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Biocontrol of Fruit Discoloring and Fruit Rot Disease in Longan (*Dimocarpus longan*) by Using Antagonistic *Bacillus* spp.

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Abstract

Fruit discoloration and fruit rot disease in longan is a serious disease caused by the pathogenic fungi *Pestalotiopsis oxyanthi* and *Lasiodiplodia pseudotheobromae*. This study investigated the potential of antagonistic bacteria as an eco-friendly alternative to traditional fungicides for managing longan diseases caused by these fungal pathogens. Two bacterial isolates, UP-JLS050 and UP-JLS067, demonstrated significant inhibitory activity against the pathogenic fungi *P. oxyanthi* and *L. pseudotheobromae*, with high inhibitory activity. The bacterial strains were isolated from leaves and soil in a longan garden, and dual culture methods showed that the isolates UP-JLS050 and UP-JLS067 exhibited the highest inhibitory activity on the fungal pathogens, with percentage inhibitions of 72.67 ± 2.31 and 70.37 ± 1.28 %, respectively. Biochemical methods and molecular techniques, including sequencing of the 16S rDNA gene, were then used to identify the isolates. This confirmed their similarity to *Bacillus subtilis* and *Bacillus amyloliquefaciens* with 100 and 99 % identity, respectively. *In vitro* tests on longan fruit indicated that these antagonistic bacteria, administered at a concentration of 10^8 CFU/mL, significantly reduced disease incidence by up to 45 % after 9 days of incubation. The incubation of *B. subtilis* (UP-JLS050) and *B. amyloliquefaciens* (UP-JLS050) reduced disease incidence by 45 ± 5.77 and 32.5 ± 5.00 %, respectively.

Keywords: Biocontrol, Discoloring disease, *Lasiodiplodia* sp., *Pestalotiopsis* sp., *Bacillus* spp., Dual Culture, Antagonistic

Introduction

Longan production in Thailand bears immense economic significance, with its exports extending across various Southeast Asia nations [1-3]. Yet, the enduring challenge of sustaining longan quality persists, besieged by the relentless threats of discoloration and fruit rot diseases, masterminded by the notorious culprits *Pestalotiopsis* sp. and *Lasiodiplodia* sp., precipitating a distressing decline in both the yield and quality of this prized fruit. The symptoms of longan discoloration disease are widespread in northern Thailand, particularly in major longan-growing regions like Chiang Mai, Lamphun, Phayao, etc., where outbreaks of discoloration disease caused by *Pestalotiopsis* sp. and *Lasiodiplodia* sp. have led to up to 80 % damage [4,5]. Documented reports of discoloration, cracking, and rot diseases spreading throughout longan orchardsacross multiple provinces in Northern Thailand emphasize the severity of this issue [6]. In 2002, there was a significant outbreak of discoloration disease in Chiang Kham district, Phayao province, resulting in financial losses for longan plantation owners. Subsequent surveys in 2017 - 2018 reaffirmed the persistence of major outbreaks, particularly impacting export-grade longan, which stringent adherence to size and fruit skin color standards. Faced with this looming threat, many farmers reluctantly resorted to the use of toxic chemicals for disease prevention, thereby ushering in an era characterized by excessive chemical use and the looming specter of drug-resistant pathogens. Predominantly, chemical fungicides have been the primary method in the fight against these diseases. However, the utilization of chemicals presents grave risks to both farmers and consumers, including potential fungicide resistance,

thereby posing significant threats to human health and the environment through the accumulation of chemical pesticides and fungicides. Consequently, a resounding call for an alternative approach arises 1 grounded in biological pest control, offering a health-conscious and environmentally friendly paradigm shift [7].

Several studies have identified the efficacy of *Bacillus* spp. as a potent biological control agent in 36various agricultural contexts, especially in combatting pests like armyworms in rice and onions [8-10]. Building upon this foundation, the current research endeavors to examine the efficacy of antagonistic bacterial strains against maladies causing discoloration and rot in longans, with a specific focus on counteracting the fungal pathogens *Pestalotiopsis oxyanthi* and *Lasiodiplodia pseudotheobromae*. This investigation also seeks to discern the potential of these bacterial agents as sustainable, environmentally friendly alternatives to conventional fungicides in the management of longan diseases. To ensure a holistic approach and address the multifaceted challenges faced by the agricultural sector, an integrated collaborative framework has been established, encompassing academic researchers, industry experts, and community enterprises. This joint effort aims not only to advance scientific understanding but also to empower farmers to sustain the production of premium-quality longans that meet the stringent demands of the global market.

Materials and methods

Disease fungal isolation and detection by Scanning Electron Microscope (SEM)

Samples of Longan var. Daw Kan-On were collected from the garden within the Longan-planted community enterprise of Ban Tam Nai, Mueang Phayao district, Phayao province, Thailand, where an outbreak had occurred. These samples underwent a systematic isolation process involving tissue transplantation, achieved by cutting 5×5 mm^2 sections from the wound periphery. Subsequently, the samples were immersed in 95 % ethyl alcohol (AR1380, RCI-Labscan) for 3 min, followed by a 3-minute immersion in 10 % sodium hypochlorite and a 1-minute rinse with sterile distilled water. After blotting with sterile tissue paper, the treated samples were placed on Potato Dextrose Agar (PDA) (MH096, Himedia), with 4 pieces on each plate, and incubated at room temperature for 24 - 48 h to encourage fungal growth from the tissue. Mycelium from each colony was then aseptically collected using a needle and transferred to fresh PDA medium to establish pure cultures. Finally, the isolated fungus was subjected to testing and evaluation for pathogenicity using Koch's postulates method.

