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Antagonistic Bacteria Isolated from *Apis cerana* for Control of Black Rot Disease of Crucifer Caused by *Xanthomonas campestris* and Development of Potential Antagonist Products

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Abstract

Crucifers are one of the most important groups of vegetables in Thailand. *Xanthomonas campestris* can cause devastating disease in crucifers particularly black rot disease. Biological control is now widely used in many crops. This research was conducted to screen antagonistic bacteria capable of inhibiting the growth of *X. campestris* in crucifers. One hundred and six bacterial isolates were isolated from bee samples collected from different sites in northern Thailand. After testing their abilities to inhibit the growth of pathogenic bacteria, 12 isolates showed high ability to inhibit the growth of *X. campestris* TISTR 1100. From the hemolysis test, 9 isolated strains did not possess the capability of hemolysis. The isolates KUKPS-C3BN2, KUKPS-C3AN5 and KUKPS-C16HM4 were *Bacillus* spp., while the isolates KUKPS-C2AN2, KUKPS-C16HN7 and KUKPS-C17AN3 were *Kluyvera* spp., *Enterobacter* spp. and *Enterobacter* spp., respectively. The antagonistic bacteria *Bacillus tequilensis* KUKPS-C3AN5 and *Bacillus thuringiensis* KUKPS-C16HM4 were developed into antagonist products using soybean-mungbean, and rice kernels as 2 separate carriers. The survival rates and the ability to inhibit plant pathogens were evaluated. It was found that the survival rates of antagonists in both the products were 99.10–108.34% and the antagonists could inhibit the growth of pathogenic bacteria after 2 months of incubation. This research demonstrated that 2 antagonistic bacteria had high potential to control black rot disease in crucifers and could be developed for application in the agricultural sector.

Keywords: Antagonist; *Bacillus*; Bee; Biocontrol; Black rot disease; Crucifer; Plant pathogen

Introduction

The cruciferous vegetable is a large family of vegetables. There are more than 300 genera divided into more than 3,000 species originating and distributed globally. Cruciferous vegetables are one of the most important vegetables in Thailand because they are used for daily consumption, have commercial value and are also exported to various countries. The cabbage is a plant in the family Cruciferae (Brassicaceae) that is important to the food system and economy of the country. Cultivation of cabbage is distributed throughout Thailand with the quantity and quality of production dependent on the terrain and climate. The northern region of Thailand is considered more suitable for growing this vegetable than other regions (Nath *et al*. 1999). Currently, the cultivation of cruciferous vegetables in Thailand is facing a serious problem from microbial infection, resulting in substantial economic losses (Nath *et al*. 1999). Plant diseases are important factors affecting plant growth and agricultural productivity and may result in reduced production that is insufficient to meet the demands of the world's ever-increasing population. It may also cause a shortage of food products for export.

Black rot disease of cruciferous plants is a common disease that is caused by *Xanthomonas campestris* pv. *campestris*. The infection begins in the seedling stage, causing the plant to appear stunted and, the lower leaves wither and V-shaped yellow lesions are formed on the leaf margins. The yellowing spreads until the midrib and the veins turn black. The contaminated area will become brown and dry, with the injured leaves falling off before they reach maturity. When the leaf tissues are cross sectioned, the alimentary canal is black and a large number of bacteria are present in yellow slime and occasionally in the mid-stem gap (Agrios 1997). When infected seeds germinate, the bacterium on the seed coat penetrate to the cotyledons and

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juvenile leaves through natural openings or wounds on the roots and leaves. This causes considerable damage to crops during warm weather and high rainfall during seedling growth (Français 2022). The exopolysaccharide produced by pathogens is called xanthan a sticky substance leading to clogging of the xylem and phloem and damaged cells turn black (Qian *et al*. 2006). The bacterial pathogen can migrate from leaves to stems through the xylem under hot and humid conditions. Consequently, it can move up and down the plant stem (Français 2022).

Pesticide residues in vegetables are now recognized as having a direct impact on the consumer health and the country's economy (Ruanpanun and Nimnoi 2020). However, the chemicals are necessary to control the spread of diseases caused by microorganisms, where often the spread is rapid, having a severe impact on crops across a vast area. As a result, farmers choose to apply pesticides for pest control and prevention because it is a convenient, fast and highly efficient control method. However, the increased use of chemicals coupled with a lack of knowledge and suitable care regarding the choice and application by farmers, ecosystems and natural environments are being destroyed or disrupted (Abo-Elyousr *et al.* 2022). The plant pathogen's natural enemies and microorganisms are eliminated until their numbers decline, resulting in outbreaks of severe plant disease. There are numerous issues to consider regarding the long-term effects of applying chemicals to control plant diseases (Sánchez-Hernández *et al*. 2022).

