Biological Control of *Bipolaris oryzae* With *Bacillus Subtilis* and the Development of a Formulation for Rice Seed Treatment

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ABSTRACT

One of the most important diseases that affects rice yield is brown spot disease, which is caused by *Bipolaris oryzae*. In this study, antagonistic bacteria that inhibit *B. oryzae* were evaluated, and a formulation for seed treatment was developed. The effect of antagonistic bacteria was assessed on *B. oryzae* growth inhibition by a dual culture method. Antagonistic bacteria were developed as a formulation and evaluated on *B. oryzae* growth inhibition in artificial media, infection of treated seeds and brown spot disease on the seedling. The shelf life of antagonistic bacteria in formulations was investigated for 6 months. The most effective antagonistic bacterium, which showed 66.1% growth inhibition, was identified as *B. subtilis.* In poisoned food method, a formulation of Talc+SCMC1 was amended in Potato Dextrose Agar showed the highest inhibited mycelial growth of *B. oryzae* at 73.5%. Seeds treated with a Talc+PVP1 formulation during 0, 3 and 6 months of storage showed the highest reduction natural infection of *B. oryzae* at 67.3, 94.3 and 72.3%, respectively. Seedlings treated with 2 g of a Talc+PVP3 formulation showed the highest reduction in the development of brown spot disease at 92.5% during 3 months of product storage. The initial population of each formulation was 13.5−13.7 log CFU/g at 0 months. The population of *B. subtilis* decreased with each month of storage and was the lowest at 6.5−6.7 log CFU/g after 6 months of storage.

Keywords: Rice, biocontrol, *Bipolaris oryzae*, formulation, antagonist

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INTRODUCTION

Rice is an important food crop worldwide. In 2016, rice production in Thailand was ranked as the sixth in the world after China, India, Indonesia, Bangladesh and Vietnam with an export volume of 9.91 million tons, valued at 154.73 billion baht (Office of Agricultural Economic, 2016). Brown spot disease caused by *Bipolaris oryzae* (Telemorp: *Cochliobolus miyabeanus*) is one of the most important causes of rice yield loss. The disease is seed borne, and its transmission causes seed discolouration, seedling blight, and necrotic spots on leaves. Ba and Sangchote (2006) reported that *B. oryzae* infected the embryo, endosperm, palea, lemma, rachilla and sterile lemma but was mostly found on the rachilla at 82%. The disease symptoms appeared on the coleoptile and roots after 7−14 days of planting. Brown spot disease has generally been found in South and South−East

Asian countries, including Thailand. Klinmanee *et al*. (2013) reported brown spot outbreak in rice varieties CHT1, PSL2, PTTi and Sang Yod Phatthalung at 30−100% incidence and 5−40% severity (Temp. 26.4−29.7°C, 67.0−87.0% RH) in Phatthalung and Songkhla Province, Southern of Thailand. Savary *et al*. (2000) reported that sheath blight (*Rhizoctonia solani*), brown spot (*Cochliobolus miyabeanus*) and leaf blast (*Pyricularia oryzae*) were rice diseases that reduced the yield by 1−10% in tropical Asia. The control of brown spot disease, seed treatment and foliar spray with effective fungicides such as iprodione, propiconazole, azoxystrobin, trifloxystrobin, dithane and carbendazim has been reported (Gupta *et al*., 2013; Wongcharoen, 2013; Ahmed *et al*., 2002; Sparks *et al*., 2017). However, the improper use of these fungicides is harmful to humans and the environment. Recent studies have proposed alternative ways to control brown spot disease. Benzoic acid and salicylic acid at 9 mM completely inhibited mycelial growth of *B. oryzae in vitro.* A 20−mM benzoic acid spray reduced the disease severity (1.0−2.0%) and disease incidence (4.3−21.8%) on rice leaves at 7−21 days after application. Najed *et al*. (2014) reported that *Streptomyces* spp. isolated from soil in the rice field showed antifungal activity against *B. oryzae.Trichoderma harzianum* and *T. viride* were reported to inhibit the growth of *B. oryzae* at 71.2 and 67.9%, respectively by a dual−culture test (Manimegalai *et al*., 2011). *Bacillus* spp. showed a 52.2% mycelial growth reduction of *B. oryzae* (Nalk *et al*., 2016). The objective of this study was to evaluate antagonistic bacteria against *Bipolaris oryzae* and develop a formulation for using it as a seed treatment.

