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Selection of carrier materials for formulation of the antagonistic *Bacillus* spp. against rice bacterial leaf blight

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ABSTRACT

Bacillus pumilus ST-115, B. safensis AG-131, B. stratosphericus AG-62 and B. subtilis TG-71 showed strong antagonistic effects against Xanthomonas oryzae pv. oryzae causing rice bacterial leaf blight. This research aims at evaluating the effects of carrier materials on cell densities, the antagonistic effects and disease-reducing effects of these four bacterial strains and selecting the suitable carrier material(s) for each of them. Five carrier materials, i.e., talc powder, rice bran, rice husk powder, rice grain powder and rice kernel powder, were used to store the Bacillus spp. in a six-month period at room temperature. Results show that after six-month storage, formulations of Bacillus spp. using talc powder, rice bran and rice husk powder could remain the cell densities at over 10^6 CFU/g formulation, the antagonistic effects on agar plates and the disease-reducing effects under greenhouse conditions. Rice bran was the suitable carrier material for B. safensis AG-131, rice husk powder for B. pumilus ST-115 and talc powder for both B. stratosphericus AG-62 and B. subtilis TG-71.

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1 INTRODUCTION

Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a common rice disease which reduces rice quality and could cause yield loss of 10-20% (Mew *et al.*, 1993). Although using chemicals is the commonly used control means for BLB in Vietnam, it reveals many disadvantages, e.g., causing adverse effects on the environment and ecosystem, having harmful impacts on human health, stimulating the resistance of pathogens and being costly (Khoa *et al.*, 2016). Therefore, efforts have been made to find alternative control methods that are more effective, economic, and environmentally friendly. Two approaches to be considered are the uses of host resistance and bio-control agents (antagonists). Host resistance, however, requires long-term study, substantial investment and modern techniques while using antagonistic microorganisms is more affordable, sustainable and environmentally friendly (Nguyen Dang Ngoc Giau, 2014). Indeed, the antagonists could persist in the environment to continuously inhibit pathogens (Mew *et al.*, 2004).

Five antagonistic *Bacillus* spp. against *Xoo* were isolated, i.e., *B. aerophilus* HG-33, *B. pumilus* ST-115, *B. safensis* AG-131, *B. stratosphericus* AG-62 and *B. subtilis* TG-71 (Vo Thi Phuong Trang, 2013; Nguyen Dang Ngoc Giau, 2014; Tran Kim Thoa, 2015). Their disease-reducing effects were confirmed under field conditions (Nguyen Hoang Thong, 2014; To Anh Khoa, 2014; Hang Anh Tai, 2015; Nguyen Mong Huyen Trang, 2015; Nguyen

Ngoc Loc, 2018). The disease-reducing effects of antagonistic bacteria depend on their cell densities during application (Heijnen and Van Veen, 1991), hence commercial products should be able to maintain high cell density in a long time of storage. The product components play an important role here, among those the carrier materials (Vidhyasekaran and Muthamilan, 1995). The main functions of carrier materials are to ensure the growth and maintain the appropriate bacteria cell densities in adequate time (Smith, 1992). This paper presents the effects of five carrier materials on cell densities, antagonistic effects and disease-reducing effects of the four strains of *Bacillus* spp. to select the suitable carrier material(s) for each of them.

2 MATERIALS AND METHODS

2.1 The antagonistic bacteria and carrier materials

The antagonistic *B. stratosphericus*, *B. safensis*, *B. subtilis* and *B. pumilus* used in this research were provided by the Plant Pathology Research Group of the Biotechnology Research and Development Institute, Can Tho University. Five different carrier materials were commercial talc powder, rice bran, rice kernel powder, rice grain powder and rice husk powder processed from rice cultivar IR50404.

2.2 Forming formulations of *Bacillus* spp. and evaluating bacteria viable cell densities in formulations monthly during storage time

Forming formulations of *Bacillus* spp.

The experiment was arranged in a completely randomized design with two factors: solid carrier materials (talc powder, rice bran, rice kernel powder, rice grain powder, and rice husk powder) and antagonistic bacteria (*B. stratosphericus*, *B. safensis*, *B. subtilis*, and *B. pumilus*). Therefore, there were 20 treatments with three replications.

