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Original Article

Screening of rhizosphere soil bacteria for biocontrol of *Lasiodiplodia theobromae*

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ABSTRACT

Rhizobacteria capable of inhibiting *Lasiodiplodia theobromae* were screened. In total, 890 rhizobacterial strains were isolated and initially screened for their antagonistic activity using a dual culture test, resulting in 149 isolates being able to inhibit the fungal growth. The antagonistic activity was then rechecked using the culture supernatant. In this step, there were 78 bacterial isolates yielding supernatant that could inhibit the fungal growth. Of these, the isolate named JN15 showed maximum inhibition of the fungus *L. theobromae* (approximately 60%). The antifungal activity of the JN15 culture supernatant was stable in the pH range 4–10 (29–42% inhibition) and remained active at 40 °C. The bacterial strain JN15 isolated from *Senna siamea* was then characterized in terms of its phenotypic and genotypic properties including morphology, biochemical profiles, and 16S rRNA gene sequence. Based on this analysis, the bacterium JN15 was identified as *Bacillus amyloliquefaciens*.

Introduction

The rhizosphere is defined as the specific zone of contact between soil particles and plant roots (Dessaux et al., 2016). The rhizosphere is dynamic and unarguably one of the most complex microbial habitats on Earth. In the rhizosphere zone, plants and microbes interact and form a unique ecosystem; this includes carbon and water cycling, nutrient and mineral trapping. Considering the plant-microbe interactions, their co-metabolism generates a wide range of metabolites, which are of great interest and are widely known to facilitate various purposes including energy sources and signaling compounds, among others (Prashar et al., 2014). More importantly, such metabolites can be used as signaling molecules, and hence act as either chemical attractants or repellents in this niche among microbiomes (Estabrook and Yoder, 1998). Rhizobacteria present in the rhizosphere zone have been shown to produce a variety of useful metabolites (Lugtenberg and Kamilova, 2009). One of the potential applications of these rhizobacteria is to utilize them as biocontrol agents (Whipps, 2001). To date, several researchers have shown that many rhizobacteria possess antagonistic properties toward plant pathogens (Compant et al., 2005; Bhattacharyya and Jha, 2012).

Lasiodiplodia theobromae (Pat.) Griff. & Maubl. (syn. *Botryodiplodia theobromae* Pat.) is one of the key pathogens causing postharvest diseases of many fruits. The fungus is widely distributed in tropical and subtropical regions and has been reported as a pathogen of banana (Thangavelu et al., 2007), mango (Mascarenhas et al., 1995), papaya (Netto et al., 2014) and other tropical fruit plants (Coutinho et al., 2017). Generally, an infection of *Lasiodiplodia* spp. is diagnosed using diseased signs including branch dieback, stem cankers, necrotic lesions, seed and fruit decay (Lima et al., 2013). Fungicides have become the common means of controlling fungal pathogens; however, the use of fungicides can cause serious issues related to health hazards and environmental pollution (Aktar et al., 2009). Biological control is an alternative approach as it is considered safe and environment-friendly (Bale et al., 2008). Although study of *Lasiodiplodia* biocontrol is limited, some microbes have been described for their potential in controlling the fungus, including *Brevibacillus brevis* (Che et al., 2015), *Bacillus subtilis* and *Paenibacillus polymyxa* (Sajitha et al., 2014), *Trichoderma* species (Mortuza and Ilag, 1999) and yeast strains (Mohamed and Saad, 2009). Based on this information, the present study was undertaken to explore potential rhizobacterial isolates for the control of *L. theobromae*. It is believed that this is one of the first works highlighting the antagonistic activity of rhizobacteria on the fungus *L. theobromae*.

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Materials and methods

Fungal culture

The fungus *Lasiodiplodia theobromae* CMUL, isolated from rotten longan fruit, was obtained from the Postharvest Technology Institute, Chiang Mai University, Chiang Mai, Thailand. For routine cultivation, the fungal culture was grown on potato dextrose agar (PDA) and incubated at 30 °C for 2 d. For long-term storage, the fungal culture was kept on the PDA slant at 4 °C.