MATERIALS AND METHODS

Antagonistic Bacteria and Pathogen Isolation

Rice seed samples were obtained from Suphan Buri Rice Research Center, Central of Thailand. The antagonistic bacteria were isolated from the rice seed surface using a dilution spread plate technique. Rice seed, 1−g, was crushed and mixed with 9 mL of sterile water, then diluted at 10^{-3} 10^{-4} and 10^{-5}. Twenty microlitres of the suspension was dropped and spread on the surface of nutrient agar (NA; 5 g of peptone, 3 g of beef extract and 15 g of agar per 1 L of distilled water) and incubated at 28 ± 2°C for 48 h. Individual bacterial colonies from dilution plates were purified on NA plates by streak plating. The purified bacterial isolates were preserved dry on sterile silica gel at −20°C (Leben and Sleesman, 1982) to further evaluate their antagonistic activity against *Bipolaris oryzae*. The pathogen, *B. oryzae,* was isolated from rice seed samples using the blotter method. The seeds were placed on water−soaked blotter filter papers in Petri dishes and incubated for 7 days at 25°C under alternating 12−h NUV light and darkness. After incubation, the fungi developed on each seed were examined under a stereo microscope. The fungus was transferred to potato dextrose agar (PDA; 200 g of potatoes, 20 g of dextrose and 15 g of agar per 1 L of distilled water) plates, incubated at 25°C for 7 days, sub−cultured on PDA slants and kept at 25 °C for further experiment.

in vitro and *in vivo* Antagonism Tests

Antagonistic activity against *B. oryzae in vitro* was performed by a dual−culture technique (Khabbaz and Abbasi, 2014). The bacterial isolates were streaked on one side of PDA plates (1 cm from the edge), and 5 mm of mycelium plug of *B. oryzae* from a 7−day−old culture was placed on the opposite side of the plates and incubated at 25°C for 5 days. A single mycelium plug of *B. oryzae* was placed on a PDA plate as a control. Four replications were used for each isolate. Inhibition of the growth of *B. oryzae* was measured as percentage of inhibition of radial growth by the following formula (Matić *et al*., 2014):

$$(R1-R2)/R1 \times 100$$

Where R1 = radial growth of fungus on the control plate, R2 = radial growth of fungus towards the antagonist. The ability of antagonistic bacteria to reduce *B. oryzae in vivo* was investigated. Each bacterial isolate was cultured on nutrient broth (NB; 5 g of peptone and 3 g of beef extract per 1 L of distilled water) at 25°C for 48 h on 130 rpm of the rotary shaker. The antagonistic bacterial suspension was adjusted to a concentration at 10^6 CFU/ml. Naturally infected of *B. oryzae*, discoloured seeds were soaked in a bacterial suspension for 10 min, air−dried in a laminar flow hood, then placed in Petri dishes on blotter filter papers, and incubated for 7 days at 25°C. Discoloured seeds soaked in NB for 10 min were used as a control. Four replications, with 100 seeds per replicate, were arranged in a completely randomized design. The number of *B. oryzae* infection on the seed surface was examined under a stereomicroscope.

Antagonistic Bacterial Identification

The most effective antagonistic bacteria showing growth inhibition of *B. oryzae* were selected to develop formulations. The antagonistic bacteria were identified by morphological characteristics through microscopic observations of the bacterial cells, colonies and molecular techniques. Pure colonies of each bacterial species were cultured on NA for 24 h. DNA was extracted using heat treatment (Dashti *et al*., 2009). The colonies were placed in a sterile Eppendorf tube containing one mL of distilled water, boiled for 10 min in a water bath, and then centrifuged for 5 min at 1000 rpm. Five microliters of the supernatant was used for the polymerase chain reaction. The quantity of the DNA product was determined using NanoDrop−1000 (Thermo scientific, USA). Polymerase chain reaction amplification of the 16s rDNA gene was performed. Each PCR reaction mixture (25 µL) contained 0.1 µL of *Taq* polymerase, 2.5 µL of 10x PCR buffer, 2.0 µL of dNTPs, 0.5 µL of each primer (forward primer 27F: 5'AGAGTTTGATCMTGGGAAGTC3' and reverse primer 1389R: 5'ACGGGCGGTGTGTACAAG3'; Hongoh *et al.,* 2003), 18.4 µL of sterile reverse osmosis water and 1 µL of DNA template. The PCR conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 2 min and finally 72°C for 4 min. The amplification was checked for purity by electrophoresis on 1.0% agarose gel and stained with ethidium bromide. The PCR products were sequenced at the Macrogen Int. (Seoul, Korea). The nucleotide sequences were BLAST searched in the NCBI GenBank database (http://blast.ncbi.nlm.nih.gov) for finding similar sequences. For phylogenetic analysis, data were generated from the 16s rDNA data .The phylogenetic tree was constructed from multiple alignment from ClustalW (Thomson 1994) included in the MEGA6 software (Tamura 2013). The analysis was conducted using the neighbor−joining (NJ) likelihood method in MEGA 6.0. All positions containing gaps and missing data were eliminated.

