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Full Length Article

Bacillus subtilis N3 as a Biocontrol Agent for *Curvularia lunata* and its Antifungal Protein Properties

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Abstract

Bacillus subtilis N3 was able to inhibit the growth of *Curvularia lunata*, the causal agent of flower rusty spot disease in orchids. The optimal condition for this strain to produce the highest amount of antifungal compound was to culture the bacteria in tryptic soy broth (pH 6.0) at 37°C for 21 h. The active compound in the culture filtrate was stable for at least 20 min at pH 2 to 10 and at 20 to 121°C. The minimum inhibitory concentration (MIC) of the culture filtrate was determined to be 7.81 µg/mL. Ammonium sulfate precipitation followed by anion exchange column chromatography revealed an approximate 600-fold increase in purification with 7,700 AU/mg of antifungal activity. SDS-PAGE demonstrated that the molecular weight of the purified protein was about 39.88 kDa. Protein fingerprinting by LC/QTOF-MS-MS and the Mascot search algorithm revealed that it was highly similar to flagellin A protein of *Bacillus amyloliquefaciens* at the coverage of 69%. The purified protein was able to delay conidial germination and induce abnormal germ tube elongation of *C. lunata*. The bacterial isolate and/or their proteins show potential for use in sustainable agriculture. This is the first report showing that a flagellin-like protein is able to inhibit the growth of *C. lunata*. © 2018 Friends Science Publishers

Keywords: Antifungal; *Bacillus*; Biocontrol; *Curvularia*; Flagellin; Orchid

Introduction

Orchids are important cut flowers of high commercial value. They account for 8% of the world floriculture trade (Chugh *et al*., 2009). Of serious concern in orchid production is the frequent infection by bacterial, viral and fungal pathogens, which impacts on the commercial value (Liu *et al*., 2013; Lin *et al*., 2015; Swett and Uchida, 2015). The diseases caused by fungi may readily spread owing to the production of large amounts of airborne conidia that are easily dispersed between plants. Pathogenic fungi that cause diseases in orchids include *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Curvularia lunata*, *Fusarium proliferatum* and *Rhizoctonia solani* (Uchida, 1994).

Curvularia spp. are commonly found in plants and soil of tropical and temperate zones. These fungi can infect a variety of plants and cause diseases such as leaf spot disease in maize and palm (Sunpapao *et al*., 2014; Gao *et al*., 2015), leaf blight in tomato (Iftikhar *et al*., 2016) and stem blight in cassava (Msikita *et al*., 2007). *C. lunata* is a fungal pathogen of orchids that causes rusty brown spot symptoms on the tepals (Maketon *et al*., 2015). The spots can occur on both the abaxial and adaxial surface of the tepals. Development of the spots renders the flowers unsaleable.

Chemical fungicides are widely used to protect and/or prevent plant diseases (Leadbeater, 2014). Excessive or inappropriate application of fungicides often leaves residues in the surrounding environment and on crops, which can be a potential health risk not only for growers, but also for consumers (Wightwick *et al*., 2010). In addition, chemical fungicides can adversely affect beneficial microorganisms, such as orchid mycorrhizae (Bayman *et al*., 2002; Wightwick *et al*., 2010). Moreover, repetitive and prolonged use of fungicides in the field has led to the emergence of fungicide-resistant populations (Hollomon, 2015; Panebianco *et al*., 2015).

Biological control, which utilizes beneficial microorganisms and/or different kinds of antimicrobial metabolites, is an alternative strategy to biochemical control that has been used to inhibit the growth and suppress infection by phytopathogens (Pliego *et al*., 2011). It is an environmentally friendly approach and can provide prolonged and effective protection of plants (Fernando *et al*., 2005; Liu *et al*., 2011). Bacterial groups that are reported to be effective as biocontrol agents against phytopathogenic fungi include *Pseudomonas* spp., *Streptomyces* spp. and *Bacillus* spp. (Velivellil *et al*., 2014).