Preparation of carrier materials

Each type of carrier material was finely mixed with CMC and $CaCO_3$ as the following formula: 1,000 g carrier material + 10 g CMC + 15 g $CaCO_3$ (Vidhyasekaran and Muthamilan, 1995). The mixture of each treatment was delivered to plastic bags (5 g/bag). These bags were then sterilized twice in two consecutive days at 121^{o}C, 1 atm, 20 min.

Preparation of antagonistic *Bacillus* suspension

A loop of *Bacillus*, grew on nutrient agar (NA) medium for two days, was inoculated to 10 mL nutrient broth (NB) in falcon tubes. These tubes were incubated two days on a biological shaker before inoculating 1 mL bacteria suspension to 100 mL NB in Erlenmeyer flasks. The flasks were then placed on a biological shaker for two days at 150 cycles/min to obtain the final suspension. Based on the OD_{600} value and viable cell density standard line, the final suspension of each strain was diluted to get the cell density at 2×10^8 CFU/mL (Đặng Hoài An, 2016).

Inoculation of *Bacillus* suspension to the carrier materials

The sterilized carrier material bags were inoculated with 2 mL of antagonistic inoculant (2×10^8 CFU/mL) and covered with cotton lids to prevent contaminants and allow transpiration before drying in an oven at 40^{o}C. Every 6 hrs. during the drying time, three formulation bags of each treatment were selected randomly for moisture measuring using moisture balance Ohaus B25 – USA. Formulation bags got moisture under 20% were sealed, labeled and stored at room temperature (28 ± 2^{o}C).

Evaluating viable cell densities in formulations monthly during storage

Three formulation bags from each treatment were selected randomly to examine the viable cell densities monthly using plate counting method on (NA) medium.

Plate counting method for formulation

One g of each carrier material from a random formulation bag was added to 9 mL sterilized distilled water to get a stock solution. The stock was continued to dilute by adding 1 mL into 9 mL sterilized distilled water. A serial ten-time dilution was done until obtaining appropriate liquescency. The final solution was then spread on NA plate and incubated 48 hrs at room temperature (28 ± 2^{o}C). The number of colonies observed after 48 hrs. was used to calculate the cell density in the formulation bag as the following formula (Reynolds, 2011):

$$M = (D\times A\times V2)/(V1/m)$$

Where: M is the bacteria viable cell density in the formulation (CFU/g)

A is the average counted colonies on NA medium after two-day incubation

D is the liquescency of the final solution

V1 is the volume of the final solution that spread out on a plate (mL)

V2 is the volume to dilute m (g) formulation (mL)

m is the weight of formulation (g)

2.3 Evaluating the antagonistic effects of *Bacillus* spp. in formulations after storage

The antagonistic effects of *Bacillus* spp. after storage were determined monthly by the diameter of inhibition zones on agar plates using dual-culture test.

Preparation of pathogen suspension

The pathogen *Xoo* was grown on modified Wakimoto's medium agar plate (20 g sucrose, 5 g peptone, 5 g $Ca(NO_3)_2.4H_2O$, 0.82 g Na_2HPO_4, 0.05 g $FeSO_4.7H_2O$, 15 g in 1 L distilled water, pH 7.0) (Karganilla *et al*., 1973) at 28 ± 2°C for 48 – 72 hrs. The suspension was prepared by adding two loops of *Xoo* colonies to 10 mL sterilized distilled water and homogenized by vortexing. The final cell density in *Xoo* suspension was adjusted to 2×10^9 CFU/mL (OD_{600} = 0.3) (Võ Thị Phương Trang, 2013).

Setting up dual-culture test

Fifty microliter of *Xoo* suspension was spread on modified-Wakimoto medium agar plate. *Bacillus* colonies were then inoculated into three spots on the surface of the medium. The plates were stored at room temperature (28 ± 2°C). The inhibition zones were measured after 48 hrs. on upside down plates.

2.4 Evaluating disease-reducing effects of *Bacillus* spp. in formulations after storage under greenhouse conditions

This experiment was carried out at the third and the sixth month after storage for formulations that remained cell densities at 10^6 CFU/g and the antagonistic effects on *Xoo*.

The experiment was arranged in a completely randomized design with three rice plants per pot as one replication; each treatment involves three replications. Negative control was treated with distilled water while the positive one was treated with Starner 20 WP. The disease-reducing effects were determined by comparing the means of lesion lengths on leaves treated with *Bacillus* suspension to that on leaves of control treatments.