Development of Antagonistic Bacterial Formulations and Their Shelf−Life

Formulations of the selected antagonistic bacteria were prepared. Antagonistic bacteria were grown on LB plus 50 mg/L $MnSO_4.H_2O$ for inducing endospores and incubated on a rotary shaker at 37°C, 250 rpm for 48 h (Forma Orbital Shaker, Thermo Scientific, USA). Endospores of bacterial suspension were investigated usingmalachite green staining. The initial antagonistic bacterial suspension was adjusted at 10^{13} colony forming unit (CFU/ml) and was used in formulations. Under sterile conditions, talc with sodium carboxymethylcellulose (SCMC; as adhesive) formulations were prepared as follows: 100 g of sterilized talc powder was added to 40 mL of antagonistic bacterial suspension, 1 and 3 g of SCMC. The talc with polyvinylpyrrolidone (PVP; as adhesive) formulations, 100 g of sterilized talc powder was added to 40 mL of antagonistic bacterial suspension, 1 and 3 g of PVP (Khabbaz and Abbasi, 2014). The formulated products were dried in an oven at 60°C for 2 h, kept in polyethylene bags. The products were stored at room temperature (28 ± 2°C) for 6 months. The viability of the antagonistic bacteria in the products was determined at 1, 2, 3, 4, 5 and 6 months of storage using a dilution plate method. Four replications of each formulation at each time were analysed.

Efficacy of Antagonistic Bacterial Formulations

The effectiveness of antagonistic bacterial formulations to *B. oryzae* was tested using a poisoned food method. PDA plates were amended with 1% of antagonistic bacterial formulations. A mycelial plug (5 mm of *B. oryzae*) was placed on the centre of the plates and incubated at 25°C for 5 days. PDA plates with sterile water served as control. The radius of the fungal growth was measured, and inhibition growth (%) was estimated as mentioned above. The experiment was arranged in a completely randomized design with four replications for each formulation. Seed treatments using antagonistic bacteria formulations were conducted. Naturally infected seeds were treated with 1, 2, 3 and 4 g of antagonistic bacteria formulations per 1 kg of rice seed. Rice seeds treated with 0.5 g of mancozeb 50% WP served as a fungicide control. Naturally infected rice seeds were used as a control. The experiment was arranged in a completely randomized design with four replications (100 seeds per replicate). The number of *B. oryzae* infection on seed was observed using a blotter method. Brown spot disease symptoms on rice seedling were determined using a sand method (Mathur and Kongsdal, 2003). Treated rice seeds with antagonistic bacteria formulations were sown in autoclaved, moistened sand, and incubated under light fluorescence at room temperature (28 ± 2°C) for 14 days. The experiment was arranged in a randomized complete block design with four replications (50 seeds per replicate). The reduction of brown spot symptom on seedlings was evaluated as a percentage compared with control.

Statistical Analysis

Data were analysed for multiple comparisons by analysis of variance (ANOVA) with Duncan's multiple range test (DMRT) at a significance level $P \leq 0.05$ using SPSS for Windows.

RESULTS AND DISCUSSION

Antagonist Screening

Antagonistic bacteria were isolated from the surface of rice seeds. Twelve isolates showed inhibited mycelial growth of *B. oryzae* by a dual culture method. Antagonistic bacterial isolate RTS10 was the most effective, showing 66.1% inhibition followed by the isolate RTS11, CSC6 and RWP41 at 61.3, 56.6 and 13.3% of inhibition, respectively (Figure 1 and 2).