Screening and isolation of antagonistic rhizobacteria

Soil samples were collected from the rhizosphere region (0–10 cm deep) of 21 different plant species (Table 1). Bacterial cultures were then isolated using a serial dilution method. For this, a soil sample (10.0 g) was mixed with 90 mL of 0.1% (w/v) peptone and a decimal dilution series were performed. Aliquots (0.1 mL) of appropriate dilutions (usually between 1×10^{-4} and 1×10^{-8}) were plated in triplicate on nutrient agar (NA), and the culture plates were incubated at 37 °C for 24 h. Total viable counts were then recorded and expressed as colony forming units (CFU) per gram of the sample. Representative colonies from the plate counts were then randomly selected, purified by repeated streaking and subsequently tested for their antagonistic activity using dual culture assay against the fungus *L. theobromae* (Idris et al., 2007). For this, a mycelial agar plug of the fungus *L. theobromae* was placed at the center of the PDA plate. Spots of each rhizobacterial isolates were then made on the edge of the plate (Fig. 1B) and the plates were incubated at 30 °C for 24 h. Inhibition of the fungal growth was evaluated as the percentage reduction of the diameter of the fungal mycelia in the treated plate compared to that in the control plate. The antifungal activity was calculated as growth inhibition (%) = [(a − b)/a] × 100 (where a = radial growth measurement of the pathogen in the control plate and b is that of the pathogen in the treated plate). To confirm the results, the bacterial culture supernatants were also used. For this, the rhizobacterial isolates exhibiting antagonistic activity were cultured in 5 mL of nutrient broth (NB), and incubated at 37 °C for 24 h. After 24 h of incubation, 1 mL of the cell suspension was transferred to a microfuge tube and centrifuged at 14,000 revolutions per minute (rpm) for 15 min. The supernatant was collected and 10 μL of the supernatant was used in the dual culture assay as mentioned earlier. The rhizobacterial isolate JN15 exhibiting the greatest zones of inhibition was then selected for the further experiment.

Table 1
List of plant species used for rhizosphere soil collection and rhizobacterial strains isolated.

Plant species	TVC[a]	R/A[b]	% Inhibition[c]
Acacia concinna	0.85–14.10	60/1	42.85
Alpinia galangal	1.56–20.30	51/3	33.33–34.55
Ananas comosus	0.29–2.20	24/0	0
Artocarpus heterophyllus	0.56–1.90	32/8	33.33–57.14
Baccaurea ramiflora	0.46–6.70	26/0	0
Camellia sinensis	0.47–2.20	30/0	0
Capsicum frutescens	0.97–14.50	56/10	29.17–41.33
Citrus aurantifolia	0.81–12.20	70/6	23.33–52.30
Citrus hystrix	1.12–4.00	61/1	55.56
Citrus maxima	1.15–6.20	29/0	0
Citrus reticulate	0.78–22.40	56/8	26.39–48.89
Cymbopogon citrates	1.16–16.50	63/9	18.67–40.74
Mangifera indica	1.44–18.20	32/0	0
Metha cordifolia	0.88–13.60	28/7	29.17–55.00
Nicotiana tabacum	1.65–52.00	30/2	54.41–54.90
Ocimum tenuiflorum	0.76–12.80	54/7	25.76–57.47
Punica granatum	0.57–7.40	24/2	57.81–58.82
Sandoricum koetjape	1.36–3.40	29/0	0
Senna siamea	0.93–7.50	67/11	20.00–58.89
Solanum aculeatissimum	0.50–9.60	41/3	37.78–42.22
Tamarindus indica	1.54–23.80	27/0	0

[a] TVC = Total viable count; Data presented are multiplied by 1×10^4 colony forming units (CFU) per gram of soil sample.
[b] R/A = number of rhizobacterial strains randomly selected (R) that were antagonistic using their culture supernatant (A).
[c] Data of % inhibition were calculated based on the mycelial growth reduction using the culture supernatant of the antagonistic rhizobacteria.