Soil preparation

Soil collected in Campus of Can Tho University was smashed, plowed, harrowed and pretreated with calcium hydroxide. Then, two kg of soil was transferred to a round pot (16 cm × 14 cm), soaked in water for three days and surface-dried before sowing.

Inoculum preparation

One gram of the formulation was added to 10 mL distilled water and mixed well to get the stock suspension. The stock suspension was diluted with distilled water to get the inoculum with cell density at 10^7 CFU/mL based on the cell densities had been calculated.

Rice cultivation

Rice seeds were soaked 30 minutes in water at 55°C before incubated at 28°C for 48 hrs. to germinate. The germinated rice seeds were then soaked with inoculum in 2 hrs. before sowing. The positive and negative treatments were treated with distilled water.

Three rice plants were grown in a pot and watered daily. In addition, each pot was also provided with the recommended dose of fertilizers from Can Tho Agriculture Extension Center: 2.4 g of P_2O_5 (superphosphate, Lam Thao Fertilizers and Chemicals JSC, Vietnam) at a day before sowing, 0.5 g of urea (46% of nitrogen, Dam Phu My, Vietnam) and 0.12 g of potassium chloride (61% of K_2O, Vinacam JSC, Vietnam) at 10 days after sowing (DAS) and 1 g of N and 0.12 g of K_2O at 20 and 40 DAS.

Pathogen inoculation and measurement of lesion lengths

Rice plants were inoculated at 45 DAS by leaf-clipping method (Kauffman *et al*., 1973). The scissors, sterilized with 70% (v/v) ethanol, were submerged in the bacteria suspension and used to cut five fully expanded leaves (at 2-3 cm from the leaf tip) per plant. The positive control was sprayed Starner 20WP (1 mg/mL) at 3, 8 and 13 days after inoculation (DAI). The disease was assessed by measuring the actual lesion lengths on the inoculated leaves at 5, 10 and 15 DAI.

2.5 Data analyses

The mean and standard error of the data were calculated using Microsoft Excel version 2016. The difference among treatments was analyzed by one - way analysis of variance (ANOVA), followed by Duncan's multiple range test of IBM SPSS Statistics v22.0, and all hypotheses were rejected at $P \leq 0.05$.

3 RESULTS AND DISCUSSIONS

3.1 Formulations and cell densities of *Bacillus* spp. during storage

3.1.1 Formulation of Bacillus pumilus ST-115

The cell densities of *B. pumilus* in formulations were about $1.4–1.6\times10^8$ CFU/g when they were sealing for storing. Table 1 shows the cell densities of *B.*

pumilus in five carrier materials during six-month storage.

In the first two months, bacterial cell densities in all carrier materials dropped rapidly, excepting rice bran and rice husk powder (remaining 10^7 CFU/g). They continued to get the highest cell densities among treatments from the third month to the sixth month. At the second month for rice kernel powder and the fourth month for rice grain powder, cell densities in formulations only got 10^3 CFU/g, thus they would not be tested further. After six months, talc powder, rice bran and rice husk powder remained cell densities over 10^6 CFU/g with rice bran had the highest one at 13.6×10^6 CFU/g.

Table 1: Viable cell densities of *Bacillus pumilus* ST-115 in formulations during six-month storage

Treatments	Bacterial cell densities (10^6 CFU/g)					
	1	2	3	4	5	6
Talc powder	14^c	6.1^b	5.8^b	4.1^b	4.1^b	3.8^b
Rice bran	32^b	26^a	24^a	23^a	23^a	13.8^a
Rice husk powder	54^a	26^a	25^a	23^a	23^a	13.6^a
Rice grain powder	14^c	5.8^c	2.01^c	0.12^c	-	-
Rice kernel powder	3.4^d	0.71^d	-	-	-	-

For each column, means with the same letters are not significantly different at $P \leq 0.05$. Cell densities were converted to log_{10} values before statistic analysis.

3.1.2 Formulations of Bacillus safensis AG-131

The cell densities of *B. safensis* in five carrier materials were about $1.4 – 1.6\times10^8$ CFU/g when they were sealing for storing. Table 2 shows the cell densities of *B. safensis* in five carrier materials during six-month storage.

