

Identification of Antagonistic Bacteria Isolated from Thai Fermented Soybean (*Thua Nao*) for Biocontrol of *Lasiodiplodia theobromae*

E. CHUKEATIROTE^{1,*}, K. NIRAPHAI¹, U. SARSDUD² and S. POPLUECHAI¹

¹School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand

²Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

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In this study, bacterial biocontrol agents against the phytopathogen *Lasiodiplodia theobromae* were screened from *Thua Nao*, a Thai fermented soybean product. A total of 170 bacterial strains were isolated and initially screened for their antagonistic activity by dual culture test. Of these, 39 isolates were able to inhibit the fungal growth showing the percentage of inhibition ranging from 25.0–67.5. Among them, the isolate TN79 was selected as a potential antagonistic strain for further study. For this, the bacterial strain TN79 was cultured on nutrient agar for 2 weeks and its crude extracts were prepared using phosphate buffer pH 7.0. The bacterial crude extracts prepared were active and could inhibit all four fungal strains of *L. theobromae*. The optimum pH for antifungal activity of the extracts was 7. In addition, the extracts were also active when exposed to the UV light (254 nm) up to 1 hour and to proteinase K treatment (1 mg/ml). The bacterial strain TN79 was then characterized in terms of their phenotypic and genotypic properties including morphology, biochemical profiles, and rRNA gene sequence. Based on this analysis, the bacterium TN79 was closely related to *Bacillus velezensis*.

Keywords: *Bacillus*, biocontrol, fermented soybean, *Lasiodiplodia*, *Thua Nao*.

Lasiodiplodia theobromae (Pat.) Griff. and Maubl. (syn. *Botryodiplodia theobromae* Pat.) is an important phytopathogenic fungus causing a threatening disease for several host plants. The fungus has been associated to both tropical and subtropical plants, and causes leaf spots, necrosis, and even death of many plants including banana (Thangavelu et al., 2007), citrus (Adesemoye et al., 2014), cocoa (Mbenoun et al., 2008), mango (Mascarenhas et al., 1995), papaya (Netto et al., 2014), and other tropical fruit plants (Coutinho et al., 2017). At present, synthetic chemicals have been widely used to inhibit the phytopathogens including the fungus *L. theobromae*. Such a treatment is effective, however its use cause serious concerns related to human health and environmental issues. Besides, these chemicals can also induce the pathogen resistance, and thus future prevention can be problematic.

Biocontrol is a process of managing pests (i.e., microbial plant pathogens) using other living organisms. This is an alternative means to chemical fungicides and this treatment offers many advantages to human beings considering that it is safe and environmentally friendly. Biocontrol of the *Lasiodiplodia* fungus has been reported which includes *Brevibacillus brevis* (Che et al., 2015), *Bacillus subtilis* and *Paenibacillus polymyxa* (Sa-

* Corresponding author; e-mail: ekachai@mfu.ac.th

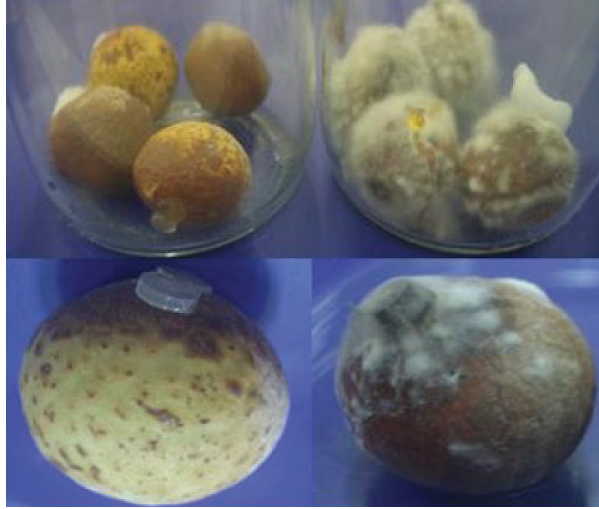


Fig. 1. Diseased symptom of longan fruit caused by *Lasiodiplodia theobromae* CMUL. After 7 day incubation, the peel of the longan fruit became dark brown and covered with the fungal mycelia (right), compared with the untreated control fruits (left)