Longan fruit samples displaying symptoms of discoloration disease were collected for diagnostic and causative agent identification purposes. The areas with visible disease symptoms were carefully examined using a stereo microscope, and subsequent tissue analysis was conducted utilizing a scanning electron microscope (SEM) JEOL JSM-5910LV from Japan. To facilitate this analysis, small sections were excised from both the afflicted longan tissue and adjacent healthy tissue. These tissue samples were initially immersed in a glutaraldehyde solution (MB222, Himedia) and subsequently rinsed with a phosphate buffer solution (M1452, Himedia). Following this, the samples underwent immersion in an osmium tetroxide solution (RM6346, Himedia) and were again rinsed with a phosphate buffer solution. A progressive series of ethanol concentrations was employed to gradually remove water from the samples. Subsequently, the samples were dried at a critical point using a critical point dryer (CPD) (Quorum K850), affixed to a sample support platform, and finally coated with a layer of gold before undergoing SEM analysis [11].

Isolation and identification of antagonistic bacteria from soil and longan leaves

Antagonism screening was conducted on a total of 120 bacterial isolates obtained from both soil and longan leaves. To initiate this process, 10 g of soil sample were placed in separate 250 mL Erlenmeyer flasks, each containing 100 mL of sterile distilled water. The resulting liquid suspension was subjected to serial dilution, and 0.1 mL of each dilution was plated onto Potato Dextrose Agar (PDA). These plates were then incubated at 25 °C for a duration of 48 to 72 h to optimize the method. Longan leaves measuring 0.5×0.5 cm^2 were harvested for the purpose of identifying antagonistic bacteria. Typical colonies from all isolates were streaked on Nutrient Agar (NA), cultured, and subsequently stored at 4 °C for use in the antagonistic screening. Subsequently, all isolates underwent the Dual Culture Method to assess their capability to inhibit fungi such as *Pestalotiopsis oxyanthi* and *Lasiodiplodia pseudotheobromae* [12]. For each of the 3 replicates, the growth of the fungus towards and away from the bacterium was allowed for a duration of 7 days following incubation. The assessment of inhibition was performed using the formula for Percent Inhibition of Radial Growth (% PIRG) [13]:

$$\text{The percentage of inhibition} = \frac{[(R1-R2)]}{R1} \times 100 \quad (1)$$

where R1 is the radius growth of the mycelium in control medium, and R2 is the radius growth of the mycelium in paired culture medium. Those antagonistic bacteria showing the highest percentage (> 70 %) of fungal growth inhibition were selected for further suppression of *P. oxyanthi* or *L. pseudotheobromae* in planta.

Bacterial characterization involved several approaches, encompassing the examination of characteristic features, biochemical tests, and DNA identification through the sequencing of the 16S rDNA gene, using the primer pairs 8F [5'- AGAGTTTGATCMTGGCTCAG -3'] and 1522R [5'AAGGAGGTGATCCRCCGCA-3'], following a previously established protocol [14]. The PCR conditions consisted of an initial denaturation step at 94 °C for 1 cycle lasting 1 min, followed by denaturation at 94 °C for 1 min, annealing at 69 °C for 1 min, extension at 72 °C for 1 min (repeated for 35 cycles), and a final extension at 72 °C for 1 cycle lasting 10 min. Subsequently, the DNA samples underwent agarose gel electrophoresis for analysis and were sent for sequencing analysis at First BASE Laboratories, Malaysia, utilizing the Sanger DNA sequencing method. The obtained sequences were then compared with data from the NCBI database for further analysis. The construction of a phylogenetic tree was carried out using MEGA 7.0 software, employing the neighbor-joining method and the p-distance model, with a bootstrap value of 1,000 replications [15]. Nucleotide sequence data of the potential bacterial strains were deposited in GenBank to obtain accession numbers for future reference.

Detection of antagonistic activities on longan fruits

Antagonistic bacteria inhibitory effects on longan fruit were tested against pathogenic fungi, *P. oxyanthi* and *L. pseudotheobromae*, using 10^6 spores/mL. of spore concentration [16]. The longan fruits underwent preparation, including washing, drying, and surface sterilization with 70 % ethyl alcohol for 30 min, followed by washing with sterile distilled water, drying, and sizing. The fruits were then inoculated with *P. oxyanthi* and *L. pseudotheobromae* by aseptic needle insertion to 2-3 mm depth and incubated at room temperature for 1 h. Subsequently, the fruits were divided into treatment groups and sprayed with 20 mL of the respective bacterial concentrations (1×10^2, 1×10^4, 1×10^6, 1×10^8 CFU/mL). Each treatment consisted of 4 replicates with 10 longan fruits. The experiment results were recorded every 3 days until spore growth occurred, and the percentage of pathogenesis was calculated using the formula:

$$\text{The percentage of pathogenesis} = \frac{\text{number of pathogenic longan fruit}}{\text{all longan fruit}} \times 100 \quad (2)$$

The percentage values of disease incidence were subjected to analysis using statistical software. The effects of the treatments were analyzed using analysis of variance (ANOVA), with the Completely Randomized Design (CRD) test employed for further assessment.

Results and discussion

Disease fungal isolation and detection by Scanning Electron Microscope (SEM)

Pestalotiopsis sp. and *Lasiodiplodia* sp. are recognized as pathogenic fungi responsible for inflicting discoloration and fruit rot diseases in longan fruits, leading to considerable postharvest losses [17]. Various preservation techniques have been explored to extend the shelf life of longan, including ozone and sulfur dioxide (SO_2) fumigation [18] and chlorine dioxide (ClO_2) fumigation [19]. Nevertheless, these methods can incur substantial costs and leave behind residual substances on the fruit. The discoloration and fruit rot diseases pose significant threats to the commercial production of fresh longan, particularly due to issues such as pericarp browning, which is exacerbated by disease and senescence. The progression of these diseases triggers the accumulation of phenolic substrates and activates enzymes like polyphenol oxidase and peroxidase, ultimately resulting in the oxidation of phenolic compounds and the formation of brown polymers [20].