Table 2: Viable cell densities of *Bacillus safensis* AG-131 in formulations during six-month storage

Treatments	Bacterial cell densities (10^6 CFU/g)					
	1	**2**	**3**	**4**	**5**	**6**
Talc powder	21^d	21^a	13^{ab}	12^b	9.8^a	5.8^{ab}
Rice bran	53^b	23^a	21^a	19^a	7.9^b	6.9^a
Rice husk powder	62^a	28^a	18^a	17^{ab}	8.3^b	5^b
Rice grain powder	43^c	9.2^b	4.4^b	0.13^d	-	-
Rice kernel Powder	23^d	1.6^c	0.77	-	-	-

For each column, means with the same letters are not significantly different at $P \leq 0.05$. Cell densities were converted to log_{10} values before statistic analysis.

After four-month storage, cell densities in talc powder, rice bran and rice husk powder still remained over 10^7 CFU/g. Otherwise, rice kernel powder and rice grain powder can only remain cell densities at 10^4 CFU/g for three months and four months, respectively. In the last two months of the storage time, talc powder, rice bran and rice husk powder had cell densities over 10^6 CFU/g. Among these, rice bran treatment can preserve *B. safensis* at the highest cell density (6.9×10^6 CFU/g) at six-month storage.

3.1.3 Formulations for Bacillus stratosphericus AG-62

The cell densities of *B. stratosphericus* in five carrier materials were about $1.4 – 1.6\times10^8$ CFU/g when they were sealing for storing. Table 3 shows the cell densities of *B. stratosphericus* in five carrier materials during six-month storage.

Table 3: Viable cell densities of *Bacillus. stratosphericus* AG-62 in formulations during six-month storage

Treatments	Bacterial cell densities (10^6 CFU/g)					
	1	**2**	**3**	**4**	**5**	**6**
Talc powder	51^a	48^a	41^a	40^a	31^a	22.4^a
Rice bran	38^b	36^b	22^b	8.2^c	8.1^b	7.9^b
Rice husk powder	58^a	38^{ab}	23^b	22^b	5.7^c	3.4^c
Rice grain powder	28^b	21^c	9.8^c	0.4^d	-	-
Rice kernel powder	15^d	1.1^d	0.08^d	-	-	-

For each column, means with the same letters are not significantly different at $P \leq 0.05$. Cell densities were converted to log10 values before statistic analysis.

Overall, talc powder had the ability to preserve *Bacillus* better than other treatments. Cell density in talc powder formulation still got 22.4×10^7 CFU/g throughout six months. Besides talc, rice bran and rice husk powder could remain cell densities up to 10^6 CFU/g during the storage period, while rice grain powder could store *B. stratosphericus* for four months (4×10^5 CFU/g) and three months in case of rice kernel powder (8×10^4 CFU/g).

3.1.4 Formulations of Bacillus subtilis TG-71

The cell densities of *B. subtilis* in formulations were about $1.4 – 1.6\times10^8$ CFU/g when they were sealing for storing. Table 4 shows the cell densities of *B. subtilis* in five carrier materials during six-month storage.

Table 4: Viable cell densities of *Bacillus subtilis* TG-71 in formulations during six-month storage

Treatments	Bacterial cell densities (10^6 CFU/g)					
	1	**2**	**3**	**4**	**5**	**6**
Talc powder	75^a	38^a	24^a	18^a	9.2^a	8.4^a
Rice bran	65^b	15^b	13^b	13^b	7.3^b	6.9^b
Rice husk powder	62^b	32^a	11^c	7.4^c	6.8^b	5.3^c
Rice grain powder	11^c	5^c	3.1^d	0.07^d	-	-
Rice kernel powder	6.6^d	0.03^d	-	-	-	-

For each column, means with the same letters are not significantly different at $P \leq 0.05$. Cell densities were converted to log_{10} values before statistic analysis.

Talc powder and rice bran got cell densities over 10^7 CFU/g after four months. Then, they remained bacteria at 10^6 CFU/g up to six months with talc had the highest one at 8.4×10^6 CFU/g. Similar to talc powder and rice bran, rice husk powder also remained cell densities at 10^7 CFU/g for three months before dropped to 10^6 CFU/g at the fourth month and remained this density to the sixth month (5.3×10^6 CFU/g). For rice grain and rice kernel powder, cell densities decreased to 7×10^4 CFU/g in the fourth month and 3×10^4 CFU/g in the second month, respectively.

The results showed that cell densities of all four strains of *Bacillus* spp. decreased rapidly in the first month of storage and continued to decrease gradually during storing period. The bacterial cell densities of the endospore-forming bacteria stored in inert carriers decreased during storing period (Omer, 2010). In the first month of storage, bacterial cell densities decreased sharply because they were sensitive with new habitat in carriers as well as did not have enough time for forming endospores.