Biological control of plant pathogens is the use of living organisms to reduce the population of plant pathogens or to reduce the activity of pathogenic plant pathogens to a level that does not cause economic damage (Khan and Javaid 2020, 2021, 2022). There are principles that can be carried out to control plant pathogens using biological approaches by: (1) introducing beneficial microorganisms from the local source of disease causative agents (Javaid *et al*. 2021; Sharf *et al.* 2021); (2) optimization of the environment for population growth and its role in the antagonism; (3) maintaining the population and its role by adding beneficial microorganisms or improving the surroundings; and (4) using biological control integrated with other plant disease management practices (Maleki *et al*. 2010; Ali *et al.* 2020). Therefore, it is necessary to study biological control using antagonistic microorganisms that can inhibit plant pathogenic bacteria in solving the problem of excessive chemical use.

The Asian cavity-nesting honey bee *Apis cerana* is widespread in temperate and tropical Asia including Thailand. *A. cerana* naturally occurs on an Asian landscape of some 30 M km^2 encompassing a series of climatic zones from tropical moist rainforest, wet-dry tropical savanna, mid-latitude steppe, dry mid-latitude grasslands, moist continental deciduous forest, and taiga (Radloff *et al*. 2010). *A. cerana* is one of the rare native bees found in Thailand. There are few studies on antagonistic bacteria associated with honey bee (*Apis*) species in Thailand. Some actinobacteria were isolated from giant honey bee (*Apis dorsata*) showed the ability to inhibit the growth of *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas campestris* pv. *campestris*, *Ralstonia solanacearum* and *Pectobacterium carotovorum* (Promnuan *et al*. 2020). It is interesting that novel bacterial species with high antibacterial activities have been found in *A. cerana*.

Successful application of biological control agents can improve the health of farmers and consumers with the advantage of being a low-cost method that encourages environmental sustainability (Abo-Elyousr *et al*. 2022). However, to successfully apply knowledge of biological control in practical plant agriculture, product formulations must be developed that are effective at managing the plant. Therefore, the current study investigated antagonistic microorganisms that can suppress plant pathogenic bacteria, delved into the appropriate media for antagonistic bacteria to grow, used the antagonists to control plant disease in plots, and developed them to be useful to farmers.

Materials and Methods

Plant pathogenic bacteria

Xanthomonas campestris TISTR 1100 was obtained as a standard culture from Thailand Institute of Scientific and Technological Research (TISTR). It was inoculated in cabbage pots (*Brassica oleracea*) before its use in this study. The lesions of black rot disease were shown after pouring the 24 h pathogen culture onto of 24 day-old cabbage seedlings.

Sample collection

Combs of the Asiatic cavity-nesting honeybee (*Apis cerana*) were collected from the Mae-rim district, Chiang Mai province, Thailand in April 2014. The three hive samples were collected from local villages in private areas. Adult bees, brood cells, pollen and honey were collected, kept in sterile tubes and stored at -20°C.

Isolation of bacteria from bees

Bacterial strains were isolated from 1 g of each bee sample (adults, brood cells and pollen using the serial dilution technique as described by Promsai *et al.* (2018) with some modifications. The bee samples were ground, added with 10 mL sterile water and vortexed for 1 min, before being diluted in about 10 times sterile water and vortexed again for 1 min. One milliliter of successive decimal dilutions was spread on nutrient agar (Merck®, Germany) containing 25 mg mL^{-1} cycloheximide and then incubated at 37°C for 24–48 h. One colony was randomly picked for streaking on agar plates.

Characterization of isolated bacteria

The bacterial strains that were isolated from native-Thai bees were incubated at 37°C for 24 h using nutrient broth. They were basically characterized for surface, color, Gram staining, cell arrangement and spore formation under a light microscope (CX31; Olympus®, Japan) as described by Forbes *et al*. (2002). The catalase activity was tested for oxidative metabolism using the method of Holt *et al*. (1994).

Screening of antagonistic bacteria capable of growth inhibition of plant pathogens

The inhibition of pathogen growth was evaluated using the agar disc diffusion method (Balouiri *et al*. 2016). The pathogenic bacterium was inoculated in 50 mL of yeast extract-malt extract (YM) broth and incubated at 30°C until the turbidity of the culture equaled a standard McFarland value of 0.5 (approximately 10^7–10^8 CFU mL^{-1}). The culture was spread on the surface of YM agar. Concomitantly, the isolated bacteria were inoculated in NB and incubated at 37°C for 24 h. The culture broths were centrifuged at 8,000 rpm and 4°C for 15 min. Then, 40 μL of supernatants were dropped on a paper disc that was placed on a YM agar surface that contained pathogenic culture. NB was used as a negative control and 1 mg mL^{-1} of streptomycin was used as a positive control. Then, the agar plates were incubated at 30°C for 48 h. The inhibition zones were observed by measuring the diameter of the clear zone in millimeters. The experiment was conducted in triplicate.