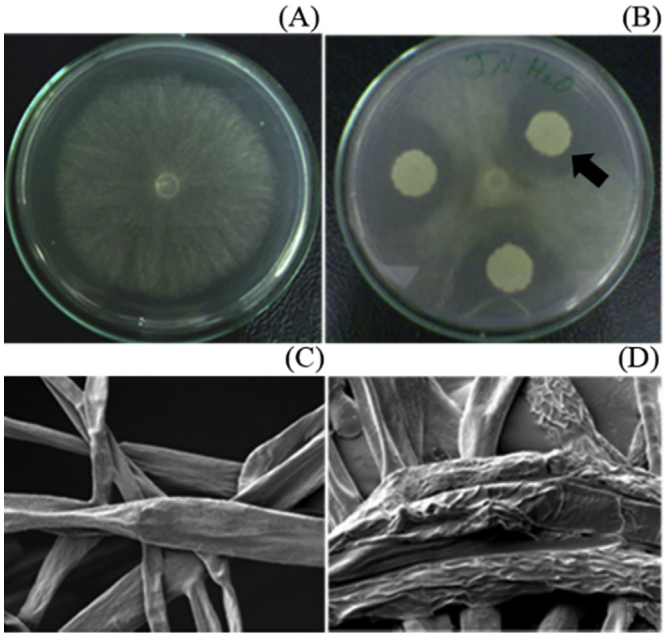

Fig. 1. Antifungal activity tested using dual culture assay (top) and scanning electron microscopy (SEM) micrographs of *Lasiodiplodia theobromae* hyphae (bottom): (A) control fungus *L. theobromae*; (B) inhibitory effect of isolate JN15 against fungus *L. theobromae* (arrow indicates area where fungal mycelia sample taken for SEM analysis); (C) SEM image (30,000X) of fungus L. theobromae hyphae in the absence of isolate JN15; (D) SEM image (35,000X) of fungus L. theobromae hyphae in the presence of isolate JN15.

Identification of antagonistic bacteria

The rhizobacterium strain JN15 had the highest antifungal activity and was selected for identification. Conventional methods were adopted: Gram-staining, presence of spore, oxygen requirement, catalase test, lecithinase test, oxidase test, ability to growth in 5% and 7% NaCl, growth at 50 °C and 65 °C, IMViC test, nitrate reduction, acid from mannitol, fermentation of glucose, arabinose, xylose and sucrose, and starch hydrolysis (MacFaddin, 2000). Further characterization was undertaken using 16S rRNA gene sequence analysis. For this, the genomic DNA of the isolate JN15 was extracted using 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 (PCR) was performed in a 25 μl reaction consisting of 10× PCR buffer, 50 mM $MgCl_2$, 2 mM of each dNTP, 1 mM of each primer, 5 Units Taq polymerase, and bacterial DNA (1 μL). The amplification protocol consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, 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,

Shiga, Japan). The purified products were then sent to Bioservice Unit (Biotec, Bangkok, Thailand) for sequence determination. 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 number of the bacterial isolate JN15 was FM200773. Sequence alignment and phylogenetic analysis were then carried out using the Phylogeny.fr software (Dereeper et al., 2010).

Assessment of the stability of the JN15 culture supernatant

The culture supernatant of the rhizobacterium isolate JN15 was assessed to determine whether pH and temperature affected its stability. For this, the bacterial strain JN15 was cultured in NB at 37 °C for 24 h. The culture supernatant was separated using centrifugation at 14,000 rpm at 4 °C for 10 min. The supernatant was then collected and used in stability tests. For pH stability assay, the culture supernatant was adjusted to pH 2–10, and incubated for an hour at 30 °C. For temperature stability assay, the culture supernatant was incubated at 30–90 °C for 15 min. The culture supernatant treated was then subjected to a bioassay for antifungal activity.

Scanning electron microscopic examination

From the dual culture tested plates, the fungal mycelia samples in the area of inhibition zones (in direct contact with the metabolite of the bacteria JN15 as shown in Fig. 1B) were cut and submitted to the Scientific and Technological Instrument Center (Mae Fah Luang University, Chiang Rai, Thailand) for scanning electron microscopy (SEM) analysis. The samples were examined using an LEO 1450 VP scanning electron microscope (ZEISS, Ramsey, New Jersey, USA) and photographed.

