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Biological control of bacterial wilt in pathumma; Curcuma alismatifolia

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ABSTRACT

BACKGROUND AND OBJECTIVES: In Thailand, bacterial wilt is one of the most severe diseases that affects pathumma, also known as *Curcuma alismatifolia* Gagnep. Biological control was proposed to control this disease with antagonistic bacteria. The current study was conducted to screen for antagonistic microorganisms capable of inhibiting the pathogenic bacteria and to evaluate the beneficial effect of antagonistic bacteria on pathumma *in vivo*.

METHODS: Antagonistic bacteria were isolated from soil samples obtained from several locations in Thailand and screened for antibacterial activity. Next, the optimal conditions for the growth of antagonistic bacteria were determined. The production of anibacterial substances were then characterized. The potential of antagonistic bacteria to reduce the growth of plant pathogens was evaluated under greenhouse conditions.

FINDINGS: In total, 102 bacterial isolates were isolated using tryptic soy medium. After evaluating their capacity to inhibit the growth of the wilt-causing bacteria using the paper disc diffusion assay, it was found that three bacterial isolates, *Bacillus subtilis* SP15, *Pseudomonas mosselii* SP38, and *Pseudomonas mosselii* SP46 showed high ability to inhibit growth of the wilt-causing bacteria *Enterobacter asburiae* JK1, JK2, JK3, JK4, *E. dissolvens* JK5 and E. hormachei JK6. The optimal conditions for all antagonistic bacterial isolates were 25 or 30 degrees Celcius, at potential of hydrogen 7-8 in modified tryptic soy medium containing 0.5 percent (weight /volume) glucose or sucrose and 1.5 or 2 percent (weight/volume) peptone. The antagonists were able to produce siderophores and phenazines. Under greenhouse experiments, the mixed cultures of antagonistic bacterial isolates could reduce the wilt disease incidence, and the number of pathogenic bacteria declined compared with the diseased control plants. In addition, it was discovered that soil materials provided the best carrier materials for the successful formulation of the mixed culture of antagonistic bacteria.

CONCLUSION: This study revealed that the selected antagonists were beneficial for controlling wilt disease in pathumma. This is the first scientific study on the control of wilt-disease causing *Enterobacter* spp. in *C. alismatifolia* Gagnep. in Thailand using antagonistic bacteria. It is expected that these antagonistic bacteria be useful in wilt disease management in the field for friendly and sustainable agriculture.

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INTRODUCTION

Curcuma alismatifolia Gagnep. (Gagnepain) is commonly referred to as the Siam tulip or pathumma, and it is a native plant of Southeast Asia. An attractive flower, it is reminiscent of a cluster of tulips (Ruamrungsri, 2015). Pathumma belongs to the family Zingiberaceae and is a plant that is frequently found in northern and northeastern Thailand. Both in Thailand and worldwide, the popularity of these beautiful plants has increased. Rhizomes are exported for around United Stae Dollar (USD) 38.1 million each year (Boontiang, 2021). Moreover, scientific documents have revealed that *C. alismatifolia* leaves have effective antidiarrheal and antioxidant properties as do other Curcuma plants (Ruamrungsri, 2015). Pathumma is susceptible to wilt disease, which has caused widespread destruction. The wilt bacteria overwinters in plant debris in soil, seeds, vegetative propagative materials, or insect vectors. They enter the plants through wounds that expose vascular elements and multiply and spread in the latter. They spread from plant to plant through the soil, handling and tools, direct contact of plants, or insect vectors. Control of bacterial vascular wilt is difficult and depends primarily on the use of crop rotation, resistant varieties, bacteria free seed or other propagative material, removal of the infected plant debris, and proper sanitation. Among several wilt diseases causing Gram-negative plant pathogens, *R. solanacearum* has been intensively studied both in biochemical and genetical aspects and has long been recognized as a model system for the analysis of pathogenicity. *Ralstonia* (formerly *Pseudomonas*) *solanacearum*, the causal agent of bacterial wilt, was ranked as one of the world's most important phytopathogenic bacteria prevalent in tropical, subtropical and some warm regions of the world. Moreover, it can also occur in cool temperate areas. The original habitat of this organism is probably the tropics, but its increasing occurrence in geographical regions with a prevailing temperate climate has resulted in speculations about its adaptation to these conditions, and its establishment. The bacteria can cause devastating problems in agriculture resulting in major losses to farmers (Promsai *et al*., 2012). *Enterobacter* sp. is a Gram-negative, rod-shaped, and facultative anaerobic bacteria. Based on molecular and taxonomy investigations, it was previously assigned to the genus Erwinia but has since been reassigned to the genus *Enterobacter*. According to several findings, *Enterobacter* spp. could cause bacterial wilt in crops globally. Some strains of *E. mori* have been associated with wilt disease of strawberry in China (Ji *et al*., 2023). Internal papaya fruit yellowing triggered by *E. sakazakii*. *Enterobacter* sp. causes patchouli wilt disease in Indonesia (García-González *et al*., 2018; Zulfadli *et al.,* 2023). Soil-borne pathogens are difficult to control because they attack plants via soil over long periods of time. Chemical control has several limitations, including a negative public attitude and associated environmental concerns. Agrochemical treatment adversely impacts the environment, with residue persistence, the reduced variety of nontarget organisms, resistance, and the shifting of the farming area (Rodelo-Torrente *et al*., 2022). The use of biological control agents in sustainable agriculture is growing as a result of the practice to limit the use of pesticides and the environmental residues they produce (Ehzari *et al*., 2022). Future sustainable agriculture will increasingly rely on the integration of biotechnology with traditional agriculture (Abo-Elyousr *et al*., 2022; Samimi *et al.,* 2023). The most sustainable and environmentally acceptable control may be achieved using biocontrol agents due to the effort to reduce the use of agrochemicals (Ilmasari *et al*., 2022) and their residues in the environment and in food (Sánchez-Hernández *et al*., 2022). Plant pathologists have been fascinated by the idea that such microorganisms could be used as environmentally friendly biocontrol agents that have no effect on nontarget organisms (Ruanpanun and Nimnoi, 2020; Sivakumar *et al.,* 2022; Dhayalan and Sudalaimuthu, 2021). Antagonistic microorganisms have emerged as an alternative management strategy. Among several biological control agents, *Bacillus* and *Pseudomonas* are potential agents to use against plant pathogens, since they can produce various substances such as antibiotics and siderophores (Promsai *et al*., 2023; Zhang *et al*., 2023). Complete eradication of wilt disease in the pathumma production chain has still not been achieved. Despite extensive studies on the pathogenesis, epidemiology, and control, bacterial wilt remains an important problem worldwide. The objectives of the current study were to screen for antagonists that could inhibit wilt-causing bacteria and to evaluate the beneficial effect of antagonistic bacteria on pathumma *in vivo* in 2012.

MATERIALS AND METHODS

Wilt-causing bacteria cultures and plant materials

Wilt-causing bacteria were isolated from infected pathumma rhizomes. The Triphenyltetrazoliumchloride (TZC) medium containing 0.1% weight per volume, (w/v) peptone, 0.05 percent (%) (w/v) glucose, 0.01% (w/v) pancreatic digest of casein, and 0.005% (w/v) 2, 3, 5 – triphenyltetrazoliumchloride (Sigma–Aldrich, USA) was used as the culture medium. The selected wilt-bacteria were used to reinfect pathumma plants and were conventionally and molecular identified described by Promsai *et al*. (2012). The pathogenic bacteria used in this study were *Enterobacter asburiae* JK1, JK2, JK3, and JK4, *E. dissolvens* JK5, *E. hormachei* JK6, and *Ralstonia solanacearum* R227 as standard cultures obtained from the Ministry of Agriculture and Cooperatives, Thailand. Pathumma rhizomes were obtained from the Bua Lai Pathumma Garden, Chiang Mai, Thailand. Pathumma rhizomes were cultivated at the beginning of May in greenhouse conditions at the Faculty of Science, Chiang Mai University.