Bacillus is a genus of Gram-positive, rod-shaped, endospore-forming bacteria. Given their potential to produce a variety of bioactive compounds and their ability to produce endospores, *Bacillus* spp. are ideal candidates for commercial exploitation as bacterial biocontrol agents

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(Cawoy *et al*., 2011). *Bacillus* spp. have shown promise in controlling a wide range of plant diseases. The mechanisms responsible include competition for niches and micronutrients, production of inhibitory metabolites and induction of systemic resistance in a host plant. Suppression of plant pathogens through competition for niches and micronutrients has been demonstrated for *Bacillus subtilis* in controlling *Fusarium* wilt in cucumber and pepper caused by *F. oxysporum* (Cao *et al*., 2011; Yu *et al*., 2011). Inhibitory metabolites produced by *Bacillus* are biochemically diverse and include peptides, lipopeptides, proteins, enzymes and organic volatile compounds (Kim and Chung, 2004; Huang *et al*., 2005; Romano *et al*., 2011; Zhao *et al*., 2013b; Asari *et al*., 2016; Xu *et al*., 2016). *Bacillus* spp. may also trigger systemic resistance in a host plant against multiple pathogens by stimulation of certain plant hormone signaling pathways (Li *et al*., 2015).

Previously, isolates of soil bacteria were shown to inhibit the growth of diverse phytopathogenic fungi (Lertmongkonthum, 2006). In the present study, these bacteria were screened for their abilities to inhibit the growth of the phytopathogenic fungus *C. lunata*. Subsequently, the optimal conditions for antifungal substance production were determined for the most effective isolate. The active substance was purified and characterized. The aims of the study were to determine the optimal conditions for production of the active compound by the selected bacteria, to purify and identify the active substance as well as to verify its mode of action against *C. lunata*.

Materials and Methods

Microbial Strains and Growth Conditions

Six isolates of bacteria that were isolated previously from soil in Kanchanaburi Province, Thailand, and shown to inhibit the growth of several phytopathogenic fungi (Lertmongkonthum, 2006) were used in the present study. The isolates were maintained on Nutrient Agar (NA) at 37°C. The phytopathogenic fungus, *C. lunata*, was kindly provided by Asst. Prof. Netnapis Khewkhum, Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Thailand. *C. lunata* was grown on Potato Dextrose Agar (PDA) at 30°C for 5 days. Conidia of *C. lunata* were suspended in 0.1% Tween 20 to yield a final concentration of 5×10^4 conidia/mL.

Identification of Bacteria

The 16S rRNA gene fragment was amplified and sequenced using universal primers 10F (5′-GAG TTT GAT CCT GGC TCA G-3′), 1500R (5′-AGA AAG GAG GTG ATC CAG CC-3′) (Ogata *et al*., 1997), 341F (5′-CCT ACG GGA GGC AGC AG-3′) (Nakamura *et al*., 2011) and 1114R (5′-GGG TTG CGC TCG TTG C-3′) (Kumar *et al*., 2011). The amplified products were purified and sent to 1st BASE (Seri Kembangan, Malaysia) for sequence determination. The 16S rRNA gene sequences were compared with sequences present in the GenBank databases using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/).

Bioassay for Antifungal Activity

One hundred microliters of the conidia suspension (5×10^4 conidia/mL) were spread on PDA plates and air dried for 1 h. Subsequently, the isolated bacteria were streaked on the plates, and all plates were incubated at 30°C for 3 days. Each treatment was performed in four replicates. The isolated bacteria that showed the largest average inhibition zone diameter was selected for further investigation.

Optimal Conditions for Bacteria to Produce Antifungal Substance

The agar diffusion plate method was used to verify the optimal condition of each factor for antifungal substance production. Briefly, bacteria were cultured in liquid media at 37°C for 18 h as a starter culture, and then 1% inoculum was inoculated into the new medium and incubated at 37°C with shaking at 200 rpm for 21 h. The culture was centrifuged at 8,000 rpm for 20 min; the supernatant was filtered through a 0.45 µm membrane filter, and the culture filtrate was collected. One hundred microliters of the conidia suspension (5×10^4 conidia/mL) of *C. lunata* were spread on PDA plates and air dried for 1 h. Subsequently, five wells in the agar were made using a sterile cork borer no. 3 (diameter 0.8 cm). One hundred microliters of Potato Dextrose Broth (PDB) were added to the central well, which served as a negative control. The same amounts of culture filtrate were added to the other four wells. All plates were incubated at 30°C for 3 days after which the diameter of inhibition zones was measured. Each treatment was performed in four replicates, and the average inhibition zone diameter was calculated.