Results and discussion

Isolation and screening of antagonistic rhizobacteria

Rhizobacterial strains were obtained from the rhizosphere soil samples of 21 different plant species (Table 1). The TVC of the rhizobacteria from each soil sample is shown in Table 1 and ranged from 0.29×10^4 CFU/g (from the soil of *Ananus comosus*) to 23.80×10^4 CFU/g (from the soil of *Tamarindus indica*). It has been hypothesized that 1 g of soil could contain around 4000 different bacterial "operational taxonomic units (OTUs)" based on the DNA-DNA reassociation data (Torsvik et al., 1990). This is not surprising considering that the rhizosphere soil area is dynamic and strongly influenced by plant-microbe interactions. For example, Berg et al. (2002) showed that the TVC of the rhizobacteria ranged from 1×10^3 CFU/g in the soil of potato to 1×10^8 CFU/g for the soil of strawberry and oilseed rape. There are many factors affecting the rhizobacteria population including soil and plant type (Dey et al., 2012).

Rhizobacteria are of great interest although it should be noted that plant-associated bacteria can be classified into beneficial, deleterious and neutral groups depending on their effects on plant growth. The present study was mainly focused on the isolation and identification of the potential rhizobacteria that were able to inhibit the growth of the fungus *L. theobromae*. For this, 890 rhizobacterial strains were randomly selected and screened for their ability to suppress *L. theobromae* using dual culture assay. Initially, 149 isolates were active against the fungus. However, when using the culture supernatant, only 78 bacterial isolates could inhibit the fungal growth. It is known that environmental conditions (nutrients, pH, temperature) have varying effects on secondary metabolite production and some studies have even shown that the production of secondary metabolites could be strongly influenced by merely the use of agar media or broth (Baxter et al., 1998; Miao et al., 2006). This is because of many possible reasons including 1) the strong impact of low water activity on the microbial growth; and 2) diluted amounts of the metabolites produced in the liquid state. The current findings further confirm this fact and thus emphasize the importance of optimization of the culture conditions used in microbial metabolite production.

Although there was no specific reason for plant selection, the plants used in the current study were widely distributed; some were herbs, medicinal plants (*Alpinia galangal*, *Cymbopogon citrates*, etc.), and economic tree species (*Ananas comosus*, *Camellia sinensis*). In this work, the antagonistic rhizobacteria were obtained from 14 different plants. However, the rhizobacteria isolated from *Ananas comosus*, *Baccaurea ramiflora*, *Camellia sinensis*, *Citrus maxima*, *Mangifera indica*, *Sandoricum koetjape,* and *Tamarindus indica* showed no activity in this study. Table 1 also shows the numbers of all rhizobacterial strains randomly selected from different plant species and those with antagonistic activity obtained from their culture supernatants (see the R/A column). It should be noted that although similar numbers of bacterial isolates from each plant sample were tested, the numbers of bacterial isolates showing antagonistic activity were different. For example, the proportion of isolates with antifungal activity was highest for *Artocarpus heterophyllus* and *Metha cordifolia* rhizosphere soil (25.0%). In addition, 17 isolates were strongly active, with a percentage inhibition larger than 50 (Table 2).

Identification of rhizobacteria isolate JN15

As described above, the rhizobacterial strain JN15 having the highest antagonistic activity (58.89%) was then studied for its identity by studying its morphology and subjecting it to a series of biochemical tests as shown in Table 3. Isolate JN15 was a Gram-positive, endospore-forming, rod-shaped bacteria. Based on its biochemical profiles (facultative anaerobic, catalase, oxidase, and nitrate reductase positive reactions), isolate JN15 had a similar profile to those of *Bacillus subtilis* and *B. amyloliquefaciens* (Priest et al., 1987).