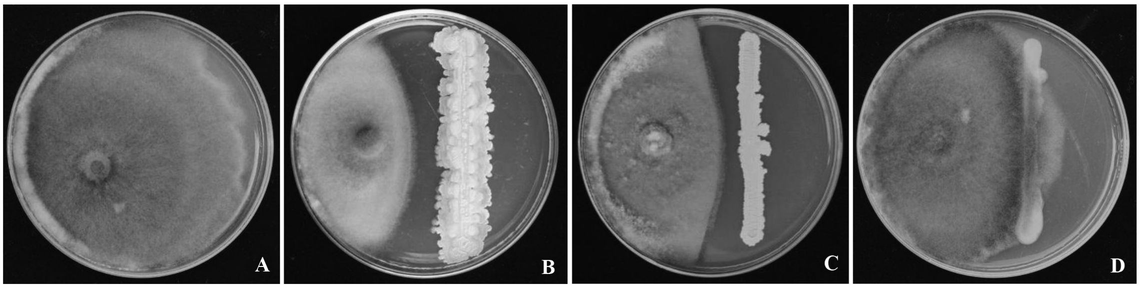

Figure 1 Inhibition of mycelial growth of *B. oryzae* by antagonistic bacterial isolates RTS10 (B), RKK1 (C) and RWP41 (D) compared with *B. oryzae* grown alone as a control on PDA plate (A) at 25°C for 5 days

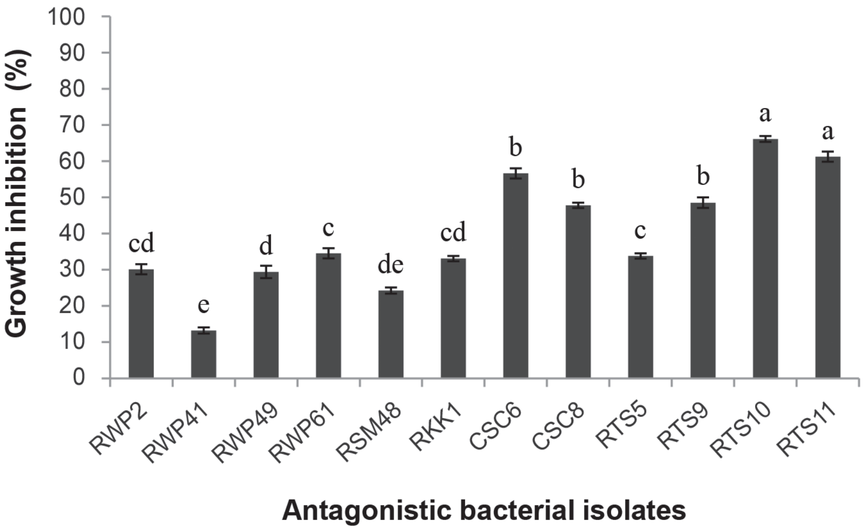

Figure 2 The percentage of mycelial growth inhibition of *B. oryzae* by antagonistic bacteria; 12 isolates were grown with a dual culture method on PDA plates at 25 °C for 5 days. The means with the same letter were not significantly different from each other, according to the DMRT ($P \leq 0.05$). The vertical bars represent the standard error of the means for four replications

The selected antagonistic bacteria, isolate RTS10 against *B. oryzae* demonstrated a potential to develop seed treatment formulation. In the experiment, the suppression of the mycelial growth of *B. oryzae in vitro* might be related to antibiotics that produced by the tested bacteria. Leifert *et al*. (1995) reported *B. subtilis* CL27 produced antibiotics against *Alternaria brassicicola* and *Botrytis cinerea in vitro*. Antibiotic production from *Bacillus* spp. depended on pH and nutrient concentration of media. *Bacillus subtilis* MTCC−8114 produced antibiotics in trypticase soy broth medium at 37°C, pH 7 and 48 h of incubation, the minimum inhibitory concentration of antifungal antibiotic was 135 µg/ml (Kumar *et al*., 2009). Athukorala *et al*. (2009) found that *B. subtilis* 3057, *B. amyloliquefaciens* BS6, *B. mycoides* and *B. thuringiensis* BS8 showed the presence of bacillomycin D, zwittermicin A and fengycin biosynthetic gene using specific primers in PCR. Moreover, Huang *et al*. (2012) reported that *Bacillus pumilus* SQR−N43 inhibited the mycelial growth of *Rhizoctoniasolani* by inducing hyphal deformation, enlargement of cytoplasmic vacuoles and cytoplasmic leakage.