Optimal culture medium: The selected bacteria were cultured in three types of culture media, namely Nutrient Broth (NB), Luria Bertani (LB) and Tryptic Soy Broth (TSB), pH 7 at 37°C with shaking at 200 rpm for 21 h. The culture broth was sampled to test antifungal activity by the agar diffusion plate method as described above.

Optimal pH: The selected bacteria were cultured in the optimal media with pH 6, 7, 8 or 9 at 37°C with shaking at 200 rpm for 21 h. The culture broth was sampled to test antifungal activity by the agar diffusion plate method as described above.

Optimal temperature: The selected bacteria were cultured in the optimal media and pH. The cultures were incubated at three temperatures, namely 30, 37 or 40°C, with shaking at 200 rpm for 21 h. The culture broth was sampled to test antifungal activity by the agar diffusion plate method as described above.

Optimal incubation time: The selected bacteria were cultured in the optimal media and pH and incubated at the optimal temperatures with shaking at 200 rpm for 24 h. The culture broth was sampled every 3 h to determine the growth of bacteria by measuring the optical density at OD_{660} and to test antifungal activity by the agar diffusion plate method as described above.

Effects of pH and Temperature on Stability of the Culture Filtrate

The culture filtrate obtained from the optimal condition for the selected bacteria was adjusted to various pH values ranging from 2.0 to 10.0 using 1 M HCl or 1 M NaOH and maintained at room temperature for 20 min. Antifungal activity was tested by the agar diffusion plate method after the culture filtrate was readjusted to the initial pH of the culture filtrate. A similar procedure was performed to evaluate the effect of temperature on stability of antifungal activity. Briefly, the culture filtrate was incubated at 20, 40, 60, 80, 100 or 121°C for 20 min. After cooling to room temperature, antifungal activity was tested by the agar diffusion plate method. An aliquot of 20 µL nystatin (1 mg/mL) was added to the central well to serve as a positive control.

Minimum Inhibitory Concentration of Culture Filtrate

The selected bacteria were grown under the optimal condition. Subsequently, the culture was centrifuged and the supernatant was lyophilized. The lyophilized powder was weighed and dissolved in the minimum volume of sterile distilled water. The protein concentration was measured with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories) and then was diluted to 250, 125, 62.5, 31.25, 15.62, 7.81 and 3.9 µg/mL solutions. Antifungal activity against *C. lunata* for each dilution was tested by the agar diffusion plate method. Twenty microliters of 1 mg/mL nystatin was used as a positive control. The minimum inhibitory concentration (MIC) was determined.

Purification and Identification of the Antifungal Protein

Ammonium sulfate precipitation: The selected bacteria were grown under the optimal condition. Subsequently, the bacterial cells were removed by centrifugation, and protein in the supernatant was precipitated by step-wise ammonium sulfate fractionation at 0–40%, 40–80% and 80–100% saturation. Protein sediments were dissolved in the minimum volume of 50 mM Tris-HCl (pH 7.5) buffer and dialyzed to remove salt in 50 mM Tris-HCl (pH 7.5) buffer three times, each time for 5 h, with 30% glycerol included in the last dialysis.

Ion exchange chromatography: After ammonium sulfate precipitation, the protein was further purified by ion exchange chromatography using DEAE Bio-gel A (Bio-Rad Laboratories). A linear gradient of 1 M NaCl was used for protein elution. The fractions with antifungal activity were pooled and concentrated using Blanose sodium carboxymethylcellulose (Bronson and Jacobs International Co.) as a water absorbent.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): The relative molecular mass and relative abundance of major proteins were determined by SDS-PAGE with 12% (v/v) sodium dodecyl sulfate-polyacrylamide gel. The Spectra™ Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific) was used as a molecular mass standard. Preparative electrophoresis was performed using the Mini-PROTEAN electrophoresis system (Bio-Rad Laboratories). The major band was excised from the gel and sent for tryptic digestion and protein analysis by liquid chromatography-quadrupole time-of-flight mass spectrometry (LC/QTOF-MS/MS) at the Proteomics Core Facility at the Faculty of Medicine, Chulalongkorn University. The protein fingerprint was blasted against the NCBI Protein database (http://www.ncbi.nlm.nih.gov/) using the Mascot algorithm (http://www.matrixscience.com/) with the following parameters: trypsin specificity with a maximum of one missed cleavage; carbamidomethyl cysteine as a fixed modification and oxidation of methionine as a variable modification; and peptide tolerance and mass tolerances of 1.2 Da and 0.6 Da, respectively. The selected significance threshold was 0.05 ($P < 0.05$).