From the results after six months storing, talc powder was considered as the suitable carriers for preserving cell densities of *Bacillus* spp. Besides, rice bran and rice husk powder had abilility to store *Bacillus* spp. at high sensities up to eight months. Talc, rice bran and rice husk powder contain a large amount of minerals (Ca, Mg, Si, Cu, Fe, Zn, etc.) which induce bacteria to form endospores. Rice grain powder and rice kernel powder, however, could not preserve *Bacillus* spp. cell densities longer than three months because of the high content of nutrient in these carriers, which inhibit the endospores formation of *Bacillus* spp. Furthermore, these carriers are easy to be contaminated because their nutrition is also flavorable for other bacteria and fungi. Therefore, rice grain powder and rice kernel powder is not suitable for storing five strains of *Bacillus* spp. mentioned above.

3.2 The antagonistic effects of *Bacillus* spp. in formulation after storage

The treatments that got cell densities over 10^6 CFU/g continued to be tested the antagonistic effects of *Bacillus* spp. monthly during the storage period. The antagonistic effects of *Bacillus* spp. in the formulation were determined by their inhibition zone against *Xoo* on agar plates. The diameters of the inhibition zones are shown in Table 5.

Table 5: The diameter of inhibition zones (mm) made by *Bacillus* spp. in formulation against *Xoo* on agar plates during storage

Bacteria	Treatments	The diameter of the inhibition zones (mm)					
		1	2	3	4	5	6
Bacillus pumilus	Talc powder	10.3±0.4	12.7±1.1	11.3±1.6	7.0±0.7	10.7±1.1	9.0±0.7
	Rice bran	8.7±0.4	11.0±1.3	9.7±1.6	10.0±0.7	9.0±0.7	9.0±1.3
	Rice husk powder	8.7±0.4	9.7±0.4	11.0±0.7	10.7±0.7	9.3±1.6	9.7±1.6
	Rice grain powder	9.0±0.7	8.0±0.7	6.7±0.4	-	-	-
	Rice kernel powder	8.3±0.4	9.3±0.4	-	-	-	-
Bacillus safensis	Talc powder	4.7±0.4	5.7±0.4	5.3±0.9	4.3±0.4	5.7±1.1	6.0±0.7
	Rice bran	5.3±0.4	4.7±0.4	6.3±0.4	6.0±1.3	6.3±1.1	5.7±1.1
	Rice husk powder	4.7±0.4	5.3±0.4	6.7±0.4	6.3±1.1	6.0±1.3	6.0±2.0
	Rice grain powder	4.3±0.4	4.7±0.9	4.3±1.1	-	-	-
	Rice kernel powder	3.3±0.4	4.0±0	-	-	-	-
Bacillus stratosphericus	Talc powder	5.7±0.4	6.0±0.7	6.3±0.9	6.3±1.1	6.7±0.4	6.7±1.1
	Rice bran	2.7±0.4	2.3±0.4	3.7±0.4	3.3±0.4	4.0±0.7	4.3±0.9
	Rice husk powder	3.0±0	3.3±0.4	3.7±1.1	3.7±0.9	5.3±1.1	5.0±0.7
	Rice grain powder	4.3±1.1	2.3±0.4	4.7±0.4	-	-	-
	Rice kernel powder	3.3±0.4	3.7±0.4	-	-	-	-
Bacillus subtilis	Talc powder	8.0±0.7	7.3±0.9	6.3±0.9	4.7±0.4	7.3±0.4	6.7±1.1
	Rice bran	9.3±1.1	9.0±0.7	8.3±1.1	6.0±1.3	8.7±1.8	7.7±2.2
	Rice husk powder	10.0±0.7	8.7±1.1	6.3±0.4	3.7±0.4	8.0±0.7	6.7±1.6
	Rice grain powder	5.3±0.4	6.3±0.4	5.0±0.7	-	-	-
	Rice kernel powder	7.6±0.4	-	-	-	-	-