Rice seeds soaked in nutrient broth as control showed the highest infection of *B. oryzae*, at 16%, which was not significantly different from the seeds treated with isolates RTS9 and CSC8. Moreover, the seeds inoculated with antagonistic bacterial suspension isolates RWP41, RWP49, RWP61, RSM43, CSC6, RTS10 and RTS11 RWP2 showed significant lower infection than control. However, no infection was found on seeds treated with RWP2, RKK1 and RTS5 (Figure 3). In this study, treating rice seeds with antagonistic bacterial suspension could reduce *B. oryzae* infection. According to Moura *et al*. (2014), rice seeds immersed in *Pseudomonas synxantha* suspension reduced the incidence of *B. oryzae* by 24.3% in 21−day−old seedlings. Corn seeds coated with *Gliocladium viren* isolate GI−3 decreased root rot disease severity caused of *Pythium ulimum, P. arrhenomanes* and *Fusarium graminearum* from 61−90% to 3−30%

and increased seedling stand, plant height and fresh weight (Mao *et al*., 1997). In the rice crop production should immerse seed with antagonistic bacteria at least for 10 min before planting because antagonistic bacteria can kill pathogen on seed and promote rice growth.

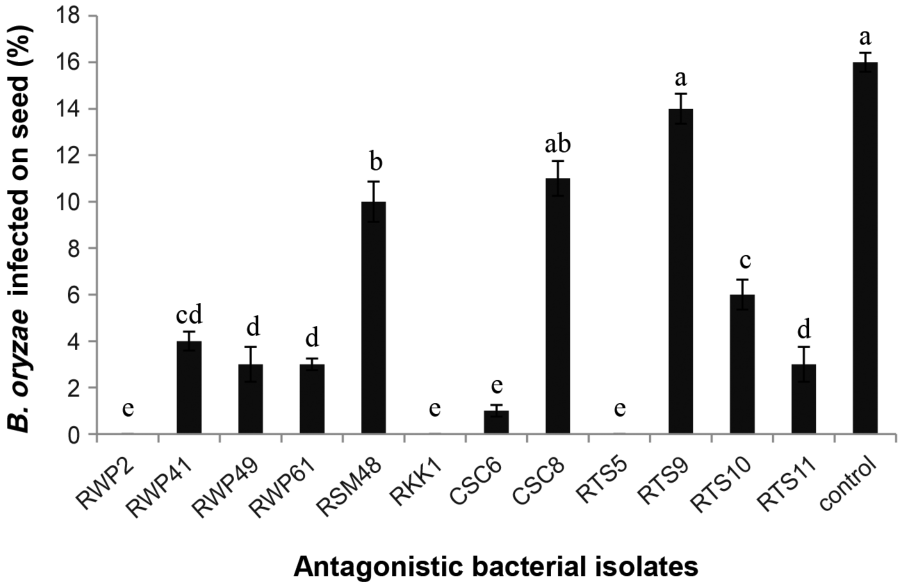

Figure 3 Brown spot incidence (%) on rice seeds after being soaked in a 12−isolate antagonistic bacterial suspension. The means with the same letter were not significantly different from each other, according to the DMRT ($P \leq 0.05$). The vertical bars represent the standard error of the means for four replications.