Recent observations have reported instances of discoloration and fruit rot disease damage in major longan-producing regions, including Phayao, Chiang Mai, Chiang Rai, and Lamphun provinces, with notable incidents of fruit cracking (48.33 % incidence) and brown spot symptoms (80 % incidence) leading to harvest losses in specific orchards at Dok-khamtai and Mueang, Phayao [21]. Building on previous research, our investigation sought to confirm the causal agents responsible for discoloration and fruit rot diseases. We isolated 50 fungal strains and conducted inoculation experiments, which revealed that 2 isolates, later identified *as L. pseudotheobromae* and *P. oxyanthi,* exhibited significant increases in brown spot and fruit cracking symptoms compared to a control group treated with sterilized distilled water. Subsequently, we employed DNA techniques for identification. The isolated fungi were then re-inoculated

onto longan fruits displaying discoloration disease symptoms and examined using scanning electron microscopy (SEM). SEM, widely employed for studying the ultrastructure of various materials, pathogens, and diseases, allowed for magnification up to thousands of times. In the realm of mycology, SEM has proven valuable for observing fungal morphology [22-24]. The primary objective of our study was to examine the original fungal isolates from longan, focusing on the outcomes of fungal inoculation experiments. For this purpose, microtome sectioning was employed to obtain microsections from the fruits subjected to *in vivo* inoculation. SEM analysis indicated that longan fruit discs inoculated with *L. pseudotheobromae* and *P. oxyanthi* exhibited fungal growth 3 days post-inoculation, with pronounced damage to the fungal hyphae and the emergence of cracks on the longan peel surface. A comparison between the outer layer of healthy longan peel, which appeared smooth with no cracks, and the surface of longan peel displaying discoloration disease symptoms revealed the presence of fungal mycelium on the surface (indicated by red arrows) and a zone of cracks (indicated by white arrows), as depicted in **Figure 1**. The normal longan fruit pericarp was thick about 630 - 700 µm and composed of 3 layers. The outer layer is an exocarp consisting of natural opening and cracking on the surface. It was covered by a thin discontinuous layer of cuticle and brown epidermal hair. SEM evaluation found that surface cracking also impairs the physiological function of the cuticle and increases water permeability, which may cause water soaking at the inner side of the peel. The injured cell would accelerate the oxidation of phenolic substances and the oxidative products resulted in dark color of inner and outer peel [25]. In this experiment, we confirmed the symptoms of discoloration disease caused by the fungal disease, when the fruit showed during fungal treatment, the dark color of the inner and outer peel of longan fruit appeared. The SEM observation showed the exocarp's surface was broken with fungal inoculation. Wax that covered the pericarp and epidermal hair was also damaged (**Figure 1(D)**).

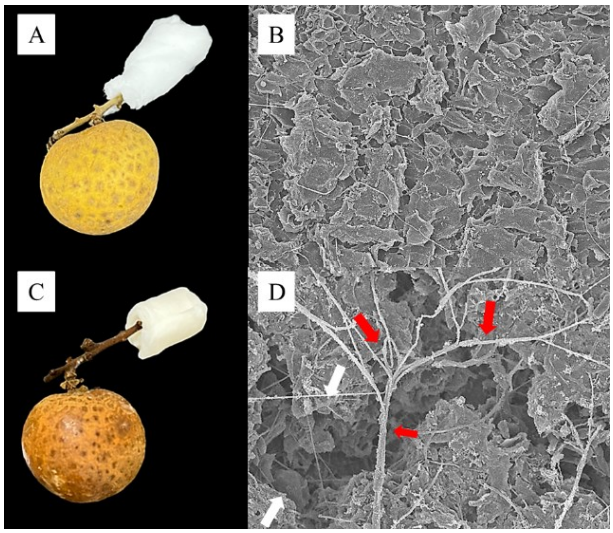

Figure 1 Longan fruit displaying symptoms of discoloration disease was examined under SEM. (A) the outer of the longan peel showed no signs of discoloration disease, (B) the outer of the normal longan peel under SEM at 200 µm, (C) the outer of the longan peel with symptoms of discoloration disease, (D) the outer of longan peel with symptoms under SEM at 200 µm (red arrow is mycelium of fungi, white arrow is longan peel cracked during mycelium growth).

Isolation and identification of antagonistic bacteria

To decrease the use of chemicals, the strains UP-JLS050 were isolated from soil and UP-JLS061 were isolated from longan leaves collected from the garden where the outbreak at longan planted community enterprise of Ban Tam Nai, Mueang Phayao district, Phayao province, Thailand. The isolates of antagonistic bacterium obtained from both the soil surface and longan leaves were screened for their antagonistic effect against *P. oxyanthi* and *L. pseudotheobromae* using the previously described dual culture method. This method of assay remains favored due to its simplicity and rapid results acquisition [26]. Among these isolates, 2 demonstrated significant efficacies in reducing the growth diameter of the fungi by more than 70 %. The growth inhibition of the fungi was assessed for all bacterial isolates after a week of incubation, and the results were recorded as decay diameter percentages representing the inhibition of mycelium growth. Isolate UP-JLS050 exhibited an impressive percentage of 72.67 ± 2.31 % inhibition against the

growth of fungal mycelium for P. oxyanthi, while isolating UP-JLS067 demonstrated a considerable inhibition percentage of 70.37 ± 1.28 % against the growth of fungal mycelium for *L. pseudotheobromae*. (**Table 1**). Remarkably, these 2 isolates stood out with the highest percentage of growth inhibition, as shown in **Figure 2**.