It is evident that bacteria in all treatments can remain the antagonistic effects against *Xoo* on agar plates. During six-month storage, the inhibition diameters varied among *Bacillus* strain, particularly 8.7 - 12.7 mm for *B. pumilus* ST-115, 3.3 - 6.3 mm for *B. safensis* AG-131, 2.3 - 6.7 mm for *B. stratosphericus* AG-62 and 4.7 - 10 mm for *B. subtilis* TG-71. Furthermore, the inhibition diameters of each strain were different during the storage period. According to Võ Thị Phương Trang (2013) and Trần Kim Thoa (2014), the diameter of inhibition zones for new-cultured *B. safensis* AG-131, *B. stratosphericus* AG-62, *B. pumilus* and *B. subtilis* were 11.3 mm, 13.3 mm, 13.6 mm and 9.0 mm respectively. Although the inhibition zones of *Bacillus* spp. decreased after storage as compared to fresh bateria, *Bacillus* spp. still remained the antagonistic effects against *Xoo in vitro*. The decrease of inhibition zone of bacteria was a result of the dormant stage of *Bacillus* spp. in storage.

3.3 Disease-reducing effects of *Bacillus* spp. in the formulation during storage under greenhouse conditions

The treatments that got cell densities over 10^6 CFU/g continued to be tested the disease-reducing effects of *Bacillus* spp. under greenhouse conditions after three- and six-month storage.

3.3.1 Formulation for Bacillus pumilus ST-115

The mean lesion lengths of *B. pumilus* after three-month storage are shown in Fig. 1. Except for rice bran treatment at 15 DAI, other treatments showed effects on disease-reducing during three assessment times. Rice bran, talc powder, rice husk powder had the mean lesion lengths similar to positive control at 5 and 10 DAI. At 15 DAI, only talc treatment had the disease-reducing effects similar to the positive control.

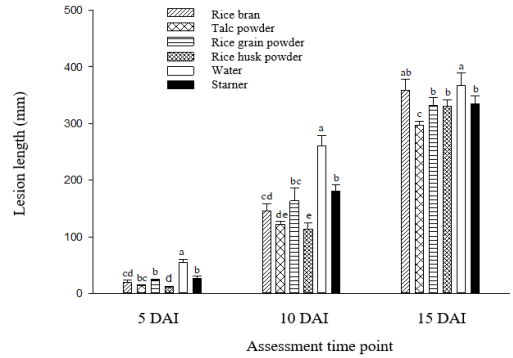

Fig. 1: Mean lesion lengths (mm) of rice bacterial blight treated with *Bacillus pumilus* ST-115 in formulations after three-month storage at 5, 10 and 15 DAI

At the same time point, bars with same letters are not significantly different at $P \leq 0.05$. DAI: days after inoculation.

The mean lesion lengths of *B. pumilus* after six-month storage are shown in Fig. 2. Only talc powder, rice bran and rice husk powder were tested in this experiment. All treatment had shorter lesion

lengths than negative control at 5 and 10 DAI. At 10 DAI, they kept remaining effective lesion length similar to the positive control.

From the results under greenhouse conditions, the cell densities of *B. pumilus* in the formulation and its antagonistic effects during storage, rice bran and rice husk powder were chosen as suitable carrier materials for storing *B. pumilus* in six months.

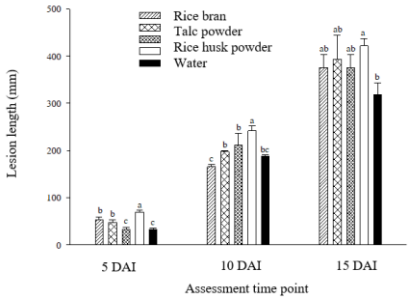

Fig. 2: Mean lesion lengths (mm) of rice bacterial blight treated with *Bacillus pumilus* ST-115 in formulations after six-month storage at 5, 10 and 15 DAI

At the same time point, bars with same letters are not significantly different at $P \leq 0.05$. DAI: days after inoculation.

3.3.2 Formulations for Bacillus safensis AG-131

The mean lesion lengths of *B. safensis* after six-month storage are shown in Fig. 3. At 5 DAI, only treatment used rice husk powder and rice grain powder had shorter lesion length than negative control. Only rice grain powder showed the disease-reducing effects that is similar to positive control. At 10 DAI, all four treatments express the same effects as Starner. However, at 15 DAI there were no differences among treatments and controls.