Hemolytic activity of antagonistic bacteria

The selected bacteria from the growth inhibition testing were evaluated for hemolytic activity using the stab inoculation method (Chumphon *et al*. 2016). The strains were cultured on blood agar, consisting of 1% tryptose, 0.5% sodium chloride, 1.5% agar and supplemented with 7% bovine blood, for 48 h at 37°C. *Bacillus cereus* was used as the positive control. Strains which showed green-hued zones around the colonies (α-hemolysis) or did not produce any effect on the blood agar (γ-hemolysis) were classified as displaying non-hemolytic activity. Strains displaying blood lyses zones around the colonies were classified as hemolytic (β-hemolysis).

Molecular identification of antagonistic bacteria using 16S rDNA sequencing

Genomic DNA samples of 12 bacterial isolates capable of inhibiting the plant pathogen were extracted (Promsai *et al*. 2018). Almost-complete 16 Svedberg units of the ribosomal ribonucleic acid (16S rRNA) gene (1.5 kb) were amplified using the universal primer pair 20F (5′AGTTTGATCCTGGCTC-3′) and 1540R (5′-AAGGAGGTGATCCAGCC-3′) (Nakajima *et al*. 1999). The 16S rDNA gene was amplified using polymerase chain reaction (PCR; Mulyigne Optimax; Labnet®; USA). The PCR products were purified using Nucleo Spin® Gel and a PCR Clean-up Kit (Invitrogen; USA), following the manufacturer's protocol. The purified PCR products were directly sequenced by the First Base Company, Malaysia using primers 20F and 1540R as sequencing primers. The identities of nucleotide sequences of the 16S rRNA gene obtained were subjected to BLAST analysis using the NCBI database (http://www.ncbi.nlm.nih.gov). The sequence information was supplied regarding the deposition of DNA sequences. The almost-complete 16S rRNA genes sequences are accessible *via* GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The relationships and diversity were studied in the evolutionary lines of the protease-producing bacteria isolated from bee samples. The bacterial sequences were studied using the Bio Edit Sequence Alignment Editor version. 7.0.5.3 Primer with Clustal_W multiple alignment and 1,000 bootstraps. The phylogenetic tree was developed by calculating the distance between the molecular sequences using the maximum-likelihood method with the MEGA X program, version 10.1.8 to present an evolution chart.

Spore potential of antagonistic bacteria

According to the results of antibacterial activities, hemolytic activities and molecular identification, *Bacillus* species which had no pathogenicity and showed potent antibacterial effects, were selected for further studies. Among KUKPS-C3BN2 and KUKPS-C16HM4, the isolate KUKPS-C16HM4 were used as representative. In this study, the isolates KUKPS-C3AN5 and KUKPS-C16HM4 were then evaluated the spore potential. The method of spore potential was modified from Foldes *et al*. (2022) and Oscariz *et al*. (1999). The isolates KUKPS-C3AN5 and KUKPS-C16HM4 were cultivated in Luria Bertani (LB) broth at 37°C for 48 h. Then, the culture broth samples were incubated in a water bath at 80°C for 15 min. Viable cell counts were determined using the dilution plate technique on LB agar and incubated 37°C for 48 h. The survival rate percentages were calculated using the following equation:

$$\text{Survival rate (\%)} = \text{Cell number in treated (CFU mL}^{-1}\text{)} / \text{Cell number in control (CFU mL}^{-1}\text{)} \times 100$$

Where treated is the treatment that was incubated at 80°C for 15 min and control is the treatment that was incubated at 37°C for 15 min.

Development of antagonist product

Preparation of antagonist inoculum: The antagonistic

bacteria were inoculated in NB and incubated at 37°C for 24 h. Each culture broth sample was measured using a spectrophotometer to adjust the optical density to 1.0 at 540 nm (approximately 10^8 CFU mL^{-1}), followed by centrifugation at 8,000 rpm at 4°C for 10 min to obtain a cell pellet. The obtained cell pellets (20–30% moist) were used for mixing with carriers.

Preparation of antagonist product using soybean and mung bean extract: The patented soybean and mung bean extract medium (Promsai and Chumphon 2021) was prepared and sterilized at 121°C for 15 min. The medium was cooled for 30 min prior to mixing with the cell pellets of the antagonistic bacteria. The antagonist products were kept at room temperature for 60 days.

Preparation of antagonist product using rice grains as carrier: The rice grains were prepared and sterilized at 121°C for 15 min. After cooling, the rice grains were mixed with cell pellets of the antagonistic bacteria and the antagonist products were kept at room temperature for 60 days.