Table 1 The percentage inhibition of *P. oxyanthi* and *L. pseudotheobromae* by Dual culture method.

Treatments	Percentage Inhibition of radial growth (±SD)	Treatments	Percentage Inhibition of radial growth (±SD
P. oxyanthi (control)	-	*L. pseudotheobromae* (control)	-
UP-JLS050	72.67 ± 2.31[a]	UP-JLS067	70.37 ± 1.28[a]
UP-JLS054	61.3 ± 1.15[b]	UP-JLS061	52.60 ± 5.13[b]
UP-JLS067	52.00 ± 3.46[c]	UP-JLS054	51.85 ± 6.42[b]

Remark: [1]a, b, c in each column means there was a statistical difference at a 95 % confidence level ($p < 0.05$)
[2](-) means there was no inhibition of mycelium growth.

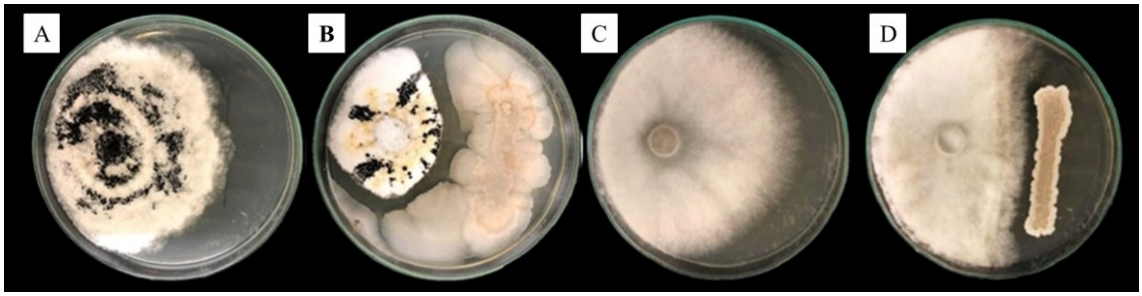

Figure 2 The highest percentage inhibition of antagonistic bacteria on a dual culture testing. (A) *P. oxyanthi* as control, (B) antagonistic bacteria (UP-JLS050), (C) *L. pseudotheobromae* as control, and (D) antagonistic bacteria (UP-JLS067).

After testing the ability of the bacterial isolates to resist *P. oxyanthi* and *L. pseudotheobromae in vitro* using the Dual culture method, the 2 isolates with the highest percentage of growth inhibition (**Figure 1**) exhibited remarkable performance, namely, isolate UP-JLS050 and isolate UP-JLS067. Isolate UP-JLS050 demonstrated the highest percentage of inhibition against *P. oxyanthi*, at 72.67 ± 2.31 %, while isolate UP-JLS067 showed the highest percentage of inhibition against *L. pseudotheobromae*, at 70.37 ± 1.28 %. Statistical analysis at a confidence level of 95 % ($p < 0.05$) (**Table 1**) confirmed significant differences between these isolates. The 2 selected isolates were identified as *B. subtilis* (UP-JLS050) and *B. amyloliquefaciens* (UP-JLS067) using conventional biochemical methods, tests with VITEK2 (data not shown), and molecular techniques with PCR and Sanger DNA sequencing. A phylogenetic tree based on the 16S rDNA gene sequence shows similarity in the genus *Bacillus* and was constructed according to the bootstrapping test values based on 1000 replications of the neighbor-joint method. The DNA of these isolates was compared with GenBank using the Basic Local Alignment Search Tool (BLAST) shown in **Figure 3**. The nucleotide sequence of isolate UP-JLS050 exhibited 100 % similarity with *B. subtilis* strain Y-10 (GenBank: MW047293.1), the antagonistic bacterial stain, as a biocontrol agent for the management of anthracnose disease of chilli [27]. In part of the isolate, UP-JLS067 displayed 99.03 % identity with *B. amyloliquefaciens* strain 21P (GenBank: KM877236.1). The results of this experiment were consistented with phylogenetic tree analysis reported, the analysis revealed the division of the *Bacillus* group into 4 clusters: cluster I contained *B. subtilis*, *B. vallismortis* and *B. mojavensis* strains; clusters II and III contained strains of *B. atrophaeus* and *B. amyloliquefaciens*, respectively; and cluster IV contained *B. sonorensis* and *B. licheniformis*. All *Bacillus* strains were recorded at more than 98 % similarity [28].

Many studies reported the potential *Bacillus* sp. exhibits antifungal activity against fungal growth [29-31]. Moreover, *Bacillus* sp. is considered one of the most widely used and studied biocontrol organisms, and 4 - 5 % of its genome is responsible for the synthesis of antibiotics such as the cyclic lipopeptides (LPs) surfactin, iturin, and fengycin [32].