Assignment of the strain to the genus *Bacillus* was confirmed by the 16S rRNA gene sequencing study. The 16S rRNA gene sequence of isolate JN15 was determined initially using a BLAST search (Altschul et al., 1990). The 16S rRNA gene sequence of strain JN15 was identical (100%) to *B. amyloliquefaciens*. A phylogenetic tree was then generated to determine the phylogeny of strain JN15 and

Table 2
List of antagonistic rhizobacteria exhibiting strong antagonistic activity (>50% inhibition) using culture supernatant.

Rhizobacterial isolate	Plant source	% Inhibition
JN15	*Senna siamea*	58.89
QN02	*Punica granatum*	58.82
QT01	*P. granatum*	57.81
GN04	*Ocimum tenuiflorum*	57.47
GT03	*O. tenuiflorum*	57.33
KN03	*Artocarpus heterophyllus*	57.14
KN02	*A. heterophyllus*	56.67
NT03	*Citrus hystrix*	55.56
UT02	*Nicotiana tabacum*	54.90
CT02	*Metha cordifolia*	54.84
JN14	*S. siamea*	54.44
KT02	*A. heterophyllus*	53.33
KT06	*A. heterophyllus*	53.33
JT03	*S. siamea*	53.09
CT03	*M. cordifolia*	52.78
EN03	*Citrus aurantifolia*	52.30
UN02	*N. tabacum*	50.49

Table 3
Morphological, physiological and biochemical characteristics of rhizobacterial strain JN15 isolated from *Senna siamea*.

Characteristic	JN15	*B. amyloliquefaciens*	*B. licheniformis*	*B. subtilis*
Gram-staining	+	+	+	+
Shape	Rod	Rod	Rod	Rod
Presence of spore	+	+	+	+
Oxygen requirement	Facultative	Facultative	Facultative	Facultative
Growth in NB, 50 °C	+	+	+	+
Growth in NB, 60 °C	−	−	−	−
Growth in 5% NaCl	+	+	+	+
Growth in 7% NaCl	+	+	+	+
Catalase	+	+	+	+
Voges Proskauer	+	+	+	+
Methyl red	−	−	−	−
Formation of indole	−	−	−	−
Starch hydrolysis	+	+	+	+
Citrate utilization	−	+	+	+
Nitrate reduction	+	+	+	+
Egg-yolk lechitinase	−	−	−	−
Oxidase	−	+	+	+
Fermentation of arabinose	+	+	+	−
Fermentation of glucose	+	+	+	+
Fermentation of sucrose	+	+	+	+
Fermentation of xylose	+	+	−	+
Acid from D-mannitol	+	+	+	+

NB = nutrient broth; + = positive; − = negative.

its related *Bacillus* species (Fig. 2). According to Fig. 2, strain JN15 was in the same clade as *B. subtilis* and *B. amyloliquefaciens*. The 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, the current study proposes that the isolate JN15 be classified in the *B. subtilis*/*B. amyloliquefaciens* group at present.

Antagonistic activity of rhizobacteria JN15

The antifungal activity of the JN15 culture supernatant was also tested for stability in terms of pH and temperature (Fig. 3). The data revealed that the culture supernatant of isolate JN15 was stable over a wide pH range (4–10) with 30–46% inhibition although it should be noted that there was no activity under strongly acidic conditions (pH 2 and pH 3). The JN15 culture supernatant was stable under mild temperatures (30 °C and 40 °C), and its stability was completely lost after treatment at high temperature (50 °C and higher). Bacterial members belonging to the *B. subtilis*/*B. amyloliquefaciens* species complex is well known not only as the good producers of a wide range of antibiotics (Stein, 2005) but also as potential biocontrol agents of many fungal pathogens (Elizabeth et al., 1999). Previous reports have suggested that *B. subtilis* and *B. amyloliquefaciens* are able to inhibit the growth of *L. theobromae* (Arrebola et al., 2010; Sajitha et al., 2014), and in the latter case, the lipopeptide iturin A was identified as the principal inhibitor of many postharvest fungal pathogens (Arrebola et al., 2010).