Investigation of cell number survival in antagonist products: The viable cell count of antagonistic bacteria was evaluated using the serial dilution plate count technique. Briefly, a sample of 1 g or 1 mL of antagonist products was added in 0.85% NaCl and diluted in 0.85% NaCl until the proper dilution was achieved. The decimal dilution was spread on NA at 37°C for 48 h. The viable cell number was determined weekly and the survival rate was calculated at day 60 using the equation (Bao *et al.* 2010):

$$\text{Survival rate (\%)} = \text{Log } N_1 / \text{Log } N_0 \times 100$$

Where, N_1 is cell number of antagonistic bacteria at day 60 (CFU mL^{-1} or CFU g^{-1}) and N_0 is cell number of antagonistic bacteria at day 0 (CFU mL^{-1} or CFU g^{-1}).

Evaluation of capacity of pathogenic inhibition of antagonist products

The inhibition of *X. campestris* by antagonist products was investigated using the paper disc diffusion method. The overnight culture of pathogenic bacteria (approximately 10^7–10^8 CFU mL^{-1}) was spread on YM agar. One milliliter of soybean-mungbean antagonist product was centrifuged at 8,000 rpm and 4°C for 10 min to obtain the supernatant. After that, 10 μL of supernatant were dropped onto a paper disc (6 mm diameter) and the immersed paper disc was placed on the YM agar. The evaluation of the rice grains used 1 g of the rice grain antagonist product with 5 mL of sterile 0.85% NaCl and was mixed well. After that, 10 μL of solution were dropped onto a paper disc (6 mm diameter) and the immersed paper disc was placed on the YM agar. Untreated soybean-mungbean extract medium or rice grains were used as the respective negative controls. Then, the agar plates were incubated at 30°C for 48 h. The inhibition zones were observed by measuring the diameter of any clear zone in millimeters. The experiment was conducted in triplicate.

Statistical analysis

The results of the inhibition of pathogenic bacteria were statistically analyzed in IBM SPSS Statistics software by analysis of variance (ANOVA), followed by a post hoc comparison of means by Duncan Multiple Range's test.

Results

Re-infection of *X. campestris* in cabbages

From the results of re-infection, the infected plants showed V-shaped yellow-brown lesions on the tips of the cabbage leaves that is a unique characteristic of black rot disease (Fig. 1).

Isolation of bacteria from native Thai bees

Using the serial dilution technique, 106 pure isolates of bacteria were recorded from bees, pollen, brood cells and honey (32, 33, 20 and 21 isolates, respectively). Most of the bacterial isolates grew well at 37°C on NA. The morphological characteristics of all 106 isolates were examined using Gram staining and observed under a microscope as well as using a catalase test and investigating growth characteristics on the medium. It was found that the growth characteristics on culture medium showed the colony sizes in the range 1–7 mm, the colony shape was round and the colonies of some isolates were amorphous. The colony surfaces were both glossy and matte. Two types of colony colors were observed: translucent white and opaque white. Cell shapes were round and rod-shaped. Some bacterial isolates produced endospores inside cells.

Antibacterial activity of antagonistic bacteria

All isolated bacteria were tested for their inhibition ability against the black rot pathogen (*X. campestris* TISTR 1100) using the agar disc diffusion method on YM agar medium. It was found that 12 isolated bacteria could inhibit growth of *X. campestris* and each of these isolates had a slightly different capacity to inhibit the growth of the plant pathogen (Table 1).

Hemolytic activity of isolated bacteria

The selected bacteria were tested for hemolytic activity to consider the microbiological safety of the bacterial strains before any development of antagonist products. The results indicated that 3 isolates, namely KUKPS-C3BN8, KUKPS-

Table 1: Growth Inhibition of bacterial isolates against *X. campestris*

Antagonistic bacteria	Gram staining	Diameter of inhibition zone (mm)*
KUKPS-C2AN1	Positive	17.7 ± 0.6 ^{BC}
KUKPS-C2AN2	Negative	17.3 ± 0.6 ^{BC}
KUKPS-C2AN3	Negative	17.3 ± 0.6 ^{BC}
KUKPS-C3AN5	Positive	22.0 ± 1.0 ^{B}
KUKPS-C3BN2	Positive	32.3 ± 1.5 ^{A}
KUKPS-C3BN8	Positive	12.0 ± 10.4 ^{C}
KUKPS-C16HN7	Negative	22.3 ± 3.5 ^{B}
KUKPS-C16HM4	Positive	21.0 ± 1.7 ^{B}
KUKPS-C17AN3	Negative	22.3 ± 4.0 ^{B}
KUKPS-C17AN4	Negative	20.3 ± 5.5 ^{B}
KUKPS-C17AN6	Negative	33.3 ± 2.3 ^{A}
KUKPS-C18AN6	Negative	24.3 ± 2.1 ^{B}