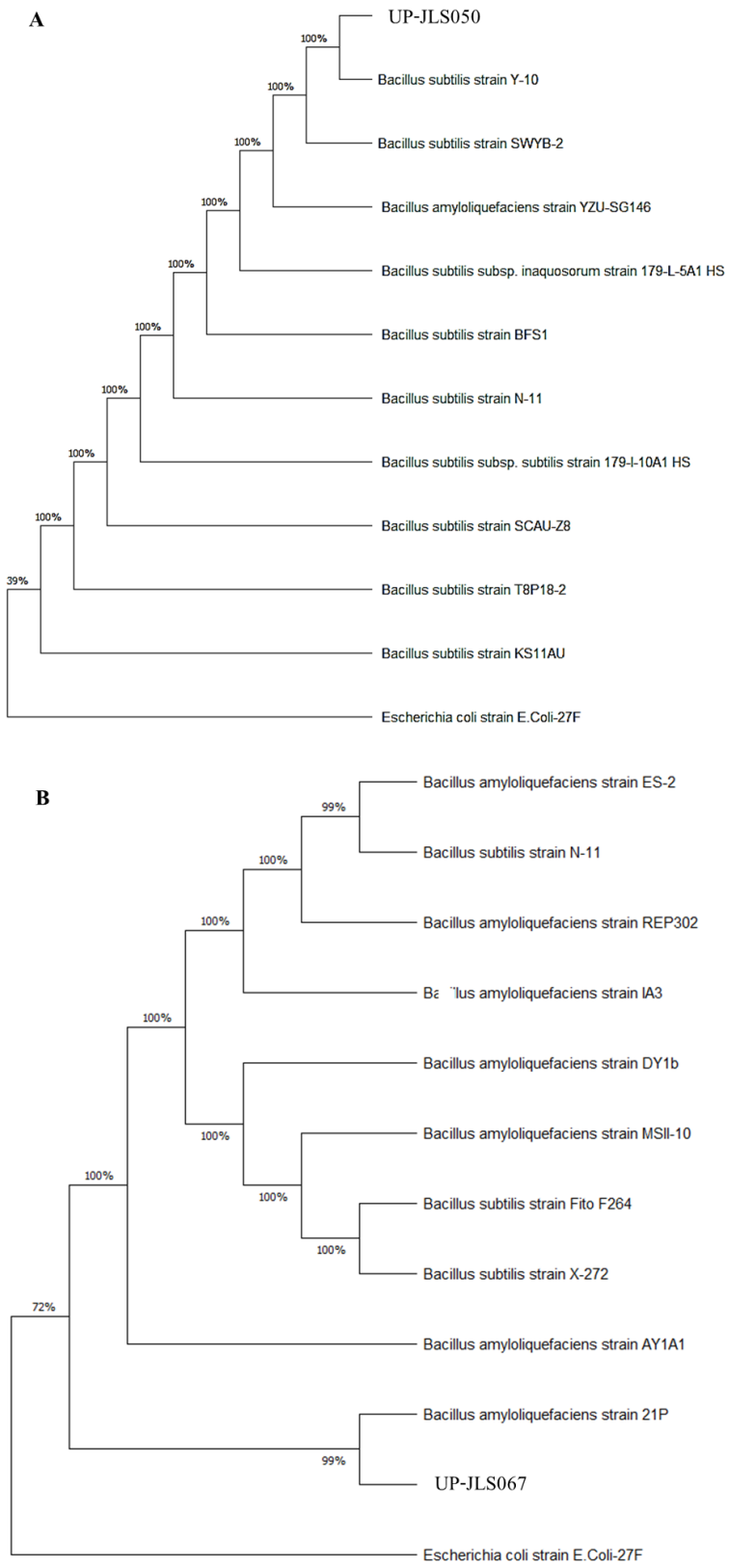

Figure 3 The neighbour-joining phylogenetic tree analysis is based on the 16S rRNA gene sequences showing the relationships of isolate UP-JLS050 as (A) and isolate UP-JLS067 as (B).

Detection of antagonistic activities on longan fruits

To assess antagonistic activities on longan fruits, the 2 bacteria, namely *B. subtilis* (UP-JLS050) and *B. amyloliquefaciens* (UP-JLS067), which exhibited substantial inhibition of fungal growth *in vitro*, were individually and collectively tested against *P. oxyanthi* and *L. pseudotheobromae* in planta using the previously described wound prick technique. Each type of fungus was separately inoculated for testing. Disease incidence averages and standard deviations were recorded for each combination of bacterial treatment, with 3 replicates of 20 longan fruits in each run. The results indicated that longan fruits in the negative control (**Figure 4(A)** and **4(G)**), treated with sterile distilled water, displayed no symptoms. However, when subjected to *B. subtilis* (UP-JLS050), the inhibition of fungal growth in planta was notably high over the 3 - 9 days growth period, followed by treatment with *B. amyloliquefaciens* (UP-JLS067) for *L. pseudotheobromae*, which exhibited no disease symptoms at 3 days compared to the control (**Figure 4(B)** and **4(H)**, which served as controls and displayed the results of disease caused by the transplanted fungus). These findings are summarized in **Figure 4**. Notably, the wound prick technique yielded the most effective disease suppression when utilizing *B. subtilis* (UP-JLS050) and *B. amyloliquefaciens* (UP-JLS067) at high concentrations (**Table 2**).

Furthermore, our study revealed that longan fruits were prone to exhibiting fruit rot symptoms even without fungal inoculation when stored in a moist chamber at room temperature. This highlights the susceptibility of longan fruits rotting under such conditions. This observation is consistent with our previous study [33]. The integration of antagonistic bacteria into biological control methods has enhanced their reliability, offering a promising avenue to reduce the usage of fungicides. The genus *Bacillus* is particularly noteworthy for its ability to produce a wide range of antibiotics, including both ribosomal and non-ribosomal peptide antibiotics [34,35], and it finds applications in agriculture [36,37]. These bacteria are predominantly employed in postharvest processes to combat fruit rot caused by fungi. Among the various strains, *Bacillus spp.* stands out due to its unique attributes, including rapid replication, resilience in adverse environmental conditions, and a broad spectrum of biocontrol efficiency [38]. Leveraging antagonistic bacteria as biological control agents against fungal phytopathogens and integrating them with other methods has enhanced their activity, making them a compelling option for reducing fungicide application [39]. Moreover, *Bacillus spp*. exhibit rapid replication, resistance to adverse environmental conditions, and superior biocontrol efficiency [40].