Isolation and screening of antagonistic bacteria

Soil samples from different parts of Thailand were collected for bacterial isolation using tryptic soy broth (TSB, Merck, Germany). One gram of each soil sample was put into 5 mL of TSB and then incubated at 30, 37, and 45 degrees Celsius (°C) for 48 hours (h). The microbial cultures were streaked on tryptic soy agar (TSA) under the previous conditions to achieve the pure isolate. Random colonies were selected from agar plates in a variety of sizes, shapes, and colors. For further investigation, each pure isolate was kept at 4°C and -20°C in 20% glycerol. Agar disc diffusion was used to test each bacterium's ability to inhibit the growth of the wilt-causing bacteria. Each isolated bacterium was cultivated in TSB at 30°C for 48 h. After that, the culture broth was centrifuged at 3,000 x*g* for 10 minutes (min.) Concomitantly, the pathogenic bacteria were cultivated in TZC broth at 30°C for 12 h (At 660 nm, the optical density was 0.5, corresponding to approximately $1x10^6$-$1x10^7$ colony forming units per milliliter (cfu/mL) prior to swabbing onto TZC agar plates. A paper disc with a diameter of 6 mm (Macherey-Nagel, Germany) was immersed into the obtained bacterial supernatant before being put onto the surface of the previously prepared plate. A negative control was performed using the TSB medium. The tested plates were incubated for 24-48 h at 30°C before being checked for the presence of clear inhibitory zones. The radius of the inhibition zone, measured in millimeters, was used to evaluate the degree of bacterial wilt inhibition. Accordingly, the antagonistic bacteria were determined from among the bacterial isolates that initially underwent preliminary screening and demonstrated positive *in vitro* inhibitory activity. The potential antibacterial agents that showed a high level of inhibition were kept and further evaluated.

Identification of antagonistic bacteria

The selected bacterial isolates that exhibited a high level of inhibition were identified using the conventional and molecular methods.

Conventional method

According to traditional morphological characteristics such as the colony growth pattern, pigment production, and spore formation, antagonistic strains (the isolates SP15, SP38, SP46, and SP58) were preliminarily identified. Biochemical characteristics were observed, i.e., catalase (Univar, Australia), lysine decarboxylase (Sigma–Aldrich, USA), phenylalanine deaminase (Sigma–Aldrich, USA), oxidase, motility, methyl red (Univar, Australia), and Voges-Proskauer (Univar, Australia) test; production of indole (Univar, Australia), lecithinase (Sigma–Aldrich, USA) and gas from glucosa; utilization of gelatin (Univar, Australia), starch (Univar, Australia) and casein (Univar, Australia); growth in TSB containing 2, 5, 7, and 10% NaCl (Univar, Australia); growth at 30, 40, 50, and 55°C; utilization of citrate, adonitol, alanine, arginine, mannitol, rhamnose, ribose, sucrose, trehalose, and xylose (Univar, Australia) (Promsai *et al*., 2018).

Molecular method

Total genomic DNA samples of the isolates SP15, SP38, SP46, and SP58 were extracted from fresh culture following Chumphon *et al*., (2022) with some modifications. A 16S rDNA (~1.5 kb) was amplified using the universal primer, 20F (5'-AGTTTGATCCTGGCTC-3') and 1540R (5'-AAGGAGGTGATCCAGCC-3') (Promsai *et al*., 2023). The PCR conditions consisted of a 1 min. initial denaturation at 94°C, followed by 35 cycles of a 1 min. denaturation at 94°C, a 2 min primer annealing at 58°C, a 2 min extension at 72°C, and a final 7 min extension at 72°C. PCR (polymerase chain

reaction) products were purified using a Gel/PCR DNA Fragments Extraction Kit (Geneaid, Catalog no. DF100). The obtained PCR products were analyzed by First Base Company, Malaysia, for nucleotide sequencing. The BLAST analysis was performed on the nucleotide sequences of the 16S rRNA gene to determine their identities. The ClustalW software was used to align the nucleotide sequences. The neighbor-joining (NJ) phylogenetic tree was constructed using the program in MEGA X. A topological study was conducted with 1,000 bootstrap repetitions.

Production of siderophores

Screening of siderophore production

Production of siderophores was preliminarily examined according to the modified universal chrome azurol S (CAS, Sigma–Aldrich, USA) assay method. Siderophore production was tested on modified Gaus No.1 medium (MGs) containing CAS. Pure isolate samples of SP15, SP38, SP46, and SP58 were stabbed on CAS-agar and incubated at 30°C for 72 h. Orange, purple, or yellow surrounding colonies were regarded as siderophore-producing isolates (Hofmann *et al*., 2021). The bacterial isolates which changed the medium color, were secondarily detected for siderophore type and concentration.

Detection of the chemical nature of siderophores

The positive isolates were cultured in MGs-1 broth and incubated at 30°C for 48 h with agitation of 150 rpm. Turbimetric monitoring of the population density at 660 nm was performed throughout the incubation. The centrifugation at 3,000 x*g*, 4°C, for 10 min was conducted to separate the bacterial cells. The obtained supernatants were concentrated using an ultrafiltration technique prior to the detection of the nature of the siderophores (catecholates and hydroxamates) using chemical assays.

Catecholates: Catecholate-type detection was performed according to Grobelak and Hiller (2017) with some modifications. A mixture comprising 50 microliters (µL) of culture supernatant, 50 µL of 0.5 molarity (M) hydrochloric acid (HCl) (RCI Labscan, Thailand), and 50 µL nitritemolybdate reagent was poured into 96-well microplates. When the yellow color appeared, 50 µL of 1 M NaOH (RCI Labscan, Thailand) was added. To allow a complete reaction, the plates were continuously incubated at room temperature for 5 min. The absorbance of red pigment was measured at 500 nanometers (nm) with the uninoculated media mixed with a reagent as a blank. The level of catecholate produced was estimated against a standard of 2,3-dihydroxybenzoic acid (Sigma–Aldrich, USA).

Hydroxamates: Hydroxamate-type siderophore detection was performed using ironperchlorate assay (Radhakrishnan *et al*., 2014) with some modifications. A total of 30 mL of the supernatant was added to 150 µL of ferric perchlorate (Univar, Australia) solution and incubated at ambient temperature for 5 min. The presence of a hydroxamate-type siderophore was shown by the development of an orange-red color. Measurement of absorbance at 480 nm was used to calculate the concentration of hydroxamate. The uninoculated medium mixed with the reagent was used as a blank. The level of hydroxamate produced was estimated against a standard deferoxamine mesylate (Sigma–Aldrich, USA).

Production of phenazines

The production of phenazine antagonistic bacterial isolates SP15, SP38, SP46, and SP58 were determined using thin layer chromatography (TLC) (Karmegham *et al*., 2020). Each isolate was streaked onto King's B (KB) agar plates. To remove the bacterial cells, sterile water was used to wash the bacterial colonies grown on agar plates. The tiny pieces of agar were excised and collected in an Erlenmeyer flask. The suspension was added with 12 mL of chloroform and was then incubated at 37°C for 2 h with shaking. Mixing the chloroform layer with an equivalent volume of 0.1 M NaOH was done. The phenazine compound shifted to the aqueous phase. The chloroform fraction was air-dried and dissolved in methanol (RCI Labscan, Thailand). For the chromatographic analysis, the methanol extract was spotted carefully on TLC plates (silica gel 60 F_{254}). The silica plates were then placed in a mobile-phase solvent containing toluene/acetone (3:1 volume per volume, v/v) (RCI Labscan, Thailand) and detected under UV light for phenazine observation.

Optimization of inhibitory substances produced by antagonistic bacteria

Four antagonistic bacteria (SP15, SP38, SP46, and SP58) were selected for optimization. Various carbon and nitrogen sources were added to the

basal medium in order to evaluate the medium optimization process. The zone of inhibition (ZOI) was used to determine the effectiveness of the improved medium (Promsai *et al*., 2023).