jitha et al., 2014), *Trichoderma* species (Mortuza and Ilag, 1999), and yeast strains (Mohamed and Saad, 2009). *Bacillus* species, Gram-positive, endospore-forming bacilli, are of great interest as they are able to produce a wide spectrum of secondary metabolites that have diversity in both structures and functions (Sansinenea and Ortiz, 2011). In addition, there are several reports describing the potential role of *Bacillus* species as the biocontrol agents (Cavaglieri et al., 2005; Melnick et al., 2008; Ali and Nadarajah, 2014). *Bacillus* species can be found in a wide range of habitats. Indeed, it has been reported that *Bacillus* species are predominant in several kinds of fermented soybean products including *Thua Nao* of Thailand (Chantawannakul et al., 2002; Petchkongkaew et al., 2008). Based on this information, an emphasis was placed on the isolation of *Bacillus* species from Thai *Thua Nao*. The present study also aimed to evaluate the potential use of the *Bacillus* strain isolated to suppress the growth of the fungus *L. theobromae*.

Materials and Methods

Fungal culture

Four *Lasiodiplodia theobromae* fungal strains (namely CMUL, LP1, LP2, and LG2) isolated from rotten longan fruit (*Dimocarpus longan* Lour.), were obtained from the Postharvest Technology Institute, Chiang Mai University, Chiang Mai, Thailand. The fungus *L. theobromae* strain 1120 was obtained from Department of Agriculture, Ministry of Agriculture and Cooperatives (Bangkok, Thailand). For routine cultivation, the fungal cultures were grown on potato dextrose agar (PDA; Criterion, USA) and incubated at 30 °C for 2 days. For long term storage, the fungal cultures were kept on the PDA slant at 4 °C.

Screening and isolation of antagonistic bacteria

Thai fermented soybean samples (*Thua Nao*) were collected from local markets in Chiang Rai, Thailand. Bacterial cultures were then isolated by serial dilution method. For this, *Thua Nao* sample (10.0 g) was mixed with 90 ml of 0.1% (w/v) peptone and decimal dilution series was performed. Aliquots (0.1 ml) of appropriate dilutions (usually between 10^{-4} – 10^{-8}) were plated in triplicate on nutrient agar (NA; Criterion, USA). In total, 170 bacterial isolates were randomly selected, then purified by repeated streaking, and subsequently tested for their antagonistic activity using dual culture assay against the fungus *L. theobromae*. The dual culture tests were performed in triplicate by placing the fungal mycelia and bacterial colony on the PDA plates. For this, a mycelial agar plug (5 mm in diameter) of the fungus *L. theobromae* was placed at the centre of the PDA plate. A loopful culture of each bacterial isolates was then made at 2 cm juxtaposed to the fungus (Fig. 2A), and the plates were incubated at 30 °C for 24 hours. Antifungal activity was then observed by the inhibition zone of the mycelial growth. Radial growth reduction was calculated as follows: $(a-b) / a$ (where a = radial growth measurement of the pathogen towards the direction in which there was no bacterial isolate (control), and b