As shown in Fig. 1 and Table 2, isolate JN15 clearly showed inhibitory activity against *L. theobromae*. Based on the dual culture assay, isolate JN15 suppressed mycelial growth with 58.89% inhibition. A fungal mycelium agar plug within the inhibition zone was also examined using SEM, which showed that there were structural changes in the fungal mycelium when antagonistic JN15 was present. The examination revealed that the mycelial sample taken from the dual culture assay plate (in the presence of JN15), was deformed (with wrinkles on the surface) and possibly ruptured (Fig. 1D). Similar morphological alterations of fungal mycelia are influenced by metabolites and degrading enzymes, and these have been reported in some fungal pathogens. For example, the morphological abnormalities of the mycelia of *Aspergillus* were observed to include deformed and swollen mycelia, when treated with *Pseudomonas* and *Bacillus* bacteria (Akocaka et al., 2015). Such

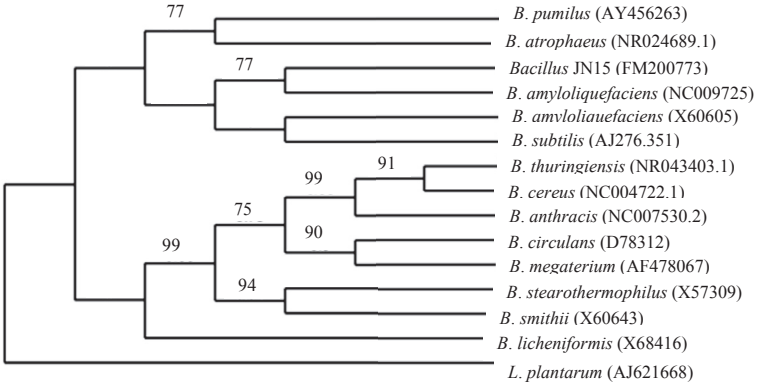

Fig. 2. Maximum-likelihood phylogenetic relationships of rhizobacterial strain JN15 with type strains of other *Bacillus* species. Cladogram was constructed based on the similarity of 16S rRNA gene sequences (accession numbers in parentheses). Bootstrap analysis was performed with 100 repeats, and the bootstrap values greater than 70 are shown.

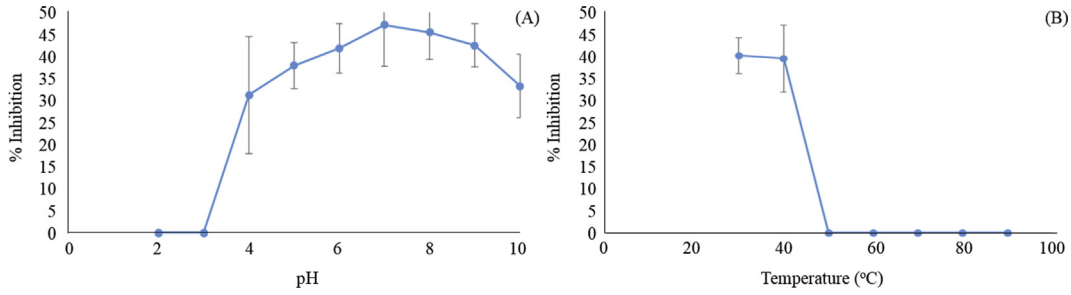

Fig. 3. Effect (mean ± standard deviation) on antifungal activity of culture supernatant of isolate JN105 on: (A) pH; (B) temperature.

deviations were affected by some cell wall degrading enzymes produced by the two bacteria. However, the mode of action of the metabolite observed in the present study is yet to be determined.

The results indicated that the rhizobacteria JN15 isolated from the rhizosphere soil *of Senna siamea* has potential biocontrol activity against the fungal pathogen *L. theobromae*. The present study is one of the few describing the antifungal activity of the rhizobacteria against *L. theobromae*. This potential should be further investigated, in particular, to identify the active components in the culture supernatant of isolate JN15. Application of isolate JN15 in the field may allow better biocontrol of the post-harvest diseases caused by this fungus.

Conflict of interest

None.

Acknowledgements

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