Carbon sources

The antagonistic strains were grown at 30°C in an Erlenmeyer flask containing 50 ml modified TSB in which the glucose was substituted with sucrose, maltose, fructose, galactose, lactose, mannitol or sorbitol (Sigma–Aldrich, USA). After 48 h of incubation, each culture was centrifuged to collect the supernatant for further use with the agar disc diffusion method against four isolates of wilt-causing bacteria *Enterobacter asburiae* JK1, JK2, JK3, and JK4 The modified TSB with various carbon sources substituted for the glucose was used as a negative control. The most effectively inhibiting carbon source was selected for further evaluation of the optimal concentration: 0.1, 0.5, 1.0, 1.5, 2.0, or 2.5% (w/v).

Nitrogen sources

The antagonistic strains were grown at 30°C in an Erlenmeyer flask containing 50 ml modified TSB in which peptone was substituted with yeast extract, tryptone, corn flour, corn steep liquor, ammonium dihydrogenphosphate, $NH_4(H_2PO_4)$, or ammonium nitrate (NH_4NO_3) (Himedia, India). The modified TSB was a negative control. The most effectively inhibiting carbon source was selected for determination of the optimal concentration; 0.1, 0.5, 1.0, 1.5, 2.0, or 2.5 % (w/v). Cultures were centrifuged to obtain the supernatant and tested for the growth inhibition of pathogenic bacteria.

Media potential of hydrogen (pH)

To determine the optimal media pH of the culture medium, antagonistic strains were cultivated in 50 mL modified medium with the pH adjusted to 4, 5, 6, 7, 8, 9, 10, or 11. The obtained supernatants were tested for the growth inhibition of pathogens. The modified TSB samples at various pH levels were used as a negative control.

Temperature

The tested temperatures were 25, 30, 37, or 45°C. The antagonistic strains were cultivated in 50 mL of modified medium. After 48 h of incubation, each culture broth was centrifuged to obtain the supernatant, which was further investigated for its activity with the ZOI. The modified TSB with the optimal carbon source, nitrogen source, and media pH was used as a negative control. The antagonistic strain was then cultivated in the most effective medium after finding the optimal medium for the optimal growth of cells and antibacterial activity.

Evaluation of antagonistic bacteria in the control of bacterial wilt under greenhouse conditions

Three antagonists were mixed and cultivated in 100 ml of modified TSB obtained from the earlier study. The antagonist cultures were grown at 30°C for 24 h. To prepare wilt-bacterial inocula, each pathogenic bacterial isolate was grown in TZC broth at 30°C for 24 h. The concentrations of the antagonistic mixed-culture and wilt-causing bacteria were approximately $1x10^7$ - $1x10^8$ cfu/mL. The rhizomes were planted in 15cm-diameter plastic bags containing sterile soil created from commercial soil mixed with 3:1:1 ratios of chaff and coir. The rhizomes and pseudostems of pathumma were wounded prior to drenching with the bacterial inocula. The experiments were conducted in a greenhouse at the Faculty of Science, Chiang Mai University. Three experiments were performed.

Experiment 1

Pathumma rhizomes and soil in pots were co-applied with antagonistic and pathogenic bacteria prior to cultivation.

Experiment 2

Samples of shooting pathumma were treated with both antagonistic and pathogenic microorganisms.

Experiment 3

Before pathumma cultivation, pathogenic bacteria were introduced to rhizomes and the soil, and after plant germination, mixed cultures of antagonistic bacteria were introduced to the plant pots.

The number of both antagonistic bacteria and pathogenic bacteria was assessed in each trial using the serial dilution method and spread on TSA and TZC agar plates. The viable cell count of bacteria and growth plant evaluation were performed every 15 days for 5 months. All treatments were replicated three times. To count the number of live bacterial cells in the soil mix, core samples from each infested bag were taken using a spatula. A test tube containing

Table 1: Assessments of antimicrobial activity, siderophores and phenazine of selected antagonistic bacteria[a,b]

Bacterial isolate	Sources	Growth inhibition of wilt bacteria (mm)	Siderophore production				Phenazine production
			Hydroxamate conc. (µM)		Catecholate conc. (µM)		
			Culture broth	Concentrated culture broth	Culture broth	Concentrated culture broth	
SP15	Hot spring	13.5±0.5a	5.00±2.12a	10.00±3.68a	1.64±0.5a	1.90±0.39a	-[c]
SP38	Rain tree	19.0±1.9b	27.12±5.68b	41.00±3.37b	3.50±0.3c	4.24±0.52c	+
SP46	Banyan tree	20.0±3.5b	45.87±6.12c	63.87±6.57c	1.75±0.1a	2.25±0.32a,b	+
SP58	Banyan tree	19.0±3.5b	49.50±7.64c	70.75±4.49d	2.69±0.9b	2.71±0.42b	+

[a] Results are shown as mean ± standard error
[b] Means followed by the same letter within a column are not significantly different (Tukey's Test, P=0.05)
[c] Phenazine production; " + " phenazine positive, " - " phenazine negative

9 mL of sterile water and 1 g of soil was added. The solution was agitated and serially diluted prior to spreading on TSA and TZC agar plates containing 100 micrograms per milliliter (µg/mL) cycloheximide (Sigma–Aldrich, USA). The agar plates were then incubated at 30°C for 48 h, and the number of bacteria was calculated in cfu units. The bacterial population data were transformed to $\log_{10}$ values (Swe *et al.*, 2023). The numbers of total bacteria and wilt-causing bacteria were counted on the TSA and TZC plates, respectively, with morphological characteristics observed. Consequently, the numbers of antagonistic bacteria were calculated by subtracting the number of wilt-causing bacteria from the total number of bacteria. The occurrences of wilt disease and the growth of plants were assessed. The disease incidence (DI) was calculated using the following equation (Vinayarani and Prakash, 2018).

DI = Number of infected plants/number of all inoculated plants x 100.

All experiments were conducted in 3 years of planting (trials 1, 2 and 3).

Designation of mixed culture products

Three carrier materials, chaff, coir, and soil were accessed for their capacity to support the growth of antagonistic bacteria. The isolates SP15, SP38, and SP46 were cultured on TSB and incubated at 30°C for 24 h. To separate the bacterial cells, the culture was then centrifuged at 3,000 x g for 10 min. The cell pellets were mixed with sterile distilled water. The individual suspension or a suspension made from a mixture of the four antagonistic cells containing $1x10^8$ cfu/mL was added into 100 g of the carrier materials and mixed well under sterile conditions. The materials were then incubated at ambient temperature for 60 days. The viable cell count was determined every 15 days of storage.

Statistical analysis

A one-way analysis of variance (ANOVA) was used to statistically analyze the data using the SPSS software version 20 for Windows. Tukey's test was conducted, and the means were compared at $P < 0.05$.

RESULTS AND DISCUSSION

Isolation and screening of antagonistic bacteria

One hundred and two bacterial isolates were isolated from seven soil samples obtained in Thailand at various locations and 55 isolates were Gram-positive and rod-shaped, and 47 isolates were Gram-negative and rod-shaped. From the result of the growth inhibition of the plant pathogens (*E. asburiae* JK1, JK2, JK3, JK4, *E. dissolvens* JK5 and *E. hormachei* JK6), only four isolates (SP15, SP38, SP46, and SP58) that were isolated from soils collected from the Sankumpang hot spring, Srinakarin Dam, and Bangsai Arts and Crafts Centre displayed a high ability to make a clear inhibitory zone of 13-23 mm in diameter (Table 1). The four bacterial antagonists showed different degrees of antagonism, and there was a slight difference between the six strains of pathogenic bacteria. The results indicated that the microorganisms isolated from soils had a high capacity to suppress the growth of wilt-bacterial strains. This conclusion was supported by the fact that plant-growth-promoting rhizobacteria (PGPR) exhibited the colonization of roots, encouraging plant

development and/or lowering the prevalence of plant disease (Vinayarani and Prakash, 2018). Dong *et al*. (2023) revealed that *Bacillus velezensis* RC116 could inhibit the growth of wilt-disease bacteria in tomato. RC116 not only produced protease, amylase, lipase, and siderophores but also secreted indoleacetic acid and dissolved organophosphorus *in vivo*. The biocontrol marker genes associated with antibiotics biosynthesis could be amplified in the RC116 genome. Extracellular secreted proteins of RC116 also exhibited strong lytic activity against *Ralstonia solanacearum* and *Fusarium oxysporum* f. sp. *Lycopersici*. This antagonist was likewise successful at preventing the pathogen's growth and survival both *in vitro* and *in vivo*. It may be possible that there was no disease in the area where the antagonists were isolated. The prospective antibacterial agents with high levels of inhibition were maintained, and further *in vivo* evaluation was undertaken in the greenhouse.