* Data are presented as the mean ± standard deviation. Values within the column followed by different uppercase superscripts are significantly different at $P < 0.05$ level

Table 2: Hemolytic activity of antagonistic bacteria on blood agar

Antagonistic bacteria	Hemolytic activity		
	Beta-hemolysis	Alpha-hemolysis	Gamma-hemolysis
KUKPS-C2AN1			✓
KUKPS-C2AN2			✓
KUKPS-C2AN3			✓
KUKPS-C3AN5			✓
KUKPS-C3BN2			✓
KUKPS-C3BN8	✓		
KUKPS-C16HN7			✓
KUKPS-C16HM4			✓
KUKPS-C17AN3			✓
KUKPS-C17AN4	✓		
KUKPS-C17AN6	✓		
KUKPS-C18AN6			✓

✓ Positive result

Table 3: Efficacy of endospore survival of antagonistic bacteria after incubation at 80°C for 15 min

Isolates	Viable cell count (cells) after incubation at		Endospore survival rate (%)
	37°C	80°C	
B. tequilensis KUKPS-C3AN5	48	10	21
B. thuringiensis KUKPS-C16HM4	34	13	38
B. subtilis (control)	31	22	71

C17AN4 and KUKPS-C17AN6 showed hemolytic activity, whereas 9 isolates, namely KUKPS-C2AN1, KUKPS-C2AN2, KUKPS-C2AN3, KUKPS-C3AN5, KUKPS-C3BN2, KUKPS-C16HN7, KUKPS-C16HM4, KUKPS-C17AN3 and KUKPS-C18AN6 did not show any hemolytic activity (Table 2).

Molecular identification and construction of phylogenetic tree

After the 16s RNA gene analysis of selected bacteria, it was found that the isolates KUKPS-C3BN2, KUKPS-C3AN5 and KUKPS-C16HM4 had similarity with *Bacillus* spp. of 96, 97 and 96%, respectively. The similarity values of the antagonistic bacterial isolates KUKPS-C2AN2, KUKPS-C16HN7 and KUKPS-C17AN3 to *Kluyvera* spp., *Enterobacter* spp. and *Enterobacter* spp. were 98%, 99% and 96%, respectively. Subsequently, the 16s RNA gene sequences of the antagonistic bacteria *Bacillus* spp. were evaluated closely using phylogenetic tree construction (Fig. 2). Based on the phylogenetic tree construction, the maximum likelihood tree confirmed the placement of the isolate KUKPS-C3AN5 to *B. tequilensis*, while the isolates KUKPS-C3BN2 and KUKPS-C16HM4 were *B. thuringiensis*.

Fig. 1: Lesions on cabbage leaves of re-infection of *X. campestris* TISTR 1100 after inoculation at leaf germination stage. Arrows indicate the V-shaped yellow lesions

Spore production of antagonistic bacteria

From the results of the study of the morphological characteristics under a microscope at 1,000x magnification, *B. tequilensis* KUKPS-C3AN5 and *B. thuringiensis* KUKPS-C16HM4 formed intracellular endospores. Thus, efficacy of spore tolerance to heat was investigated in this study. The LB culture broth was incubated at 80°C for 15 min to test the ability of spore production following heat. As shown in Table 3, *B. tequilensis* KUKPS-C3AN5, *B. thuringiensis* KUKPS-C16HM4 and *B. subtilis* (the comparable standard culture) had spore survival rates of 21, 38 and 71%, respectively.

Feasibility study of development of antagonist product

B. tequilensis KUKPS-C3AN5 and *B. thuringiensis* KUKPS-C16HM4 were selected for the development of antagonist products using rice grains and soybean-mung bean extract as carriers. From the results of survival ability and efficacy against plant pathogenic bacteria (*X. campestris*) after 60 days of storage, the survival rates of antagonistic bacteria cultivated in rice grain carrier were higher than for the soybean-mungbean extract carrier (Fig. 3). Additionally, the efficacy against plant pathogens showed that *B. tequilensis* KUKPS-C3AN5 was more effective than *B. thuringiensis* KUKPS-C16HM4 (Fig. 4).