At each step of protein purification, protein concentrations were measured, assayed by the agar diffusion plate method, after which antifungal activity (%), specific antifungal activity (%) and recovery (%) were calculated. The antifungal activity of the protein was expressed as arbitrary units (AU), defined as the reciprocal of the highest dilution of the sample that produced a zone of inhibition, and was expressed in arbitrary units per milliliter (AU/mL) (Barefoot and Klaenhammer, 1983; Mezghanni *et al*., 2012).

Microscopy

One hundred microliters of the conidia suspension (5×10^4 conidia/mL) of *C. lunata* was mixed with 50 µg purified protein. The volume was adjusted with PDB to 300 µL and the solution was incubated at room temperature for 24 h. The effect of the purified protein was determined under an optical microscope and scanning electron microscope (SEM), and compared with the control conidia suspension of *C. lunata* lacking the purified protein.

Statistical Analysis

SPSS 17.0 software was used for data analysis. Data were reported as means and standard errors for each treatment. Each experiment contained three or four replicates.

Results

Bioassay for Antifungal Activity of Active Compound on *C. lunata*

Six bacterial isolates, namely M10, M15, M23, N1, N3 and N9, were tested for antagonistic activity against *C. lunata* on PDA. The diameter of the inhibition zone for each isolate is listed in Table 1. Isolate N3 resulted in the widest inhibition zone of 4.5 mm. Thus, this isolate was selected for the subsequent experiments. Sequence analysis of 16S rRNA gene sequence of isolate N3 showed that it was 99% identical to that of *Bacillus subtilis* (gb|EF417872.1|). Therefore, the isolate N3 hereafter is designated *B. subtilis* N3. The 16S rRNA gene sequence of *B. subtilis* N3 was deposited in the GenBank database under accession no. KU973548.

Optimal Conditions for Bacteria to Produce Antifungal Substance

B. subtilis N3 was cultured in three culture media (NB, LB and TSB) for 21 h. The culture media were sampled to determine antifungal activity. The supernatant of *B. subtilis* N3 that resulted in the widest inhibition zone was TSB, followed by NB and LB with inhibition zone diameters of 6.75, 5.37 and 4.87 mm, respectively (Table 2). When TSB was adjusted to pH 6, 7, 8 or 9, the supernatant from medium of pH 6 provided the widest inhibition zone of 7.62 mm (Table 2). When *B. subtilis* N3 was cultured in TSB at pH 6 at 30, 37 or 40°C, the highest antifungal activity was observed from the culture supernatant at 37°C with an inhibition zone at about 7.69 mm (Table 2). A time-course experiment was performed by culturing *B. subtilis* N3 in TSB of pH 6 at 37°C for 24 h. The culture medium was sampled every 3 h to determine bacterial growth and antifungal activity. *B. subtilis* N3 entered the log-phase at about 3–6 h; subsequently, the culture grew slowly and entered a stationary growth phase at about 12 h (Fig. 1). The supernatant of *B. subtilis* N3 started to show antifungal activity from 9 to 24 h of growth. The highest antifungal activity was observed in the culture supernatant after growth of 21 h, which yielded an inhibition zone diameter of about 7.7 mm (Fig. 1).

Effects of pH and Temperature on Stability of Culture Filtrate

pH stability: The antifungal activity of the culture filtrate after adjustment to pH 2, 4, 6, 8 or 10 was assayed by the agar diffusion plate method. The antifungal substance produced by *B. subtilis* N3 was stable at a wide range of pH values, wherein the inhibition zones derived from supernatant treated at pH 2–6 were slightly wider than those derived from supernatant treated at pH 8–10 (Table 3).