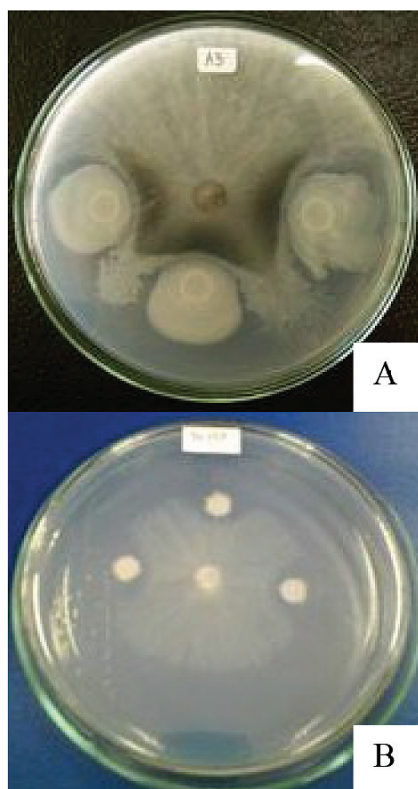


Fig. 2. Antifungal activity of the bacterial strain TN79 isolated from *Thua Nao* samples against *Lasiodiplodia theobromae* CMUL using dual culture assay. The inhibitory zones of the mycelial growth were observed when using the TN79 cells (A), and their crude extracts (B), respectively

is that of the pathogen towards the direction in which the bacterial isolate was inoculated); these values were then expressed as percentage inhibition of radial mycelial growth. The data of the percentage inhibition of the screening were derived from the three replicates being done on the same plate (as shown in Fig. 2 as a representative). When the SD value is zero (0), the mycelial lengths were the same when moving towards the direction where the antagonists (or the extracts) were present. To confirm the results, the bacterial culture supernatants were also used. For this, a loopful of each bacterial culture was transferred to 3 ml nutrient broth (NB; Criterion, USA) and incubated at 37 °C for 24 hours. An overnight bacterial culture was pelleted by centrifugation at 15,000 rpm at 4 °C for 10 min. The culture supernatant was then collected and used instead of the bacterial cells in determining an inhibitory activity (Fig. 2B). The bacterial isolates exhibiting the greatest zones of inhibition were selected for further experiment.

Characterisation of antifungal metabolites produced by the strain TN79

Initially, the bacterial strain TN79 was cultivated on NA plates for 2 weeks at 37 °C. The bacterial extracts were then prepared using a solid phase extraction method. For this procedure, the bacterial cultures were macerated with 15 ml phosphate buffer (pH 7.0; 0.144 % Na₂HPO₄, 0.024 % KH₂PO₄) using a sterile stainless blender (Waring 800BU). The agar slurry was gently vortexed and subsequently centrifuged to separate the solid and solvent phase. The solvent phase was then collected and the agar solid was re-extracted twice. All these extracts were combined, filtered (0.45 µm), and transferred to a vial. The crude extracts were then stored at 4 °C in airtight bottles until use.

The effect of pH on the antifungal activity of the crude extracts produced by the strain TN79 was also studied. The pH of the phosphate buffer was adjusted in a range of 4–9 using hydrochloric acid (1 M) and sodium hydroxide (1 M), and subsequently used for crude extract preparation. In addition, the crude extracts were also treated with proteinase K (1 mg/ml) (Merck, Germany) for 1 h, and exposed to the UV light (in the UVC range of 254 nm) at a distance of 15 cm for 30–60 min. The antifungal activity of the metabolites was then tested as previously described.

Statistical analysis

Data of percent inhibition of the fungal growth obtained from the dual culture test of the bacterial isolates and their culture supernatants were subjected to a simple descriptive statistics, and expressed as means and standard deviation (SD) values.

Identification of antagonistic bacteria

The bacterial strain TN79 exhibiting highest antifungal activity was selected for identification. Conventional methods including Gram-staining, presence of spore, oxygen requirement, catalase test, lecithinase test, ability to growth in 5 and 7% NaCl, growth at 50 and 65 °C, IMViC test, nitrate reduction, fermentation of glucose, arabinose, xylose, and sucrose, and starch hydrolysis, were adopted (MacFaddin, 2000). Further characterization was undertaken using 16S rRNA gene sequence analysis. For this, the genomic DNA of the isolate TN79 was extracted by the standard protocol of Sambrook and Green