Discussion

The result of a re-infection in cabbage of *X. campestris* TISTR 1100, which is a standard culture obtained from the Thailand Institute of Scientific and Technological Research

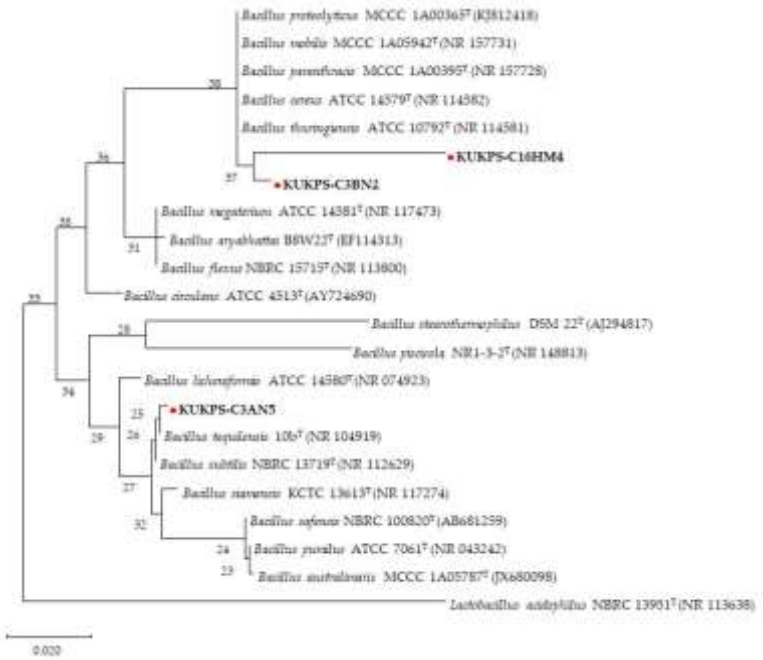

Fig. 2: Maximum likelihood phylogenetic tree of antagonistic bacteria and *Bacillus* species based on 16S rRNA sequences analysis. Scientific names in parentheses are the originally proposed names of the strains and numbers in parentheses are GenBank EMBL database accession numbers

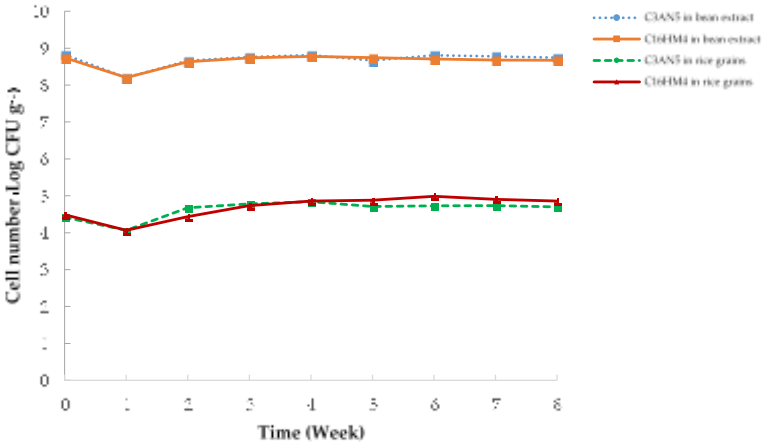

Fig. 3: Viable cell count of antagonistic bacteria after 8 weeks of storage in 2 carriers (soybean-mungbean and rice grains)

(TISTR), confirmed that this pathogen could cause the severe lesion in cabbage. Other studies reported that bacteria in the genus *Xanthomonas* were the major causative agents on vegetable crops, especially *X. campestris* pv. *campestris*, regarding black rot in cabbage, cauliflower and lettuce. Rubel *et al.* (2017) reported the occurrence of black rot disease in seeds infected with *X. campestris* pv. *campestris* as one of the most common diseases in cruciferous plants and that it could reduce the crop yield by more than 50 percent under the proper growth conditions.

In general, the application of antagonistic products is often used in the form of a spray or seed coating which means that there is a chance that consumers and farmers may come in direct contact with the microorganisms. Therefore, it is necessary to study the risk characteristics that might cause harm to consumers and farmers. Red blood cell lysis is one of the distinctive features of human pathogens. Human blood is made up of a solid part (45%) that is mostly blood cells and platelets, whereas the liquid part (55%) is plasma (Tuchin *et al.* 2004). Most human pathogens are capable of lysis of red blood cells and these pathogens can be divided into 3 groups: (1) alpha hemolytic group incomplete lysis of red blood cells (a greenish-brown zone around the colonies of the pathogens on blood agar),