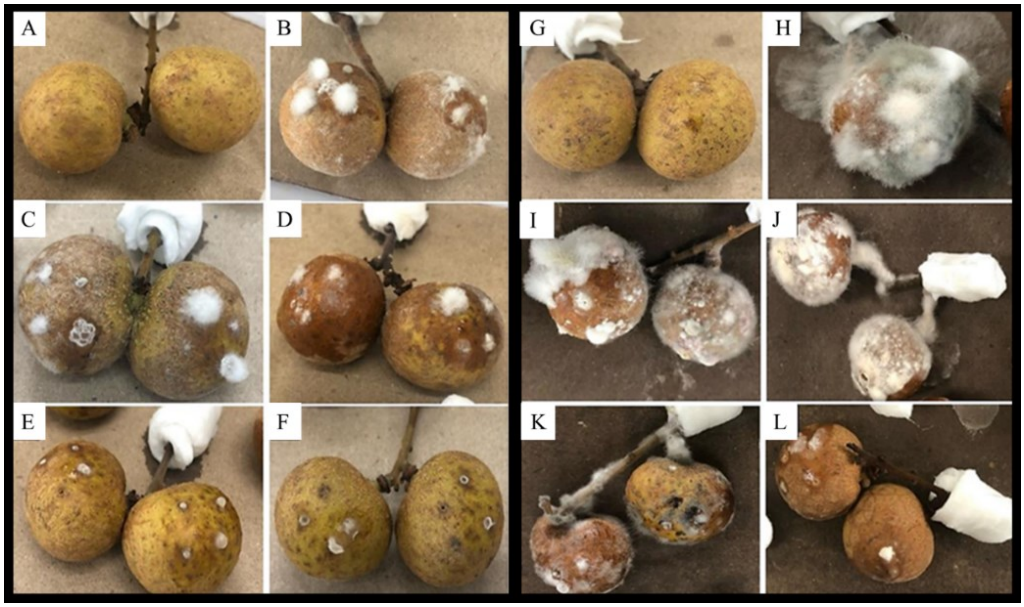

Figure 4 The antagonistic bacteria testing in planta with *Bacillus* spp. in different concentrations to resistant of disease fungal. (A) spraying with sterile distilled water as control, (B) spraying with *P. oxyanthi*, (C) spraying with *B.subtilis* (UP-JLS050) (10^2 CFU/mL), (D) spraying with *B.subtilis* (UP-JLS050) (10^4 CFU/mL), (E) spraying with *B.subtilis* (UP-JLS050) (10^6 CFU/mL), (F) spraying with *B.subtilis* (UP-JLS050) (10^8 CFU/mL), (G) spraying with sterile distilled water as control, (H) spraying with *L. pseudotheobromae*, (I) spraying with *B. amyloliquefaciens* (UP-JLS067) (10^2 CFU/mL), (J) spraying with *B. amyloliquefaciens* (UP-JLS067) (10^4 CFU/mL), (K) spraying with *B. amyloliquefaciens* (UP-JLS067) (10^6 CFU/mL), (L) spraying with *B. amyloliquefaciens* (UP-JLS067) (10^8 CFU/mL).

Table 2 Effect of *B. subtilis* (UP-JLS050) and *B. amyloliquefaciens* (UP-JLS067) on suppression of fruit discoloration and fruit rot disease in Longan.

Treatments	Percentage of disease (%)		
	3 DAT	**6 DAT**	**9 DAT**
Control	-	-	-
P. oxyanthi (10^6 spore/mL)	60.00 ± 8.17^{a}	67.50 ± 25.00^{b}	77.50 ± 17.08^{a}
B. subtilis (UP-JLS050) (10^2 CFU/mL) +P	62.50 ± 9.57a	70.00 ± 18.26^{a}	75.50 ± 5.00^{a}
B. subtilis (UP-JLS050 (10^4 CFU/mL) +P	55.00 ± 10.00^{b}	60.00 ± 14.14^{ab}	65.00 ± 12.91^{b}
B. subtilis (UP-JLS050) (10^6 CFU/mL) +P	52.50 ± 9.57^{b}	60.00 ± 8.16^{ab}	60.00 ± 8.16^{b}
B. subtilis (UP-JLS050) (10^8 CFU/mL) +P	25.00 ± 10.00^{c}	35.00 ± 5.77^{c}	45.00 ± 5.77^{c}
Control	-	-	-
L. pseudotheobromae (10^6 spore/mL)	-	75.00 ± 17.32^{a}	82.50 ± 12.58^{a}
B. amyloliquefaciens (UP-JLS067) (10^2 CFU/mL) +L	-	55.00 ± 5.77^{b}	62.50 ± 5.00^{b}
B. amyloliquefaciens (UP-JLS067) (10^4 CFU/mL) +L	-	45.00 ± 12.91^{b}	52.50 ± 9.57^{b}
B. amyloliquefaciens (UP-JLS067) (10^6 CFU/mL) +L	-	45.00 ± 12.91^{b}	52.50 ± 9.57^{b}
B. amyloliquefaciens (UP-JLS067) (10^8 CFU/mL) +L	-	17.50 ± 9.57^{c}	32.50 ± 5.00^{c}

Remark: [1] +P: *P. oxyanthi* (10^6 spore/mL), +L: *L. pseudotheobromae* (10^6 spore/mL)
[2] a, b in each column means there was a statistical difference at a 95 % confidence level ($p < 0.05$)
DAT: Day after treatment, (-): No symptoms.