(2012). The 16S rRNA encoding gene was then amplified using primers 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi et al., 1998). The polymerase chain reaction was performed in a 25 μ l reaction consisting of 10X PCR buffer, 50mM MgCl₂, 2mM of each dNTP, 1mM of each primer, 5 Units Taq polymerase, and bacterial DNA (1 μ l). Amplification protocol consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec, and a final extension of 72 °C for 5 min. The amplified products were electrophoresed in 0.8% agarose gel, and subsequently purified using TaKaRa SUPRECTM-PCR (TaKaRa, Japan). The purified products were then sequenced and the sequencing data were analyzed using BLAST (Altschul et al., 1990) and the closest known species were determined based on the percentages of sequence similarity. The accession numbers of the 16S rRNA gene sequence of the bacterial isolate TN79 deposited in GenBank was EU590118. Sequence alignment and phylogenetic analysis were then carried out using the Phylogeny.fr software (Dereeper et al., 2010).

Results and Discussion

Pathogenicity of Lasiodiplodia theobromae

Initially, *L. theobromae* fungal strains were tested for their pathogenicity. For this, a piece of mycelial agar plug was placed on the surface sterilized longan fruit, and incubated at room temperature for 7 days. It was observed that the diseased symptom was developed after incubating for 3 days. For treated longan fruit, the exocarp area where the fungal agar plug was placed, turned dark brown and became rotten. Besides, the mycelial growth was observed on the fruits. This diseased symptom did not occur on the untreated control fruits (Fig. 1).

Isolation and screening of antagonistic bacteria

In this study, *Thua Nao* samples were used to isolate the bacteria with antifungal property. A total of 170 bacterial strains were isolated, and initially characterized as endospore-forming bacilli. All bacterial isolates were then screened for antifungal activity against *L. theobromae*, using the dual culture method. Of 170 isolates, it was found that 39 isolates (22.94%) were able to inhibit the fungal growth with the percentage of inhibition ranging from 25.0–67.5 (Table 1). To confirm the antagonistic activity, the culture supernatants of these bacterial isolates were prepared and used in the dual culture assay. By this means, 36 isolates were active showing an inhibitory zone. Of these, the bacterial isolate TN79 exhibited highest antifungal activity when using both cell and culture supernatant (Table 1 and Fig. 2).

Characterisation of antifungal metabolites produced by the strain TN79

In this study, the crude extracts of the bacterial strain TN79 were prepared using the solid state condition. The antifungal activity of the TN79 crude extracts was then tested

Table 1

Preliminary screening of bacterial strains isolated from *Thua Nao* samples capable of inhibiting the growth of *Lasiodiplodia theobromae* fungus strain CMUL using dual culture test

Bacterial isolates	% Inhibition (supernatants)	% Inhibition (cells)
TN79	52.0±0	60.0±0.58
TN112	52.0±0.58	40.0±1.15
TN117	51.2±0	37.5±0
TN142	49.4±0.58	27.5±1.53
TN133	47.6±0	60.0±0.58
TN3	46.2±1.00	45.0±0
TN2	45.7±1.00	32.5±0
TN119	45.3±0	30.0±1.00
TN129	44.0±0	25.7±0
TN188	44.0±1.15	37.5±2.08
TN107	43.9±0	25.0±0
TN6	42.7±1.00	37.5±0.58
TN5	41.0±1.15	50.0±0
TN9	41.0±0	27.5±2.31
TN92	41.0±2.08	45.0±2.31
TN59	40.0±0	37.5±0
TN153	38.7±0	27.5±1.00
TN147	38.3±2.31	27.5±0
TN130	37.2±0	37.1±0
TN168	36.1±2.31	50.0±0
TN68	34.7±0	50.0±0.58
TN125	34.6±0.58	55.0±0
TN121	33.3±0.58	30.0±0
TN16	33.3±0	45.0±1.00
TN101	30.8±0	30.0±0
TN28	29.6±0.58	50.0±0
TN134	28.0±0	48.6±1.15
TN8	28.0±1.15	32.5±1.53
TN11	26.9±1.00	67.5±2.31
TN114	26.7±0	27.5±0.58
TN149	21.3±1.00	48.6±0
TN126	20.3±0.58	37.5±1.00
TN141	20.3±0	30.0±1.00
TN148	17.9±0	45.0±0
TN144	17.3±2.31	30.0±2.08
TN122	7.2±2.08	42.9±0.58
CMU3	1.3±0	45.9±0
TN103	0.0±0	37.5±0.58
TN25	0.0±0	30.0±0
TN90	0.0±0	37.5±0.58