Identification of antagonistic bacteria

The bacterial isolate SP15 was Gram-positive and rod shaped with ellipsoidal endospores located at the cell terminal, oxidase negative, catalase positive, and non-motile. Its microscopic size was 0.5-1.5 µm x 1.0-3.0 µm (width x length). The strains SP38, SP46, and SP58 were Gram negative, short-rodshaped, facultative anaerobe, oxidase positive, and motile. Their microscopic sizes were 0.5-1.0 x 1.0-2.5 µm (width x length). Three bacterial isolates, SP38, SP46 and SP58 were classified as *Pseudomonas,* since they produced oxidase and could not ferment lactose. However, they could not be identified to the species level by using the conventional method due to the complexity of the genus *Pseudomonas*. All strains were repeatedly confirmed using 16S rRNA gene determination. Partial 16s RNA sequencing was used to identify the antagonistic bacterial isolates to the species level and to determine whether there were clusters of similar organisms. From the Genbank database similarity and a neighbor-joining tree (Fig. 1), it was revealed that the isolates SP15, SP38, SP46, and SP58 were *Bacillus subtilis*, *Pseudomonas mosselii*, *Pseudomonas mosselii*, and *Pseudomonas aeruginosa*, respectively. Identification of the prospective biocontrol agents requires the efficient isolation of microorganisms from the rhizosphere of various crops. The largest potentially most promising group of rhizobacteria used in the biocontrol of plant diseases is fluorescent *Pseudomonas* spp., one of the bacterial biocontrol agents. These bacteria rapidly and actively colonize the root, thereby becoming excellent soil inocula. (de Oliveira *et al*., 2022). *Bacillus* spp. have been studied extensively for many years. *Bacillus* spp. and *Pseudomonas* spp. as biocontrolling organisms agreed with other observations. For example, these following references reported biocontrol activity. *B. licheniformis* and *B. aerius* had a high potential for antifungal activity against *Podosphaera xanthii*, a pathogen causing powdery mildew in cucumber (Abo-Elyousr et al., 2022). *B. subtilis* EA-CB0015 exhibited the growth inhibition of *Botrytis cinerea* and *Colletotrichum* sp. (Arroyave-Toro *et al*., 2017). Also, root bacteria including *Pantoea*, *Cronobacter*, and *Pseudomonas* could effectively control *Fusarium oxysporum* f. sp. *cucumerinum*, wilt-causing fungi in cucumber (Zhang *et al*., 2023). However, *Bacillus* and *Pseudomonas* usually inhibited some fungal and bacterial pathogens. The scientific documentation of these bacteria in *Enterobacter* control is limited. The current study seems to be the first report showing *B. subtilis* and *Ps. mosselii* antagonism to *Enterobacter* in pathumma and suggests that the selected isolates have excellent promise for pathumma bioprotection or for use in integrated disease management. To demonstrate effectiveness under bacterial mechanisms, more research is still required.

Production of siderophores

Four isolates of antagonistic bacteria were preliminarily screened for siderophore production on MGs-1 medium containing CAS. All antagonists were grown on CAS agar and showed yellow- and pink-zone surrounded bacterial colonies. The culture supernatants were further characterized for hydroxamate or catechol compounds. The level of hydroxamate and catecholate production was 5.0-49.5 µ*M* and 1.6-3.5 µ*M*, respectively (Table 1). Between the antagonistic isolates, the capacity to produce the siderophore was broadly distributed. Moreover, when the twofold concentrated broth cultures of each antagonistic bacterium were tested, the ultrafiltrates of bacterial supernatants demonstrated a higher level of siderophore concentration in the ranges 10.0-70.8 µ*M* and 1.9-4.2 µ*M*, respectively. These results indicated that the ultrafiltration technique was very beneficial for metabolite concentration. The results from the qualitative and quantitative estimations

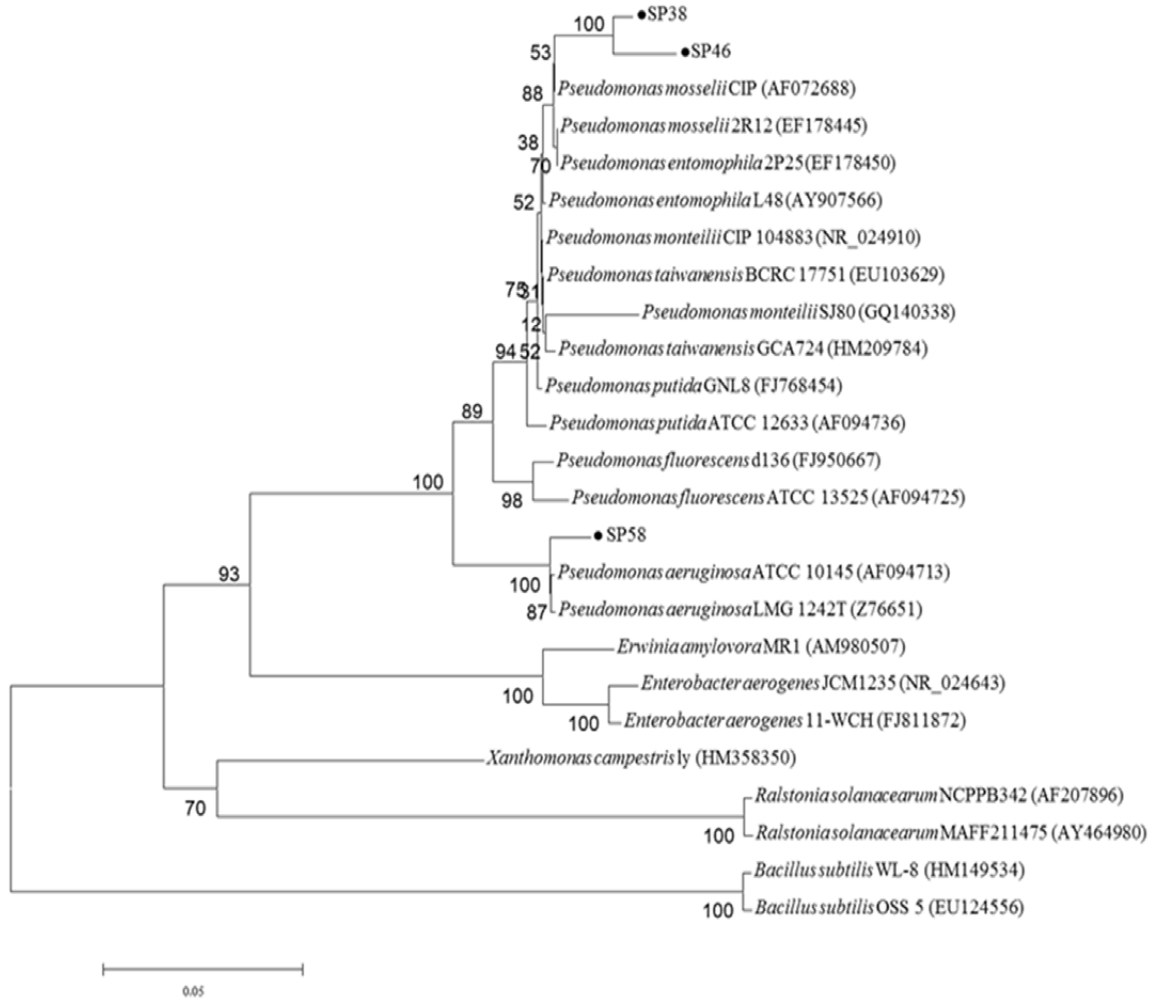

Fig 1: A neighbour-joining tree illustrating the position of the antagonistic bacterial isolates including SP38, SP46 and SP58 based on 16S rRNA gene sequences was constructed. 1,000 replicates were used to generate the bootstrap values, and the bar indicates 0.02 substitution per nucleotide position. In parenthesis, the GenBank accession numbers were displayed.