Conclusions

The research has addressed the detrimental issues of fruit discoloration and fruit rot disease in the context of longan processing, primarily caused by the fungal pathogens *P. oxyanthi* and *L. pseudotheobromae*. With the aim of reducing the reliance on chemical treatments and fungicides, our experiments have successfully demonstrated the antagonistic capabilities of *B. subtilis* (UP-JLS050) and *B. amyloliquefaciens* (UP-JLS067) against the pathogenic fungi responsible for fruit discoloration and fruit rot in longan. The study has revealed a notable reduction in disease incidence when these *Bacillus* strains were employed, particularly in cases where plants were initially heavily inoculated with fungi. This discovery implies that longan plants colonized by *B. subtilis* (UP-JLS050) and *B. amyloliquefaciens* (UP-JLS067) possess the potential to curtail fungal growth, leading to a significant decrease in disease-related damage to longan fruits and offering a sustainable alternative to the use of chemicals and fungicides in longan farming.

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References

[1] S Subhadrabandhu and C Yapwattanaphun. Lychee and longan production in Thailand. *Acta Hortic.* 2001; **558**, 49-57.
[2] K Kubo. *The geography of the labor-intensive fruit export industry in Southeast Asia: A case study of Thai longan*. *In*: S Sakata (Ed.). New trends and challenges for agriculture in the Mekong region: From food security to development of agri-businesses. Bangkok Research Center, JETRO Bangkok/IDE-JETRO, Bangkok, Thailand, 2019.
[3] N Shao, W Chaiyapa, LK Cheng. *Toward a better longan pricing policy - case study of Chiang Mai and Lumphun provinces, Thailand*. Chiang Mai University, Chiang Mai, Thailand, 2022.

[4] J Visitpanich, C Sittigul and Y Chanbang. *Diseases and pests longan, lychee, and mango*. Thanabaan interprint, Bangkok, Thailand, 2002.
[5] A Sudswang, S Somjai and S Toopgrajank. Management and agricultural technology affecting to longan security in Thailand. *World J. Eng. Tech.* 2018; **6**, 738-51.
[6] A Pipattanapuckdee, D Boonyakait, C Tiyayon, S Pimjai and O Ruangwong. *Lasiodiplodia pseudotheobromae* causes postharvest fruit rot of longan in Thailand. *Australas. Plant Dis. Notes* 2019; **14**, 21.
[7] L Ons, D Bylemans, K Thevissen and BPA Cammue. Combining biocontrol agents with chemical fungicides for integrated plant fungal disease control. *Microorganisms* 2020; **8**, 1930.
[8] MF Rabbee, B-S Hwang, K-H Baek. *Bacillus velezensis*: A Beneficial Biocontrol Agent or Facultative Phytopathogen for Sustainable Agriculture. *Agronomy*. 2023; 13(**3**):840.
[9] R Uasoontornnop, K Junniam, P Maseechan, N Lanongkan, K Chotelersak, K Nantavisai, P Tangteerawatana, M Namsa-Aid and S Puttikamonkul. Antifungal activity of *Streptomyces spectabilis* SP-O2 against aflatoxin producing mold, *Aspergillus flavus*. *Trends Sci*. 2022; **19**, 375.
[10] D Fira, I Dimkić, T Berić, J Lozo and S Stanković. Biological control of plant pathogens by *Bacillus* species. *J. Biotechnol.* 2018; **285**, 44-55.
[11] Y Paopun and P Thanomchat. *Reducing osmium tetroxide usage in preparing plant samples for scanning electron microscope analysis*. Kasetsart University, Bangkok, Thailand, 2016.
[12] T Kaur, R Rani and R K Manhas. Biocontrol and plant growth promoting potential of phylogenetically new *Streptomyces* sp. MR14 of rhizospheric origin. *AMB Expr*.2019;**9**
[13] W Naiumsawang. Soil microorganisms and their inhibition on *Curvularia* sp.; fungal pathogens using dirty panicle disease in rice seed. *Srinakharinwirot Univ. J. Sci. Tech.* 2020; **12**, 101-8.
[14] EN Esteban, M Indart, S Cerone, GD Yaniz, AG Inza, H Landi, S Mogni, M Juliarena and L Igarza. Production and biochemistry-molecular analysis of microbial community fermenting whey as a potential probiotic for use animals. *Open J. Vet. Med*. 2012; **2**, 104-12.
[15] C Ferrer, F Colom, S Frasés, E Mulet, JL Abad and JL Alió. Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. *J. Clin. Microbiol*. 2001; **39**, 2873-9.
[16] C Sittigul, P Nualbunruang and A Sottikul. *Management of major diseases and insect pests of off season longan in Northern area*. Chiang Mai University, Chiang Mai, Thailand, 2004.
[17] K Chaiporn and K Kunasakdakul. Efficacy of endophytic actinomycetes from *Sapindaceae* plants in controlling fruit rot disease of longan. *J. Agr.* 2013; **29**, 239-48.
[18] A Taimaneerak, J Uthaibutra, S Sugaya, W Kunkhum and K Whangchai. Ozone fumigation on sulfur dioxide treated longan for sulfur residue reduction and delaying of pericarp browning as well as disease control in longan fruit during storage. *Food Appl. Biosci. J.* 2018; **6**, 240-52.
[19] A Chumyam, D Kunthawun, B Bussaban, J Uthaibutra and K Saengnil. Effects of ClO_2 fumigation on postharvest fungi and disease development of longan fruit. *Acta Hortic.* 2015; **1088**, 339-44.
[20] A Pipattanapuckdee, P Seehanam, C Tiyayon, D Boonyakait, K Kunasakdakul, S Supakitthanakorn and O Ruangwong. Inhibition of *Lasiodiplodia pseudotheobromae* Causing fruit rot disease of longan by using antagonistic *Bacillus siamensis* RFCD306. *Chiang Mai J. Sci*. 2023; **50**, 1-13.
[21] S Mahadtanapuk. *Fruit discoloring and cracking disease control of export longan production in Phayao province.* University of Phayao, Phayao, Thailand, 2018.
[22] MT Oliveira, AF Specian, CG Andrade, EJ Franca, L Furlaneto-Maia and MC Furlaneto. Interaction of *Candida parapsilosis* isolates with human hair and nail surfaces revealed by scanning electron microscopy analysis. *Micron* 2010; **41**, 604-8.
[23] HLD Almeida Jr, RP Duquia, LASD Castro and NM Rocha. Scanning electron microscopy of the green nail. *Int. J. Dermatol*. 2010; **49**, 962-3.
[24] DM Silva, LR Batista, EF Rezende, MH Fungaro, D Sartori and E Alves. Identification of fungi of the genus *Aspergillus* section *nigri* using polyphasic taxonomy. *Braz. J. Microbiol*. 2011;**42**, 761-73.
[25] W Chitbanchong, V Sardsud, K Whangchai, R Koslanund and P Thobunluepop. Control of rotting and browning of longan fruit cv. biew kiew after harvested by sulphur dioxide treatment under various storage temperatures. *Pakistan J. Biol. Sci*. 2009;**12**, 1438-47.
[26] NS Nysanth, SL Sivapriya, C Natarajan and KN Anith. Novel *in vitro* methods for simultaneous screening of two antagonistic bacteria against multiple fungal phytopathogens in a single agar plate. *3 Biotech* 2022; **12**, 140.
[27] N Ashwini and S Srividya. Potentiality of *Bacillus subtilis* as biocontrol agent for management of anthracnose disease of chilli caused by *Colletotrichum gloeosporioides* OGC1. *3 Biotech* 2014; **4**, 127-36.