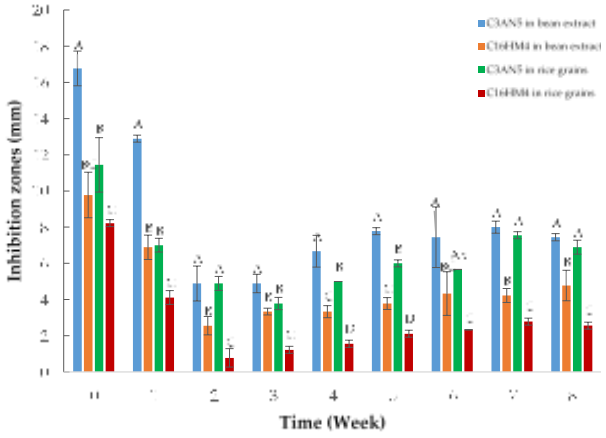

Fig. 4: Efficacy of growth inhibition of *X. campestris* by antagonistic bacteria cultured on 2 carriers (soybean-mungbean and rice grains). The storage of antagonistic products was examined for 8 weeks, and the agar diffusion method was performed every week. Values within the week followed by different uppercase letter are significantly different at $P < 0.05$ level

for example *Streptococcus viridans*; (2) beta hemolysis the complete lysis of red blood cells (a clear zone around the colony), for example, *B. cereus* and (3) gamma hemolysis or non-hemolytic group no red blood cell lysis and no zone around the colony (Chumphon *et al*. 2021). From the current study, the 6 isolates that were non-hemolytic strains were selected for molecular identification. Some strains which belonged to the genus *Bacillus*, were selected for construction of phylogenetic tree to correctly identify the bacterial strains. The evolutionary tree illustrates how different living things have evolved from members of the same species due to variations in genes or proteins. The results of these investigations are frequently represented in a graphic that shows the connection between or the site of the recent gene modifications. In the current study, based on the results of hemolytic activity and molecular identification, 2 strains of antagonistic microorganisms (*B. tequilensis* KUKPS-C3AN5 and *B. thuringiensis* KUKPS-C16HM4) that were expected to have potential, were used for the development of antagonist product.

This research was consistent with several studies in finding that several species of *Bacillus* was often used in the development of antagonistic microorganisms for the control of various plant diseases. Wulff *et al*. (2002) investigated the biochemical and molecular characteristics of antagonistic bacteria (*Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus*) capable of producing secondary metabolites and inhibiting the black rot pathogen *X. campestris* pv. *campestris* in cauliflower. Their results revealed that *B. amyloliquefaciens* could produce surfactin, iturin, bacillomucine and azalomycin F, while *B. subtilis* could produce surfactin and arthrobactin, whereas *B. pumilus* could produce surfactin, amphomycin, arthrobactin and valinomycin that could reduce the black rot caused by *X. campestris* pv. *campestris*. These results were in accordance with the findings of Todorova and Kozhuharova (2010) who evaluated the antagonistic properties and antimicrobial agents of *B. subtilis* that had been isolated from soil. The isolates *B. subtilis* TS 01 and ZR 02 could inhibit the growth of the pathogenic fungi *Alternaria solani*, *Botrytis cinerea*, *Monilia linhartiana* 869, *Phytophthora cryptogea* 759/1 and *Rhizoctonia* spp. In addition, the isolates TS 01 and ZR 02 could inhibit *Pseudomonas syringae* pv. *tomato* and *X. campestris* with zones of inhibition of 48.0 and 50.0 mm, respectively. In addition, Mishra and Arora (2012) investigated the plant root-associated bacteria *Pseudomonas* and *Bacillus* for the control of the black rot pathogen *X. campestris* pv. *campestris* (*Xcc*) in cruciferous plants. Their results indicated that 54 bacterial isolates had ability to inhibit the growth of *Xcc*. Two isolates, namely KA19 and SE produced inhibition zone diameters of more than 11 mm that increased to 18.1 mm when the mixed culture was used. From the results of 16s RNA gene and phylogenetic tree analysis, the isolates KA19 and SE were closely related with *Pseudomonas aeruginosa* and *Bacillus thuringiensis*, respectively. When these 2 antagonistic bacteria were evaluated in field conditions by spraying the leaves, soaking the seeds and kneading the soil, it was found that both strains were able to reduce the symptoms of black rot disease compared to the uninoculated plants. In addition, the growth inhibition of pathogens was more effective when mixed cultures (KA19 + SE) were used.

Endospores are thick-walled structures that are resistant to adverse environmental conditions, such as radiation, stress, chemical disinfectants, extreme heat or cold, and a food shortage. Furthermore, endospores can regenerate into vegetative cells when grown in a suitable soil and environment; these can be highly effective at controlling pathogens immediately. Consequently, endospore-forming bacteria would be suitable for application in the development of endospore products to extend product shelf life and ease of use. A *B. subtilis* endospore product was developed for the control of wilt disease in ginger and the endospores of *B. subtilis* were able to withstand temperatures up to 100°C (Udomsak 2009).