of siderophore production by *B. subtilis* SP15, *Ps. mosselii* SP38, *Ps. mosselii* SP46, and *Ps. aeruginosa* SP58 showed that they were powerful producers of siderophores. Several published studies have reported the ability of *Bacillus* spp. and *Pseudomonas* spp. to produce siderophores. Radhakrishnan *et al.* (2014) demonstrated that a bacterium producing siderophore *Bacillus* sp. SD12 had a high ability to reduce the growth of *Phythium* sp., *Aspergillus niger*, and *Fusarium solani*. This bacterium was isolated from iron factory soil. Siderophores are themselves growth inhibitors of various phytopathogenic fungi, such as *Phytophthora parasitica*, *Phythium ultimum*, and *Fusarium oxysporum* veri *dianthi*. A direct correlation was established *in vitro* between siderophores' synthesis in fluorescent pseudomonads and their capacity to inhibit the germination of chlamydospores of *F. oxysporum*. In antifungal activity testing, the partially purified siderophores inhibited the growth of phytofungal pathogens such as *Fusarium solani*, *Aspergillus niger*, and *Phythium* sp. Subramanium and Sundaram (2020) discovered that siderophore producing *Pseudomonas* spp. (*Ps. aeruginosa* PSA01 and *Ps. fluorescences* PSF02)

isolated from rhizospheric soil could inhibit the growth of *Fusarium oxysporum*. Both isolates proved to produce siderophores and solubilized phosphate. A pot experiment resulted in the promotion of the growth of *Arachis hypogaea*. The inoculated PSA01 and PSF02 significantly increased in the root length, shoot length, fresh weight, dry weight, and iron and oil content as compared to the untreated control. This realization is particularly considered to be important to understand siderophore production by *P. aeruginosa*. From the results of the siderophore characterization, although the bacteria grew well in the culture medium, and some antagonistic strains secreted trace amounts of siderophore, they were still considered positive siderophore-producing isolates. Siderophore is a secondary metabolite; a microorganism will produce secondary metabolites whenever its growing rate slows. Because of this, there is no relationship between siderophore concentration and bacterial turbidity. In addition, *Bacillus subtilis* SP15 showed a large positive reaction on CAS agar, but it produced small amounts of hydroxamate and catecholate. It is suggested that detection in liquid media should be used to confirm siderophore production. The sensitivity of the chemical reaction method was higher than the CAS agar plate assay in terms of the low accumulation of siderophores (Arora and Verma, 2017). The resulting siderophore production suggested the hypothesis that bacteria that promoted plant growth or suppressed disease were dependent on the iron concentration. The formulation of high siderophore-producing *P. aeruginosa* F2 and *P. fluorescens* JY3 strains reduced the damping-off incidences caused by *F. oxysporum* and *Rhizoctonia solani* (Abo-Zaid *et al*., 2020).

Phenazine production

By using pigment detection under UV light at 254 nm, the antagonists were initially screened for the synthesis of phenazine with KB plates. Among the four antagonists, only three strains, *Ps. mosselii* SP38, *Ps. mosselii* SP46, and *Ps. aeruginosa* SP58, showed fluorescent UV absorbance. The only positive pigment-producing isolates *Ps. mosselii* SP38, *Ps. mosselii* SP46, and *Ps. aeruginosa* SP58 were then tested on TLC and the antagonistic substance chromatographs were compared to standard chemicals. It was found that both the antagonistic and reference compounds behaved similarly. UV-light analysis of the silica plates revealed a 2-hydroxyphenazine spot (R_f = 0.39) for substances from *Ps. mosselii* SP46 and *Ps. aeruginosa* SP58. Phenazine-1-carboximide (PCA) spots (R_f = 0.82) were displayed only in the standard compound. Meanwhile, *Ps. mosselii* SP38 and the standard compound showed the spot at R_f = 0.65. It was found that only the antagonistic bacteria *Ps. mosselii* SP38, *Ps. mosselii* SP46, and *Ps. aeruginosa* SP58 could produce phenazine derivatives. However, the production and characterization of phenazine compounds should be further studied using HPLC (high-performance liquid chromatography), mass spectrometry, or NMR (Nuclear magnetic resonance spectroscopy) techniques. Earlier studies of phenazine-producing pseudomonads have been published. Karmegham *et al*. (2020) revealed that *P. aeruginosa* VSMKU1 had a strong and broad-ranging antifungal metabolite and could be applied to treat sheath blight disease in rice. The growth of *R. solani* could be inhibited by antifungal agents, and the presence of the gene encoding phenazine-1-carboxamide in the strain VSMKU1 contributes to this fact. The phenazine compound from VSMKU1 significantly arrested the growth of *R. solani* compared to carbendazim by the well diffusion method. These results emphasized that the VSMKU1 isolate can be used as an alternative potential biocontrol agent against sheath blight of rice, instead of using commercial fungicides such as validamycin and carbendazim, which cause environmental pollution and health hazards. The antibacterial substance produced by *Streptomyces* B-92 was characterized as 4-(3S,4R,5S)-3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2Hpyran-2-yloxy) phenazine-1-carboxylic acid. An *in vitro* antimicrobial assay showed that bioactive compound of *Streptomyces* sp. strain UICC B-92 exhibited antagonistic activities against two Gram-positive bacteria, *B. cereus* strain ATCC 10876 and *S. aureus* strain ATCC 25923. The bioactive compound of Streptomyces sp. strain UICC B-92 is suggested as a new compound based on glycoside structure and its position. This novel compound possesses potential in the discovery of new agrichemical and medicinal compounds (Pratiwi *et al*., 2020). Four antagonistic bacterial isolates demonstrated the capacity to synthesize siderophore or phenazine, two highly effective antibacterial substances. This suggested that the antagonistic strains have great potential

Table 2: Summary results of antimicrobial product optimization

Antagonistic isolate	Optimum compositions and conditions					
	C-source	C-source (%)	N-source	N-source (%)	pH	Temperature (°C)
SP15	glucose	0.5	peptone	2.0	8	30
SP38	sucrose	0.5	peptone	2.0	7	25
SP46	glucose	0.5	peptone	1.5	7	25
SP58	sucrose	0.5	peptone	1.5	7	25

for application as a biocontrol agent in pathumma plant *in vivo*. To demonstrate the potential of these organisms for the biocontrol of undesirable microbes and for the encouragement of plant growth, which may be important in agricultural fields in the future, additional in-depth research is needed. The inhibitory substance might be derived from a chemical technique or genetically modified microbes. The substance or an analogous one could be applied as a pesticide.

Optimization of the inhibitory substances produced by antagonistic bacteria

The optimization of the media and growth conditions can significantly improve the yields of microbial metabolites and reduce the overall cost of microbial metabolites (Bellebcir *et al*., 2023). This current study evaluated the type and concentration of carbon sources and nitrogen sources for applying the modified media that exhibited the highest capacity to inhibit the growth of plant pathogens. The pH of the media and the temperature of cultivation were also studied.

Effect of the carbon sources

The influence of carbon sources on the production of antibacterial activity was investigated using basal medium in which glucose was substituted by various carbon sources. The zone of inhibition was assessed. The best type and concentration of the carbon sources were selected under the highest value of antimicrobial activity that was significantly different. If the highest value of ZOI was not significantly different, the suitable source was selected for not only being low cost but also for being more commercially available. The highest antimicrobial activity production levels of *B. subtilis* SP15, *Ps. mosselii* SP38, *Ps. mosselii* SP46, and *Ps. aeruginosa* SP58 were obtained in media containing 0.5% glucose, 0.5% sucrose, 0.5% glucose, and 0.5% sucrose (w/v), respectively (Table 2). It is interesting to note from the optimization results that these antagonists required a simple carbon source such as glucose and sucrose at low concentration. This result was associated with the findings of Mosquera *et al*. (2014) that glucose was the best carbon sources for antimicrobial substance production against the phytopathogen *Mycosphaerella fijiensis*. The antimicrobial activity was 1.2-fold higher in the modified culture medium.