Table 1: Diameter of *C. lunata* inhibition zone in the presence of antagonistic bacterial isolates

Bacterial isolate	Inhibition zone (mm)
N1	4.0± 0.41
N3	4.5± 0.58
N9	0.0± 0.0
M10	4.0± 0.0
M15	3.5± 0.58
M23	3.5± 0.41

Table 2: Diameter of *C. lunata* inhibition zone induced by supernatants obtained from culture media after incubation under different culture conditions

Variable factor	Condition	Inhibition zone (mm)
Medium	TSB, pH 7	6.75 ± 0.29
	LB, pH 7	4.87 ± 0.66
	NB, pH 7	5.37 ± 0.78
TSB medium pH	pH 6	7.62 ± 0.25
	pH 7	6.50 ± 0.58
	pH 8	7.19 ± 0.38
	pH 9	4.90 ± 0.99
TSB, pH 6 at each temperature	30°C	6.00 ± 0.82
	37°C	7.69 ± 0.24
	40°C	6.00 ± 0.71

Table 3: pH stability of the antifungal substance isolated from *B. subtilis* N3 after treatment at different pH values for 20 min and then readjustment to the initial pH. Note: the four outer wells were filled with 100 µL culture filtrate, and the central well was filled with 20 µL of 1 mg/mL nystatin, which served as a positive control

pH	Inhibition zone (mm)	Image
2	6.55 ± 0.76	
4	7.12 ± 0.35	
6	6.62 ± 0.97	
8	5.35 ± 0.93	
10	4.85 ± 0.52	

Temperature stability: The culture filtrate was incubated at 20, 40, 60, 80, 100 or 121°C for 20 min then cooled to room temperature and antifungal activity was assayed by the agar diffusion plate method. The antifungal substance from *B. subtilis* N3 was stable under the wide range of temperatures from 20°C to 121°C (Table 4).

Table 4: Temperature stability of the antifungal substance isolated from *B. subtilis* N3 after treatment at 20 to 121°C for 20 min and then cooling to room temperature. Note: the four outer wells were filled with 100 µL culture filtrate, and the central well was filled with 20 µL of 1 mg/mL nystatin, which served as a positive control

Temperature (°C)	Inhibition zone (mm)	Image
20	7.25 ± 0.50	
40	8.40 ± 0.42	
60	6.62 ± 0.29	
80	5.72 ± 0.56	
100	6.08 ± 0.70	
121	4.70 ± 0.62	

Determination of Minimum Inhibitory Concentration of the Culture Filtrate

The antifungal activity of culture filtrate containing different concentrations of protein (250, 125, 62.5, 31.25, 15.62, 7.81 and 3.9 µg/mL) was tested by the agar diffusion plate method. The respective inhibition zone diameters were 10.00, 9.00, 8.60, 8.00, 7.00, 5.00 and 0 mm (Fig. 2). The minimum inhibitory concentration (MIC), which was the concentration that yielded 50% inhibition, was estimated from the graph. The MIC of the culture filtrate was determined to be 7.81 µg/mL (Fig. 2).

Purification and Identification of the Antifungal Protein

Proteins in the culture filtrate of *B. subtilis* N3 were precipitated by ammonium sulfate and purified by DEAE ion exchange chromatography. The antifungal protein was recovered by 40–80% saturation of ammonium sulfate, with specific activity at 504.06 AU/mg (Table 5). Anion exchange chromatography on DEAE Bio-gel A demonstrated that fraction no. 51–60 retained antifungal activity, which was eluted by 1 M NaCl (Fig. 3). The specific activity of this purified protein was 7,710.84 AU/mg with a 606.20-fold increase with purification (Table 5).