Note: The data of the percentage inhibition of the screening were derived from the three replicates being done on the same plate (as shown in Fig. 2 as a representative). When the SD value is zero (0), the mycelial lengths were the same when moving towards the direction where the antagonists (or the extracts) were present.

Table 2

Inhibitory activity on *Lasiodiplodia theobromae* strain CMUL of the TN79 crude extracts after treatment with proteinase K and UV. Data presented were the percentage of inhibition

Treatment of TN79 extracts	Percentage of inhibition
Control	34.78 ± 1.00
Proteinase K (1 mg/ml)	33.33 ± 0.58
UV – 30 min	34.72 ± 0.58
UV – 60 min	34.72 ± 0.58

Table 3

Percentage of inhibition of *Lasiodiplodia theobromae* growth by the bacterium strain TN79. Data presented were the percentage of inhibition when using the bacterial cells and the bacterial crude extracts (in parentheses)

Fungal strains	Percentage of inhibition
CMUL	60.0 ± 0 (52.0 ± 0)
LP1	40.0 ± 0 (17.39 ± 0.58)
LP2	50.0 ± 0 (26.09 ± 0)
LG2	47.8 ± 0 (34.78 ± 0)
1120	52.2 ± 0 (12.50 ± 0)

Note: The data of the percentage inhibition of the screening were derived from the three replicates being done on the same plate (as shown in Fig. 2 as a representative). When the SD value is zero (0), the mycelial lengths were the same when moving towards the direction where the antagonists (or the extracts) were present.

for stability in terms of pH (Fig. 3). Our data revealed that the crude extracts of the isolate TN79 was active in a pH range of 6–9 with ca. 15–35 % inhibition although it should be noted that there was no activity under strong acidic condition (pH < 5). In addition, there was no effect of the proteinase treatment and UV exposure on the antifungal property of the TN79 crude extracts (Table 2). It should also be noted that the TN79 crude extracts were active against all five strains of *L. theobromae* used in the study (Table 3).

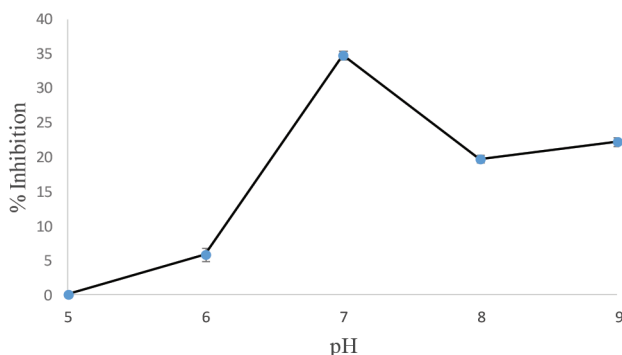


Fig. 3. Effect of pH on the antifungal activity of the TN79 crude extracts. The pH of the culture supernatant of the bacterial strain TN79 was adjusted to pH 5–9, and the extract's stability was tested against the fungus *Lasiodiplodia theobromae* CMUL

Table 4Morphological, physiological and biochemical characteristics of the bacteria strain TN79 isolated from *Thua Nao*