Effect of the nitrogen sources

Among the seven types of nitrogen sources (peptone, ammonium dihydrogen phosphate, ammonium nitrate, corn flour, corn steep liquor, tryptone, and yeast extract), peptone demonstrated the highest antibacterial activity for all antagonistic isolates. The suitable nitrogen sources of *B. subtilis* SP15, *Ps. mosselii* SP38, *Ps. mosselii* SP46, and *Ps. aeruginosa* SP58 were obtained in media containing 2, 2, 1.5, and 1.5% (w/v) peptone, respectively (Table 2). As a result of the presence of a ZOI, the organic nitrogen sources consisting of yeast extract, tryptone, and peptone had a greater advantage than inorganic sources as they also had trace minerals and ions that could enhance the production of enzymes. Similar studies have been reported; Mosquera *et al*. (2014) assessed the suitable culture medium for *B. subtilis* strain EA-CB0015 and found that the medium consisted of 32.5 g/L of yeast extract, demonstrating a 1.2 times increase in antifungal inhibition. The ability of inorganic nitrogen sources to act as direct sources of amino acids' synthesis or their potential role as antibiotic precursors may have contributed to their effectiveness.

Effect of the media pH

The suitable pH levels of the media for antibacterial production by *B. subtilis* SP15, Ps. mosselii SP38, *Ps. mosselii* SP46, and *Ps. aeruginosa* SP58 were a pH of 8, 7, 7 , and 8, respectively (Table 2). The ZOI values

of the media at pH 8, 9, 10, and 11 were significantly higher than for the other media. The medium with pH 8 was selected for cultivation because it had a near neutral pH, which was suitable for bacterial growth and easy to prepare. The results showed that the inhibition zones produced by antagonists were moderately different at various pH values. Almost all antagonistic bacteria had high antimicrobial substance production at a neutral pH ranging from 6 to 8.

Effect of the cultivating temperature

The optimum temperature for the cultivation of *B. subtilis* SP15 was 30°C, whereas for *Ps. mosselii* SP38, *Ps. mosselii* SP46, and *Ps. aeruginosa* SP58, it was 25°C. However, the antimicrobial substance production levels at 25, 30, and 37°C were slightly different at $P<0.05$. This result might indicate that all antagonistic bacteria can be cultured and can produce inhibiting substances at a temperature range around room temperature. Temperature is one of the crucial factors in biological control due to the bioproducts requiring simple physical conditions for storage. The results of the optimized production are summarized in Table 2. It was found that the antibacterial activity rose when the bacterial cell growth increased. It has been noted that differences in the culture medium's composition may have an impact on both the yield of bacteria and the production of antibiotic substances.

Evaluation of antagonistic bacteria in the control of bacterial wilt under greenhouse conditions

The isolates *B. subtilis* SP15, *Ps. mosselii* SP38, and *Ps. mosselii* SP46 were selected for further evaluation based on the high capacity to inhibit the growth of pathogens in the agar test, the ability to produce siderophores and pheanzines, and that the species identification indicated that all selected strains were not harmful to other organisms. As *Pseudomonas aeruginosa* is recognized as a pathogenic microorgasim in humans, the isolate *Ps. aeruginosa* was not used for the *in vivo* test.

Greenhouse trial 1

In three trials, the effectiveness of the mixture of *B. subtilis* SP15, *Ps. mosselii* SP38, and *Ps. mosselii* SP46 to suppress pathumma wilt was assessed in greenhouse conditions. Despite the fact that the disease incidence of all the combinations was low, none of the antagonist mixed cultures provided total protection for the plants against wilt-causing bacteria. Pathumma plants treated with bacterial antagonists displayed a decrease in the pathogen's cells in the soil. The application of antagonists following inoculation with bacterial-wilt pathogens in the pathumma's rhizomes and soil in pots on the initial day of cultivation increased the health of the pathumma plants. The average disease incidence of the antagonists was 3.3% (Table 3). The viable cell number of antagonistic bacteria increased by 3% on average, while the pathogenic bacteria *Enterobacter asburiae* JK1, JK3, JK4, and *Enterobacter dissolvens* JK5 declined by 8, 32, 31, and 10%, respectively. When antagonists were co-applied with pathogens on shooting pseudostems and soil, the antagonists declined by 4.4% on average while the pathogenic bacteria JK1, JK3, JK4, and JK5 declined by 2, 32, 35, and 19%, respectively. Similar results were recorded when the pathogens were applied to cultivated pathumma plants, while the antagonists were introduced to the shooting pseudostems. The cell numbers of antagonists declined by 3% on average, while the pathogenic bacteria JK1, JK3, JK4, and JK5 declined by 38, 34, 38, and 54%, respectively. When the mixed cultures of antagonistic bacteria were only inoculated into the pathumma pots, it was found that the plants did not show wilt symptoms. The results of the disease incidence and the survival of antagonistic and pathogenic bacteria indicated that the mixed antagonists could suppress the growth of wilt-causing bacteria.

Greenhouse trial 2

The applications of the antagonistic bacteria for the control of wilt-pathogens were repeatedly evaluated. Most treatment experiments showed no wilt incidence, while the disease incidence for the control plants was 33-66%. After the antagonists and pathogenic bacteria were co-applied to pathumma rhizomes and soil before cultivation, the final cell concentration of antagonistic bacteria declined by 12.5% on average while the pathogenic bacteria *E. asburiae* JK1, JK2, JK3, and JK4 declined by 30, 15, 31, and 16%, respectively (Table 3). In experiment 2, when the antagonists and pathogenic bacteria were co-inoculated in pathumma pseudostems, the quantities of antagonists declined by 11% on average while the pathogenic bacteria JK1, JK2, JK3, and JK4

Table 3: Effect of antagonist mixture on wilt causing bacteria in pathumma plants when co-applied with antagonistic and pathogenic bacteria prior to cultivation (experiment 1) in 3 trials (year 1, 2 and 3)

Treatment	Trial 1			Trial 2			Trial 3		
	DI (%)	Bacterial population change (%)		DI (%)	Bacterial population change (%)		DI (%)	Bacterial population change (%)	
		Antagonists	Pathogens		Antagonists	Pathogens		Antagonists	Pathogens
Antagonists+JK1	13.3	10.1	-8.9[a]	6.7	-13.2	-30.4	6.7	2.8	-24.8
JK1	33.3	NC[b]	2.8	33.3	NC	-13.1	33.3	NC	-27.4
Antagonists+JK2	-	-	-	0	-12.2	-15.5	33.3	5.4	-23.8
JK2	-	-	-	33.3	NC	-12.0	33.3	NC	-26.9
Antagonists+JK3	0	-3.7	-32.2	0	-12.7	-31.2	0	2.5	-37.7
JK3	33.3	NC	-22.2	33.3	NC	1.1	33.3	NC	-26.9
Antagonists+JK4	0	-0.9	-31.1	0	-13.5	-16.4	0	3.6	-38.6
JK4	33.3	NC	-2.6	33.3	NC	-2.4	33.3	NC	-27.9
Antagonists+JK5	0	6.8	-10.9	-	-	-	-	-	-
JK5	66.7	NC	18.2	-	-	-	-	-	-
Antagonists+R227	-	-	-	-	-	-	0	2.8	-36.9
R227[c]	-	-	-	-	-	-	33.3	NC	-27.9

[a] - value displayed the decreased of cell number
[b] NC = No antagonist counting in the treatment
[c] R227 were standard *Ralstonia solanacearum* obtained from Ministry of Agriculture and Cooperatives, Thailand
- = not determined

declined by 24, 38, 40, and 35%, respectively. The treatment of antagonists in shooting plants after the inoculation of pathogens in rhizomes resulted in a bacterial reduction by 12% on average for the antagonists while the pathogenic bacteria JK1, JK2, JK3, and JK4 declined by 37, 24, 40, and 26%, respectively.