Notably, although *B. subtilis* had a high potential spore production, it is a high risk for humans and animals. Based on the survival rates of the endospores of the selected isolates, they could readily be developed into live products due for use in a real planting environment as the highest temperature found in the plots was 38–42°C and the highest temperature at which the pathogen *X. campestris* could grow was 40°C. Furthermore, endospore-forming products would be easier to keep and to extend their shelf life compared to the live cells of bacteria.

In this research, the focus was on the development of formulas of ingredients suitable for the growth of antagonistic bacteria while maintaining the ability to inhibit

the growth of the pathogen. In the study of the suitable formulation of the medium or carrier for survival, the results showed that *B. tequilensis* KUKPS-C3AN5 strains that had been stored for 8 weeks in the soybean-mungbean medium had a survival rate of 99.10%, while the culture that was stored in rice grains had a survival rate of 106.42%. Similarly, the survival rates for *B. thuringiensis* KUKPS-C16HM4 stored in the soybean-mungbean medium and rice grains, were 99.19 and 108.34%, respectively. Accordingly, the survival rates of both strains in either of the carriers were not different. However, soybean-mung bean cost was higher than for the rice grains. However, to consider the effect on survival rate and efficacy in suppressing black rot pathogens, the soybean-mungbean was more cost-effective because of its inhibition efficacy in cases of severe black rot disease pathogen infestation in vegetable plots. It is necessary to use antagonists that can inhibit pathogens quickly and efficiently to achieve effective control of the spread of disease and the resulting damage to vegetables. From the observations on the inhibitory efficacy of *B. tequilensis* KUKPS-C3AN5 and *B. thuringiensis* KUKPS-C16HM4 cultured in soybean-mungbean and rice grain carriers for 2 months, both strains cultured in the soybean-mungbean medium were more effective at inhibiting pathogens than those cultured in rice grains. Comparing the two strains cultured in the same medium, *B. tequilensis* KUKPS-C3AN5 cultured in the soybean-mungbean medium had the better inhibition of pathogens with an inhibition zone of 16.78 mm, while the inhibition zones of *B. tequilensis* KUKPS-C3AN5 cultured in rice grains, of *B. thuringiensis* C16HM4 cultured in soybean-mungbean medium and of *B. thuringiensis* C16HM4 cultured in rice grains were 11.44, 9.78 and 8.22 mm, respectively. In addition, after 2 weeks of inoculation, the inhibition efficacy of the black rot pathogens decreased. This seemed to indicate that antagonist products should not be inoculated for more than 14 days and should be re-inoculated every time before use.

From the experimental results, it can be concluded that for inhibition efficacy of *X. campestris* pathogens of both antagonists the following factors were important: a complete supply of essential nutrients for the growth of the antagonists and water as one of the main factors essential for bacterial growth, as each strain of microorganism requires a different amount of moisture to grow. The moisture in the medium that can be used by microorganisms is in the form of available water or water activity (A_w). The free water surrounding the medium is not absorbed by the medium or ions of other substances where bacteria have a minimum A_w level of 0.91 (Barbosa-Cánovas *et al*. 2020). The optimum A_w value for bacterial growth is close to 1.0, which is a high value and at that point there will be dissolved nutrients suitable for the growth of microorganisms. Sirisoontaralak *et al*. (2013) reported that normal cooked rice had A_w values in the range 0.93–0.97, which was close to that for the rice grains used in the current study, while the soybean-mung bean medium in liquid form had an A_w value of 1.0. It may be concluded from the above information that the suitable medium should consider the nutrients and have an A_w value sufficient for culturing. Future work should include: a comparative study of the use of mixed microorganisms in the inhibition of pathogens; optimization of the nutrients or physical factors necessary for the growth of antagonistic microorganisms; and the characterization of inhibiting substances produced by antagonistic microorganisms to develop into potential products suitable to be used in planting plots.

Conclusion

According to this research, *B. tequilensis* KUKPS-C3AN5 and *B. thuringiensis* KUKPS-C16HM4 are antagonistic bacteria with potential application as a growth control product for *X. campestris*, a causative agent of black rot in cruciferous plants. Both strains could produce spores but not lysis red blood cells, which confirmed they did not harm humans and animals. From the application of soybean-mung bean and rice grains to develop into antagonist products, it was found that the survival rates for both strains of *Bacillus* were similar. Additionally, *B. tequilensis* KUKPS-C3AN5 was more effective in inhibiting black rot pathogens than *B. thuringiensis* KUKPS-C16HM4. Finally, the more suitable carrier for product development was the soybean-mungbean extract medium.

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Author Contributions

SP contributed to the conception and design of the experiments. SP, YP, and SM planned the experiments, and sample collection. SP and KK conducted and investigated the experiments. SP prepared original draft manuscript. SP, YP, and SM performed final revision and reviewed the manuscript. SP supervised and administered research.

Conflict of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable in this manuscript

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