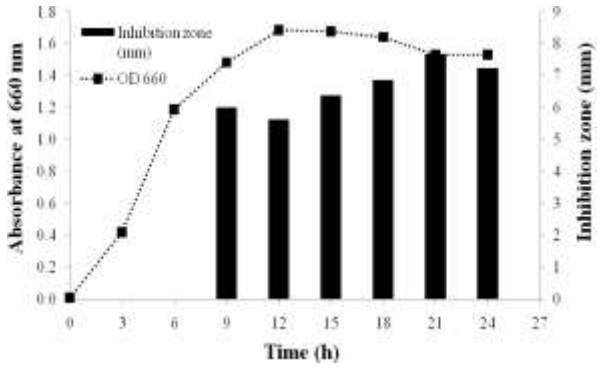

Fig. 1: Growth of *Bacillus subtilis* N3 in tryptic soy broth (pH 6) at 37°C for 24 h, as indicated by absorbance at OD_{660}, and diameter of *C. lunata* inhibition zone induced by supernatant derived from the culture medium at each time point

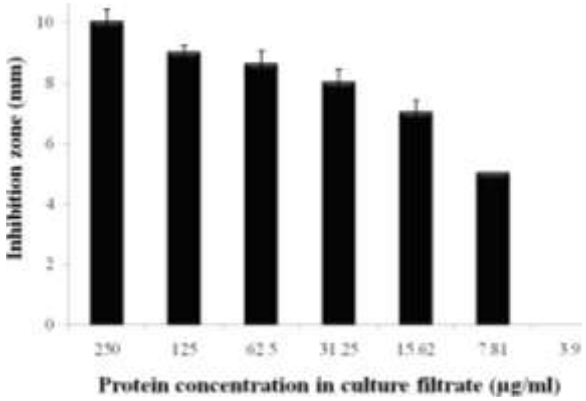

Fig. 2: Average diameter of *C. lunata* inhibition zone in response to seven concentrations of the antifungal protein

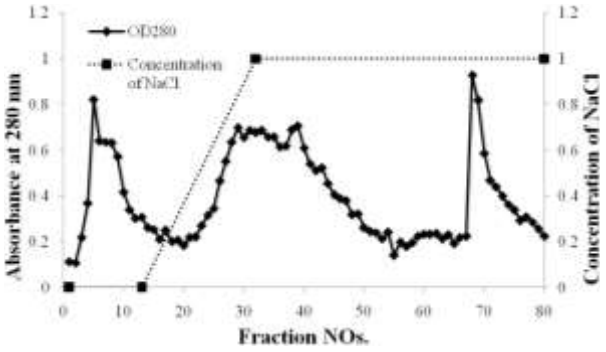

Fig. 3: Elution profile of the antifungal protein of *Bacillus subtilis* N3 purified by ion exchange chromatography using DEAE Bio-gel A

SDS-PAGE revealed that the eluted protein was purified to near homogeneity because only a single band was visualized (Fig. 4, lane 3) with a molecular mass of about 39.88 kDa. The protein band was excised from the gel and subjected to further analysis by LC/QTOF-MS/MS.

Table 5: Purification of the antifungal protein from *Bacillus subtilis* N3

Sample	Volume (mL)	Protein (mg/ml)	Total protein (mg)	Activity (AU/ml)	Specific activity (AU/mg protein)	Total activity (AU)	Purification (fold)
Culture filtrate	13,000.00	12.58	163,540.00	160.00	12.72	2,080228.00	1.00
$(NH_4)_2SO_4$ 40%–80%	46.00	40.63	1,868.98	20,480.00	504.06	942,078.00	39.63
DEAE Bio-gel A	3.32	0.42	1.28	3,200.00	7,710.84	9,854.46	606.20

The data were processed by the Mascot search engine. The Mascot score histogram revealed individual ions scores >65, which indicated identity or extensive homology ($p < 0.05$). The purified antifungal protein showed the best overall match to flagellin A protein of *B. amyloliquefaciens* group (WP_022553864) with a Mascot score of 981 with 69% protein sequence coverage (Fig. 5), which indicated extensive homology.

Microscopy

Conidia of *C. lunata* were incubated with the purified antifungal protein at room temperature for 24 h to observe conidia germination and hyphal morphology by optical light microscopy. In the control set, conidia germinated after about 3 h and subsequently normal mycelial elongation was observed (Fig. 6, top panel). In contrast, conidia in the test set did not germinate until 9 h (Fig. 6, lower panel). At 24 h, the mycelia showed abnormal growth with swollen hyphae and extensive formation of bubble hyphae. The samples from both culture conditions at 5 h were observed under a scanning electron microscope. Conidia not treated with the purified antifungal protein gave rise to straight and smooth hyphae (Fig. 7a), whereas conidia treated with the purified antifungal protein subsequently showed abnormal hyphal growth, with short germ tubes with segmented and rugose swollen hyphal tips observed (Fig. 7b).