Characteristics	TN79	<i>B. subtilis</i>	<i>B. licheniformis</i>
Shape of cells	Rod	Rod	Rod
Gram reaction	+	+	+
Spore position	Central	Central	Central
Catalase	+	+/-	+
Glucose fermentaion	+	+	+
Mannitol fermentation	+	+	+
Xylose fermentation	+	+	+
Arabinose fermentation	+	+	+
Starch hydrolysis	+	+	+
Growth in 7.5% NaCl	+	+	+
Citrate utilisation	+	+	+
Methyl red test	-	-	-
Voges-Proskeaur test	+	+	+
Indole production	-	-	-
Motility	+	+	+

Microbes are able to produce a wide spectrum of secondary metabolites. These are diverse in terms of structure and function. For example, *Bacillus* species can produce a wide range of active compounds including peptide antibiotics (Amin et al., 2012), lipopeptides (Ramarathnam et al., 2007), and other miscellaneous compounds (Sansinenea and Ortiz, 2011). Antimicrobial agents in the crude extracts of the TN79 bacilli are of great interest as they were resistant to proteinase K treatment and UV exposure. Therefore, an elucidation of the TN79 active components should be further explored.

Identification of the bacteria isolate TN79

As described above, the bacteria strain TN79 exhibiting highest antagonistic activity (Table 1) was then studied for its identity. For this, the isolate TN79 was morphologically studied and subject to a series of biochemical tests as shown in Table 4. The isolate TN79 was Gram positive, endospore-forming, rod-shaped bacteria. Based on its biochemical profiles (i.e., facultative anaerobic, catalase, oxidase, and nitrate reductase positive reactions), the isolate TN79 exhibited similar profile to those of *Bacillus subtilis* and *B. licheniformis* (Priest et al., 1987).

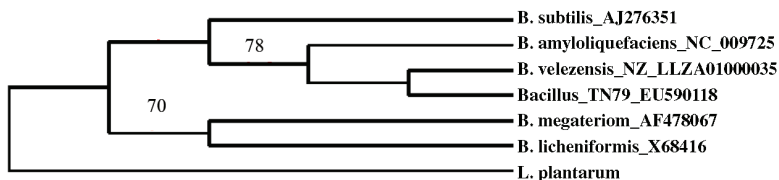


Fig. 4. Dendrogram showing relationships between the strain TN79 and other *Bacillus* species based on their 16S rRNA gene sequences. Numbers at nodes represent the percentages of occurrence of nodes in 1000 bootstrap trials

Assignment of the strain to the genus *Bacillus* was confirmed by 16S rRNA gene sequencing study. The 16S rRNA gene sequence of the isolate TN79 was determined initially using a BLAST search GenBank database (Altschul et al., 1990). The 16S rRNA gene sequence of the strain TN79 was closely related to *B. velezensis* (99.90%). Phylogenetic tree was then generated to determine the phylogeny of the strain TN79 and its related *Bacillus* species (Fig. 4). According to Fig. 4, the strain TN79 was in the same clade of *B. velezensis*. Previous study has proposed that *B. subtilis* is a species complex and probably consists of *B. amyloliquefaciens*, *B. licheniformis*, *B. mojavensis*, and *B. sonorensis* (Rooney et al., 2009). Interestingly, *Bacillus* species closely related to *B. subtilis* exhibit similar phenotypic and genotypic characteristics. Consequently, we would propose that the isolate TN79 was classified in *B. subtilis* group at present.

Conclusions

In the present study, we identified the bacteria strain TN79 isolated from *Thuja Nao*, as a potential biocontrol agent that can be used to suppress the growth of the fungal pathogen *L. theobromae*. This study is one of the few studies describing the antifungal activity of the bacteria against *L. theobromae*. This potential should be further undertaken especially for elucidating the active components in the crude extracts of the isolate TN79. A field application of the isolate TN79 should also be further explored and this would evaluate the utility of the TN79 agent for biological control of the postharvest diseases caused by this fungus.

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