Greenhouse trial 3

When the mixed cultures of the antagonistic bacteria were introduced into pathumma rhizomes and soil in pots upon cultivation along with the pathogens, the plants showed slight wilt symptoms. The disease incidences of the antagonist treatments particularly in experiment 3 increased, compared to the previous studies in trial 1 and trial 2. The amounts of antagonistic bacteria increased by 3.4% on average, while the pathogenic bacteria *E. asburiae* JK1, JK2, JK3, JK4, and *Ralstonia solanacearum* R227 declined by 24, 23, 37, 38, and 37%, respectively, after 5 months of examination (Table 3). When the antagonistic bacteria were co-applied with the pathogens on the shoots of pathumma in pots, the amounts of antagonists declined by 9% on average while the pathogenic bacteria JK1, JK2, JK3, JK4, and R227 declined by 24, 23, 30, 39, and 28%, respectively. When the pathogens were applied to the cultivated pathumma, while the antagonistic bacteria were introduced to the shooting plants, the amounts of mixed antagonists declined by 9% on average, while the pathogenic bacteria JK1, JK2, JK3, JK4, and R227 declined by 24, 12, 24, 36, and 26%, respectively. The results of the wilt-causing bacterial growth inhibition in the greenhouse assay indicated that the antagonistic bacteria isolated from various soils were effective in controlling bacterial wilt caused by *Enterobacter* spp. Moreover, the results of all trials (trial 1, 2, and 3) showed that experiment 1 could highly reduce the amounts of pathogenic bacteria; therefore, this study agreed that the antagonists should be applied in the early cultivation of plants. Several reports have demonstrated that *Bacillus* and *Pseudomonas* could be suitable biocontrol agents against plant pathogenic bacteria. Jan *et al*., (2023) proposed that *Bacillus subtilis* FJ3 isolated from soil could suppress the number of plant pathogenic fungi. The crude extract of the antifungal substance inhibited *F. oxysporum*, *A. niger*, *A. flavus*, and *R.*

oryzae at 92%, 90%, 81.5%, and 56% cell reduction, respectively. Abo-Elyousr *et al*. (2022) demonstrated that *P. xanthii*, the causal agent of the powdery mildew of cucumber could be restricted by *B. licheniformis* and *B. aerius*. Vinayarani and Prakash (2018) stated that the treatment of *Bacillus* sp. and *Pseudomonas* sp. significantly increased the plant height and fresh rhizomes while decreasing the occurrences of rhizome rot and leaf blight diseases in turmeric under greenhouse conditions. In addition to *Bacillus* spp. and *Pseudomonas* spp., actinobacteria, i.e., *Streptomyces ramulosus*, *S. axinellae*, and *S. drozdowiczii*, exhibited the inhibition of wilt-causing bacteria including *X. oryzae*, *X. campestris*, and *R. solanacearum* (Promnuan *et al*., 2020). In the current study, applications of antagonists before or together with plant cultivation were suitable for disease prevention. The main problem of biological control was its low consistency and reliability under field conditions due to a high variability in efficacy. An *in vitro* experiment was restricted by the lack of a plant that could significantly affect the antagonist's capacity to survive, colonize, and inhibit pathogens, although being simpler to perform. When field trials occur, the *in vivo* test is more accurate in simulating the environmental conditions to which the antagonistic bacteria will be subjected. One of the realized variables for the evaluation of biological control was the inoculum. A co-inoculant was proposed to use with a favorable inocula due to the synergistic effect to enhance the biocontrol. This conclusion was supported by the possibility that using a variety of strains as a biocontrol agent could be beneficial by ensuring that at least one of the biocontrol mechanisms could function under the unpredictably changing ecological conditions that the released PGPR strains will encounter (Zhang *et al*., 2023). The antagonists should not be a risk to the environment. This study showed that *Ps. aeruginosa* could be beneficial to the plant but it is an opportunistic pathogen in humans and animals. Hence, it was suggested that this strain should be replaced by other organisms or used as an antimicrobial substance producer. Further studies should evaluate the biocontrol efficacy of antagonistic bacteria in the field, particularly with an earlier finding of the disease. In comparison to applying biocontrol agents in a greenhouse, applying them in the field is intrinsically more difficult and varied.

Designation of the mixed culture products

To select the most effective carrier, several media were tested for the maximum growth of antagonistic bacteria. The preliminary results revealed that molasses and soybean whey were the most efficient medium for bacterial growth. After secondary screening, it was found that molasses was the best liquid formulation product. This medium was further evaluated for the shelf-life of antagonistic bacteria for 90 days. The cell numbers of *B. subtilis* SP15, *Ps. mosselii* SP38, and *Ps. mosselii* SP46 increased by 1.4%, 1.2%, and 1.5%, respectively. Both soybean whey and molasses are waste products from industry. They are known to be a good fermentation medium, rich in protein, fat, carbohydrates, and fiber. However, the major problem of these carriers is that it is easy to contaminate with *Bacillus* and fungi, due to the high water activity. To evaluate the optimal carrier materials for antagonistic bacteria, three materials (soil, coir, and chaff) were examined for the survival in these carriers for 60 days. Soil was the most eligible carrier material for all antagonistic bacteria. It was interesting that the population changes of all the bacteria in coir were slightly different from the soil. All bacteria survived after incubation for 2 months (Fig. 2-4). The coir could replace the soil, since coir is an agricultural waste, and its cost is low. Dry formulation is one form of solid-state fermentation that has a low water activity (A_w) value. This formulation is considered advantageous over a liquid formulation in terms of the stability of potency with regard to storage and transportation costs. The dry formulation is lower in weight and does not require low temperature for long term storage (Barbosa-Cánovas *et al*., 2020). Due to their ability to produce a number of broad-spectrum antibiotics and their prolonged shelf life as a result of their ability to create endospores, *Bacillus* spp have been found to be beneficial in the biocontrol of numerous plant diseases (Promsai *et al*., 2023). Other researchers have reported a formulation of *Bacillus* and *Pseudomonas*. Abo-Elyousr *et al*. (2022) revealed that under greenhouse conditions, a cell suspension of *B. licheniformis* and *B. aerius* decreased the disease severity of powdery mildew and raised the fresh and dry weight of the plant in comparison to an untreated plant. Using talc-based formulations of *P. putida*, *B. cereus*, and *P. aeruginosa* as biocontrol agents inhibiting *P. aphanidermatum* and *R. solani*, which cause rhizome rot and leaf

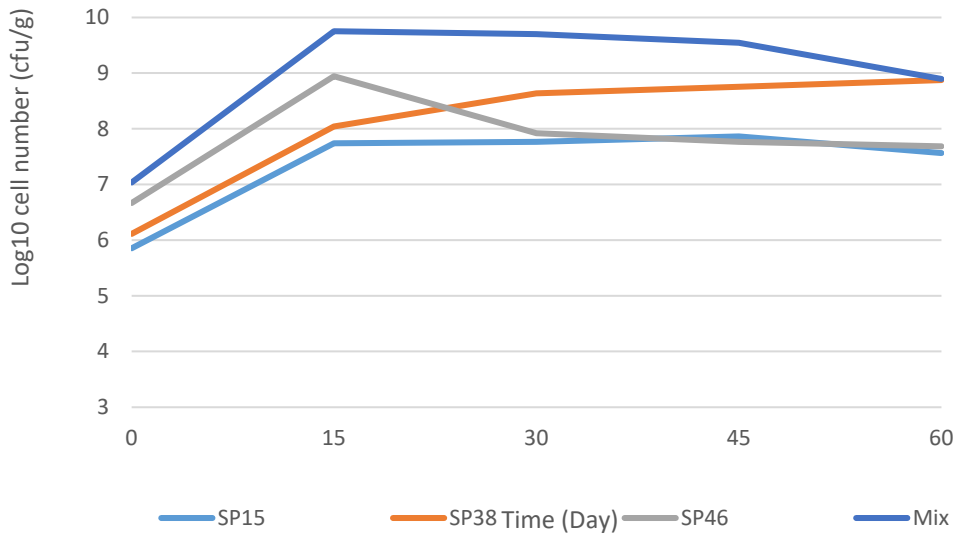

Fig. 2: Viable cell count of antagonistic bacteria in soil after incubation for 60 days at room temperature