Discussion

In this study, *B. subtilis* N3 showed antifungal activity that potently inhibited the growth of *C. lunata*, the causal agent of flower rusty spot disease in orchids. Different mechanisms have been reported to contribute to the antagonistic properties of *B. subtilis* against plant-pathogenic fungi. In the present case, production of an antifungal protein, a flagellin-like protein, which leads to abnormal growth of *C. lunata* was the main factor. Previous studies have reported that flagellin-like protein inhibited the growth of *Monilinia fructicola* and *Botrytis cinerea* (Ren *et al*., 2013; Zhao *et al*., 2013a); however, the present investigation is the first report of flagellin-like protein inhibiting the growth of *C. lunata*.

Bacillus produces diverse types of bioactive compounds, which are secreted into the culture medium. The culture conditions, such as temperature, pH and agitation, influence not only the growth of bacteria, but also the production of each bioactive compound (Moita *et al*., 2005). For instance, *B. subtilis* RB14 produced the highest amount of iturin at 25°C, whereas the highest amount of surfactin was produced at 37°C (Ohno *et al*., 1995).

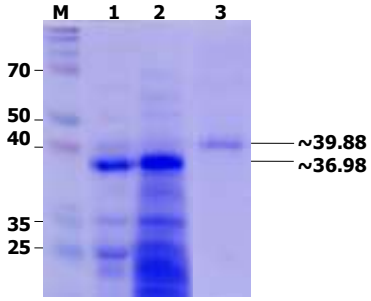

Fig. 4: SDS-PAGE gel of the protein profile from *B. subtilis* N3. Lane M, protein marker; lane 1, culture filtrate; lane 2, protein from 40–80% saturated ammonium sulfate precipitation; and lane 3, purified protein from DEAE Bio-gel A

Protein sequence coverage: 69%

Matched peptides shown in **bold underline**

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  1 MRINHNIAAL NTSRQLNAGS NSAAKNMEKL SSGLRINRAG DDAAGLAISE
 51 KMRSQIRGLD MASKNAQDGI SLIQTSEGAL NETHSILQRM SELATQAAND
101 TNTDSDRSEL QKEMDQLSSE VTRISTDTEF NTKKLLDGTA KDLTFQIGAN
151 EGQTMTLSIN KMDSESLKVG TTYTAQADGT LKSGDGNSTA TWADEEVTDG
201 KVTKEAGYYD DKGALVGSEK LEEGEKLSKG IDISSSAKAA SSALTTIKTA
251 IDTVSSERAK LGAVQNRLEH TINNLGTSSE NLTSAESRIR DVDMASEMME
301 YTKNNILTQA SQAMLAQANQ QPQQVLQLLK G
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Fig. 5: Protein sequence coverage and amino acid sequence obtained with the Mascot algorithm of the 39.88 kDa band excised from the SDS-PAGE gel. The complete amino acid sequence of the highest score match (flagellin A; WP_022553864) is shown, with the positions of the matched peptides highlighted in bold and underlined

In the current study, the type of culture medium strongly affected the antifungal activity of the bacteria, whereas variation in pH and temperature had less effect on the antifungal activity of the bacteria. Of the tested media, TSB is the richest in nutrient composition compared with LB and NB, thus it supported the most rapid growth of *B. subtilis* N3, which subsequently increased the concentration of the bioactive compound. It was reported that substitution of a monosaccharide (glucose) with a disaccharide (lactose) in TSB increases the growth and antibacterial activity of *B. subtilis* B38 (Tabbene *et al*., 2009). Henceforth, optimization of TSB composition should be investigated to further increase both growth and metabolite production. The optimal condition for production of an antifungal agent by *B. subtilis* N3 in the present study was to culture bacteria in TSB medium at

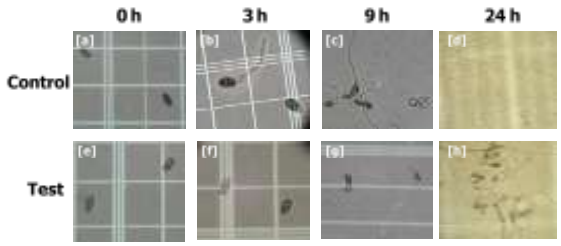

Fig. 6: Morphology of conidia of *C. lunata* observed under an optical microscope (400×) after incubation for 0 to 24 h in the absence (top panel; Control) and presence (lower panel; Test) of the purified antifungal protein

