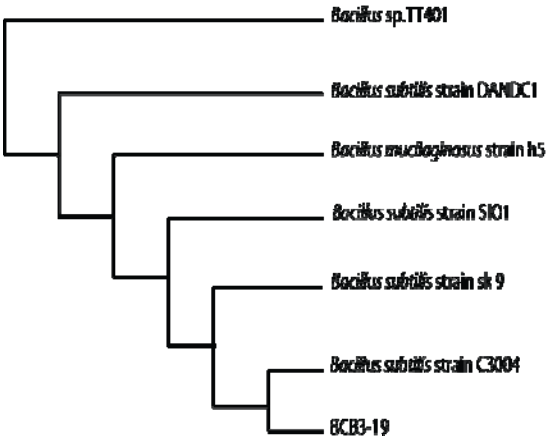

Figure 4 Partial sequence of 16S rDNA fragment (643bp) and phylogenetic analysis of strain BCB3-19.

4. Conclusions

In conclusion, all the results obtained from this study indicate the potential utilization of *B. subtilis* BCB3-19 as a biocontrol agent, especially for the control of gray mold decay of tomato during storage.

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