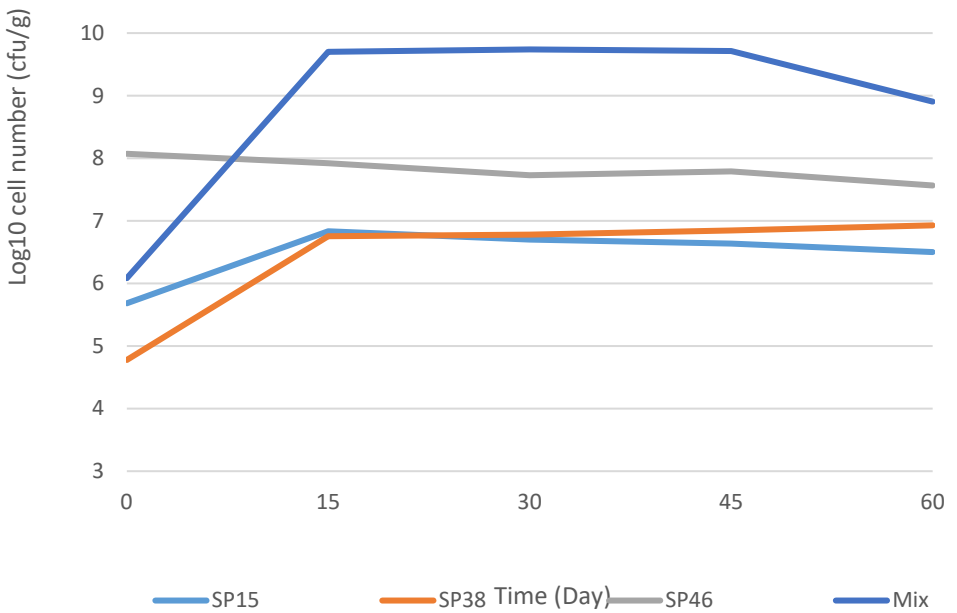

Fig. 3: Viable cell count of antagonistic bacteria in coir after incubation for 60 days at room temperature

blight diseases in turmeric, were administered to control these diseases. The results showed that the disease incidences were reduced after being treated with these formulations (Vinayarani and Prakash, 2018). A biological control agent (BCA)-based product's final efficacy is significantly influenced by its formulation, as well as by the processes of biomass discovery, manufacture, and stabilization. Even while numerous microorganisms have shown promise for their biocontrol capacity on a small scale, it is still extremely difficult to create biological control organisms that are effective. Effective formulation

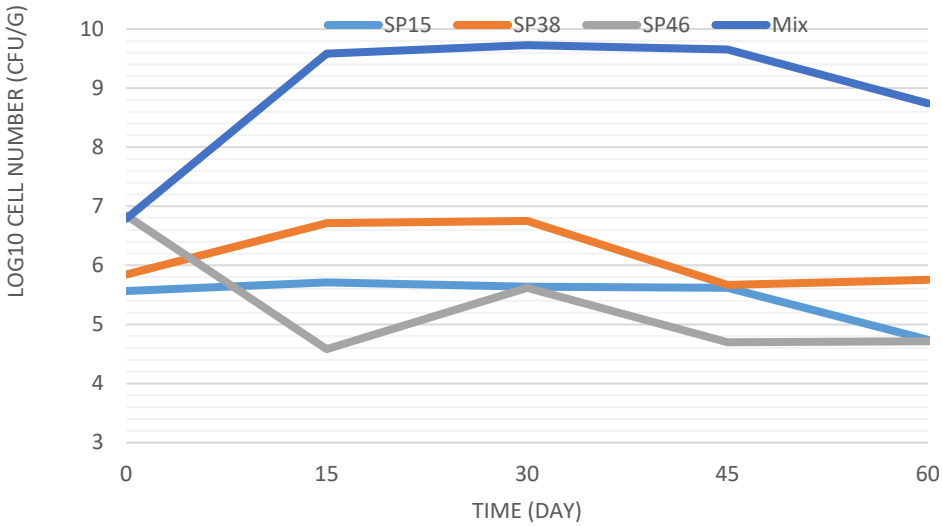

Fig. 4: Viable cell count of antagonistic bacteria in chaff after incubation for 60 days at room temperature

and successful organism scale-up are crucial for the steady and profitable development of pesticides (Ruanpanun and Nimnoi 2020). The development of an effective formulation strategy for BCAs depends on the choice of an acceptable carrier material. The carrier should help the biocontrol activity in the field survive and persist for a long time. The necessity for low cost, nonphytotoxic, and low visible residue from agriculture on saleable products must be considered in addition to the aforementioned factors (Abo-Elyousr *et al.*, 2022). The formulation plays a significant role in determining the final efficacy of a BCA-based product, as do the processes of the discovery, production, and stabilization of the biomass. Although many microorganisms have exhibited promising biological control activity on a small scale, it remains a major challenge to successfully formulate biological control organisms. To develop a successful formulation protocol for biological control agents, selection of an appropriate carrier material is important.

CONCLUSION

An agar diffusion method was conducted to screen antagonistic bacteria for the biological control of wilt disease of pathumma. Among 105 bacterial isolates, four isolates, namely SP15, SP38, SP46, and SP58, which were *Bacillus subtilis*, *Pseudomonas mosselii*, *Pseudomonas mosselii*, and *Pseudomonas aeruginosa*, respectively, displayed the highest production of inhibiting substances. The optimal conditions of the inhibiting substance from the isolate SP15 was 30°C at pH 8 in modified TSB medium containing 0.5% (w/v) glucose and 2% (w/v) peptone SP38 was 25°C at pH 7 in modified TSB medium containing 0.5% (w/v) sucrose and 2% (w/v) peptone SP46 was 25°C at pH 7 in modified TSB medium containing 0.5% (w/v) glucose and 1.5% (w/v) peptone, and SP58 was 25°C at pH 7 in modified TSB medium containing 0.5% (w/v) sucrose and 1.5% (w/v) peptone. All antagonists could produce both hydroxamate-type and catecholate-type siderophores, and three strains including *Ps. mosselii* SP38, *Ps. mosselii* SP46, and *Ps. aeruginosa* SP58 showed the ability to produce phenazine derivatives. Wilt-disease reduction using antagonistic bacteria including *B. subtilis* SP15, *Ps. mosselii* SP38, and *Ps. mosselii* SP46 was assessed in a greenhouse study. It was found that the disease incidences of pathumma plants treated with antagonists decreased by 70-100%, and the amounts of pathogenic bacteria declined by 25-40% on average, while the number of the antagonistic combination generally decreased by 5–15%. This research revealed that the use of antagonistic bacterial strains should be applied before the pathogens were dispersed. The study of the designation of mixed-culture products identified that the dry formulation bioproduct for all antagonists was soil, respectively. It is expected that these antagonistic bacteria can be useful in wilt-disease

management in agricultural fields in the future.

AUTHOR CONTRIBUTIONS

S. Promsai, the corresponding autor, contributed to original draft preparation, conceptualization, validation, compiled the data, review and editing manuscript. Y. Tragoolpua contributed to validation, review and editing manuscript. N. Thongwai contributed to supervising, validation, review and editing manuscript. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been completely observed by the authors.

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ABBREVIATIONS

%	Percent
µg	Microgram per milliliter
µL	Microliter
°C	Degrees Celsius
ANOVA	Analysis of variance
cm	Centimeter
cfu/g	colony forming unit per gram
cfu/mL	colony forming unit per milliliter
DI	disease incidence
g	Gram
Gagnep	Gagnepain
h	Hour
HCl	Hydrochloric acid
i.e.	Id est (that is)
min	Minute
mm	Millimeter
M	Molarity
nm	Nanometer
pH	Potential of hydrogen
TLC	Thin layer chromatography
TZC	Triphenyltetrazoliumchloride
TSA	Tryptic soy agar
TSB	Tryptic soy broth
v/v	Volume per volume
w/v	Weight per volume
xg	Gravitational force equivalent
ZOI	zone of inhibition

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