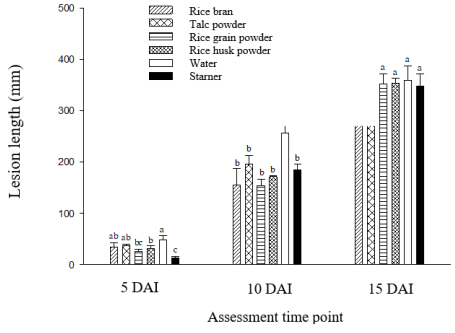

Fig. 3: Mean lesion lengths (mm) of rice bacterial blight treated with *Bacillus safensis* AG-131 in formulations after three-month storage at 5, 10 and 15 DAI

At the same time point, bars with same letters are not significantly different at $P \leq 0.05$. DAI: days after inoculation.

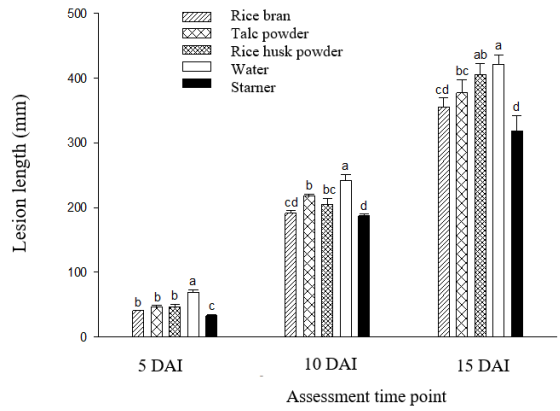

Fig. 4: Mean lesion lengths (mm) of rice bacterial blight treated with *Bacillus safensis* AG-131 in formulations after six-month storage at 5, 10 and 15 DAI

At the same time point, bars with same letters are not significantly different at $P \leq 0.05$. DAI: days after inoculation.

The mean lesion lengths of *B. safensis* after six-month storage are shown in Fig. 4. Only talc, rice bran and rice husk powder were tested in this experiment. Except for rice husk powder at 15 DAI, other treatments had shorter lesion lengths than negative control during three assessment points. However, only rice bran treatment had effective lesion length similar to positive control at 10 and 15 DAI.

Therefore, from the results under greenhouse conditions, the cell densities of *B. safensis* in the formulation and its antagonistic effects during storage, talc powder and rice bran were chosen as suitable carrier materials for storing *B. safensis* in six months. However, rice bran is much cheaper than talc powder, so it is more economic for the industry to use rice bran instead of talc for storing *B. safensis*.

3.3.3 Formulations for Bacillus stratosphericus AG-62

The mean lesion lengths of *B. stratosphericus* after three-month storage are shown in Fig. 5. All four treatments showed effects on disease-reducing at 5 and 10 DAI. At 5 DAI, talc powder and rice kernel powder treatments had mean lesion lengths similar to the positive control. At 10 DAI, all treatments had the disease-reducing effects similar to the positive control. However, at 15 DAI none treatment showed effects on lesion length.

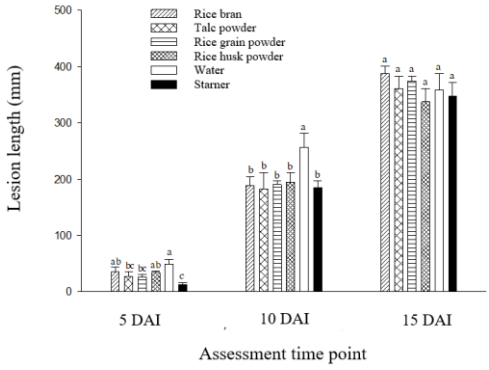

Fig. 5: Mean lesion lengths (mm) of rice bacterial blight treated with *Bacillus stratosphericus* AG-62 in formulations after three-month storage at 5, 10 and 15 DAI

At the same time point, bars with same letters are not significantly different at $P \leq 0.05$. DAI: days after inoculation.

The mean lesion lengths of *B. stratosphericus* after six-month storage are shown in Fig. 6. Only talc, rice bran, and rice husk powder were tested in this experiment. At 5 DAI, all of the three treatments had shorter lesion lengths compared to those of negative control with rice husk powder had effects that were similar to the positive control. At 10 DAI, all treatments had the disease-reducing effects similar to the positive control. However, only rice husk powder could remain effects at 15 DAI.

Therefore, from the results under greenhouse conditions, the cell densities of *B. stratosphericus* in the formulation and its antagonistic effects during storage, talc powder was chosen as suitable carrier material for storing *B. stratosphericus* in six months.