[28] LT Wang, FL Lee, CJ Tai and H Kasai. Comparison of *gyrB* gene sequences, 16S rRNA gene sequences and DNA-DNA hybridization in the *Bacillus subtilis* group. *Int. J. Syst. Evol. Microbiol*. 2007; **57**, 1846-50.
[29] M Vehapi, B İnan, S Kayacan-Cakmakoglu, O Sagdic and D Özçimen. Production of *Bacillus subtilis* soil isolate as biocontrol agent under bioreactor conditions. *Arch. Microbiol*. 2023; **205**, 52.
[30] W Ahmed, G Zhou, J Yang, S Munir, A Ahmed, Q Liu, Z Zhao and G Ji. *Bacillus amyloliquefaciens* WS-10 as a potential plant growth-promoter and biocontrol agent for bacterial wilt disease of flue-cured tobacco. *Egypt. J. Biol. Pest Contr*. 2022; **32**, 25.
[31] SP Chowdhury, A Hartmann, X Gao and R Borriss. Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42. *Front. Microbiol.* 2015; **6**, 780.
[32] T Stein. *Bacillus subtilis* antibiotics: Structures, syntheses and specific functions. *Mol Microbiol*. 2005; **56**, 845-57.
[33] Y Jiang, Z Zhang, D C Joyce, S Ketsa. Postharvest biology and handling of longan fruit (*Dimocarpus longan* Lour.). *Postharvest Biology and Technology*. 2002; **26**, 241-252.
[34] M N Michiko and P Zuber. Molecular biology of antibiotic production in *Bacillus*. *Biotechnology* 1990; **10**, 223-40.
[35] T Shigeo, T Yukinori and O Kozo. Activation of antibiotic production in *Bacillus* spp. by cumulative drug resistance mutations. *Am. Soc. Microbiol.* 2015; **59**, 7799-804.
[36] N Sawatphanit, W Sutthisa and T Kumlung. Bioformulation development of *Bacillus velezensis* strain N1 to control rice bacterial leaf blight. *Trends Sci*. 2022; **19**, 6315.
[37] DT Tuyen, NT Trung, NT Thao, NSL Thanh, NPD Nguyen, NTA Tuyet, NT Cuong, SS Chan, KS Khoo, PL Show. Antifungal activity of secondary metabolites purified from *Bacillus subtilis* isolated in Vietnam and evaluated on *in vitro* and *in vivo* models. *Int. Biodeterioration Biodegradation* 2023; **179**, 10588.
[38] K Ntushelo, LK Ledwaba, ME Rauwane, OA Adebo and PB Njobeh. The mode of action of bacillus species against fusarium graminearum, tools for investigation, and future prospects. *Toxins* 2019; **11**, 606.
[39] CH Jiang, F Wu, ZY Yu, P Xie, HJ Ke, HW Li, YY Yu and JH Guo. Study on screening and antagonistic mechanisms of *Bacillus amyloliquefaciens* 54 against bacterial fruit blotch (BFB) caused by *Acidovorax avenae* subsp. *citrulli*. *Microbiol. Res*. 2015; **170**. 95-104.
[40] FC Ayduki, LC Rozwalka, MAC Zawadneak and FL Cuquel. *Bacillus subtilis* and *Trichoderma harzianum* to control postharvest pathogens of strawberry fruits *in vitro. Acta Hortic*. 2016*;* **1117**, 181-4.