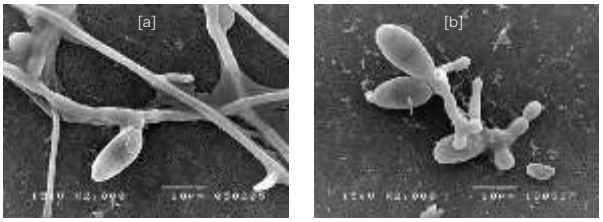

Fig. 7: Morphology of conidia of *C. lunata*o observed under a scanning electron microscope (2000×) after incubation for 5 h in the absence (**a**) and presence (**b**) of the purified antifungal protein

pH 6 at 37°C for 21 h. Time-course experiments revealed that the antifungal agent was a non-growth-associated metabolite produced when the cells reached the stationary phase, which is similar to most metabolites produced by *B. subtilis* (Sansinenea and Ortiz, 2011).

A cell-free supernatant isolated from *B. subtilis* N3 showed antifungal activity, which confirmed that the antifungal compound is an extracellular agent secreted from the cells. The antifungal compound in the culture filtrate was stable over a wide range of pH values and temperatures. The active substance in the culture filtrate especially displayed high antifungal activity, even when the filtrate was treated at 121°C for 20 min. The antifungal agent in this research was indicated to be a proteinaceous substance. A protein concentration in the crude supernatant as low as 3.9 μg/mL retained antifungal activity, which revealed the high potency of the protein.

The antifungal protein purified in this study was indicated to have a strong negative charge as it strongly bound to DEAE beads and was eluted by 1 M NaCl (Cummins *et al*., 2011). This property is in contrast to most antifungal proteins, which contain a positive charge and thus react with negatively charged cell surfaces of microorganisms (Ouedraogo *et al*., 2011; Hegedus and Marx, 2013). Peptide fingerprinting using LC/QTOF-MS/MS indicated 69% protein sequence coverage, which was most similar to flagellin A of *B. amyloliquefaciens*. Flagellin is a fundamental constituent of bacterial flagella, a structure primarily found on most motile bacteria. The proteins assemble in the bacterial flagellum and can be collected in the environment of bacteria as they leak from bacterial cells during flagellum construction (Komoriya *et al*., 1999). The flagellin-like protein from *B. subtilis* has been demonstrated previously to show antifungal activity (Ren *et al*., 2013; Zhao *et al*., 2013a). In the present study, the flagellin-like protein from *B. subtilis* N3 showed noticeable antifungal growth inhibition against *C. lunata*. Light and scanning microscopic observations indicated that the flagellin-like protein is responsible for the delay in conidia germination and hyphal tip elongation as well as abnormal morphology of conidia and hyphae, which might subsequently give rise to a decrease in frequency of rusty brown spots on orchid tepals. However, the mechanism for the antifungal activity of this protein has not been determined and thus requires evaluation. It is also important to perform additional studies to test the efficacy of the bacteria to control rusty spot disease of orchids under commercial production conditions and to compare its efficacy with that of chemical fungicides.

B. subtilis is a well-studied organism and is recognized by the US Food and Drug Administration as a "generally regarded as safe" (GRAS) microorganism (Cawoy *et al*., 2011). After consideration of the utility of *B. subtilis* N3 and/or the flagellin-like protein, it should be possible to develop the bacterium as a biocontrol agent for use in sustainable agriculture.

Conclusion

B. subtilis N3 produced antifungal protein against *C. lunata*, the causal agent of flower rusty spot disease in orchids. The antifungal compound in the culture filtrate was stable over a wide range of pH and temperatures. The purified protein from *B. subtilis* N3 was able to delay conidial germination and induce abnormal germ tube elongation of *C. lunata*. The antifungal protein was identified as aflagellin-like protein of *B. amyloliquefaciens* group. The bacterial isolate and/or protein has potential for use in sustainable agriculture.

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