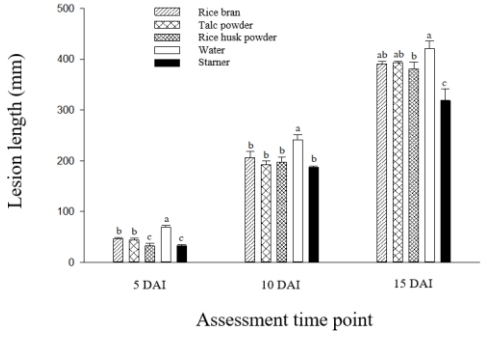

Fig. 6: Mean lesion lengths (mm) of rice bacterial blight treated with *Bacillus stratosphericus* AG-62 in formulations after 6-month storage at 5, 10 and 15 DAI

At the same time point, bars with same letters are not significantly different at $P \leq 0.05$. DAI: days after inoculation.

3.3.4 Formulations for Bacillus subtilis TG-71

The mean lesion lengths of *B. subtilis* after three-month storage are shown in Fig. 7. At 5 and 10 DAI, all four treatments had similar effects on disease-reducing to the positive control. At 15 DAI, however, there were no differences among treatments and negative control.

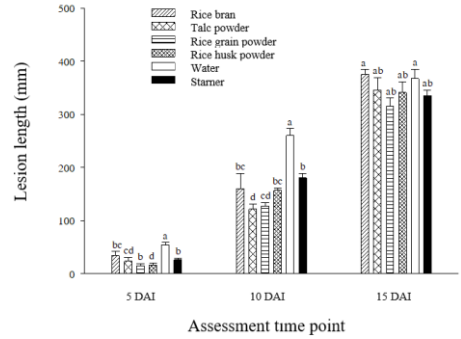

Fig. 7: Mean lesion lengths (mm) of rice bacterial blight treated with *Bacillus subtilis* TG-71 in formulations after three-month storage at 5, 10 and 15 DAI

At the same time point, bars with same letters are not significantly different at $P \leq 0.05$. DAI: days after inoculation.

The mean lesion lengths of *B. subtilis* after six-month storage are shown in Fig. 8. Only talc, rice bran, and rice husk powder were tested in this experiment. All treatment had shorter lesion lengths than negative control during three assessment points. At 10 DAI, talc powder and rice husk powder had similar effects on BLB compared to those of Starner. However, only talc powder could remain its effects at 15 DAI.

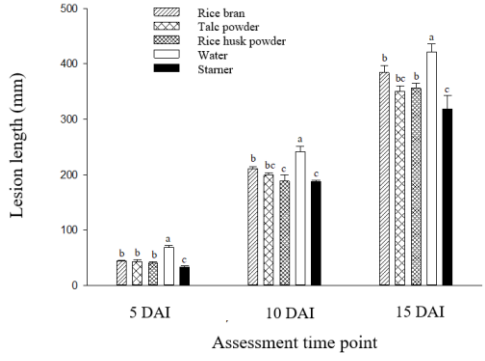

Fig. 8: Mean lesion lengths (mm) of rice bacterial blight treated with *Bacillus subtilis* TG-71 in formulations after six-month storage at 5, 10 and 15 DAI

At the same time point, bars with same letters are not significantly different at $P \leq 0.05$. DAI: days after inoculation.

From the results under greenhouse conditions, the cell densities of *B. subtilis* in the formulation and its antagonistic effects during storage, talc powder was chosen as suitable carrier material for storing *B. subtilis* in six months. Talc powder has nutrient limitation but high mineral composition, especially magnesium and silicon which could improve the sporulation, supporting long-term storage.

4 CONCLUSION

Effects of the five carrier materials (talc powder, rice bran, rice husk powder, rice grain powder and rice kernel powder) on cell densities, antagonistic effects and disease-reducing effects of the four strains of *Bacillus* spp. were tested to select the suitable carrier material(s) for each of them. After six-month storage, formulations of *Bacillus* spp. using talc powder, rice bran and rice husk powder still remained the cell densities at over 10^6 CFU/g as well as the antagonistic effects against *Xoo in vitro* and the disease-reducing effects to BLB under greenhouse conditions. After combining the results from three experiments, rice bran was the suitable carrier material for storing *B. safensis* AG-131 in six months, rice husk powder for *B. pumilus* ST-115 and talc powder for both *B. stratosphericus* AG-62 and *B. subtilis* TG-71.

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