Antagonistic Bacterial Identification

The antagonistic bacterial isolate RTS10 was selected to develop a formulation based on the highest inhibited mycelial growth of *B. oryzae* by dual culture method. Isolate RTS10 was a white colony, 0.46−0.94 × 1.67−3.17 µm of rod shape, Gram−positive and formed endospores on NA. Sequence analysis of the 16s rDNA gene, isolate RTS10 showed a 99% similarity to *Bacillus subtilis* strain HRBS−10TDI13 (Accession No. CP015222.1). Based on morphological and sequencing, antagonistic bacteria isolate RTS10 was identified as *B. subtilis.* The phylogenetic tree showed the isolate RTS10 was included in *B. subtilis* group which separated from the out group (*Xanthomonas campestris*) by using NJ. (Figure 4)

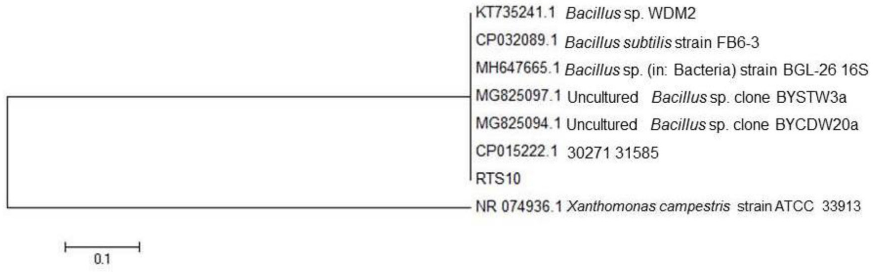

Figure 4 The phylogenetic tree constructed by using the neighbor−joining method of 16s rDNA sequences from isolate RTS10 and reference species. Branch lengths are drawn proportionally to genetic distances and the bar at the bottom of the tree indicates a length corresponding to 0.1 nucleotide substitutions per site

Shelf−Life of Antagonistic Bacteria

The initial population of each formulation was 13.5−13.7 log CFU/g at 0 months. The number of viable bacteria declined significantly differently at each month of the storage period (Figure 5). After 1 month of storage, the populations of antagonistic bacteria were at 12.1−12.3 log CFU/g, and the lowest was 6.5−6.7 log CFU/g at 6 months of storage. However, talc powder formulations with SCMC and PVP adhesive showed no significant difference in population densities at each month of storage.

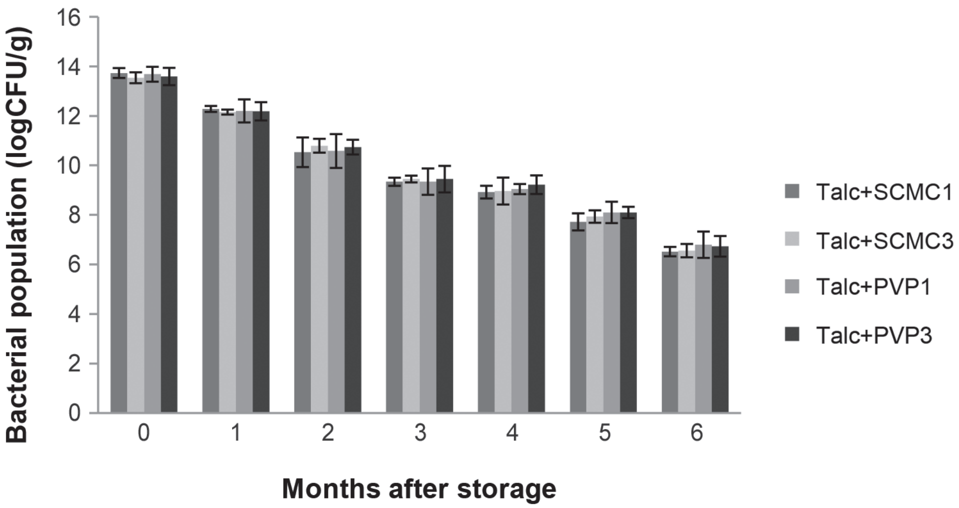

Figure 5 Population densities of antagonistic bacteria, *B. subtilis* in talc powder formulations with SCMC and PVP adhesive during 6 months of storage at room temperature (28 ± 2°C). Error bars represent the standard errors of the means from 4 replications.

Probability, antagonist bacteria can survive on talc powder with SCMC and PVP adhesive more than 6 months, thus in the next experiment should storage formulation more than 6 months and test shelf life.

Efficacy of Antagonistic Bacterial Formulations on Mycelial Growth of *B. oryzae*

The inhibitory effect of talc powder formulations on the mycelial growth of *B. oryzae* was investigated. Talc+SCMC1 formulation showed the highest inhibited mycelial growth of *B. oryzae* at 73.5%, but there was no significant difference compared with Talc+SCMC3, Talc+PVP1 and Talc+PVP3 (Figure 6.).

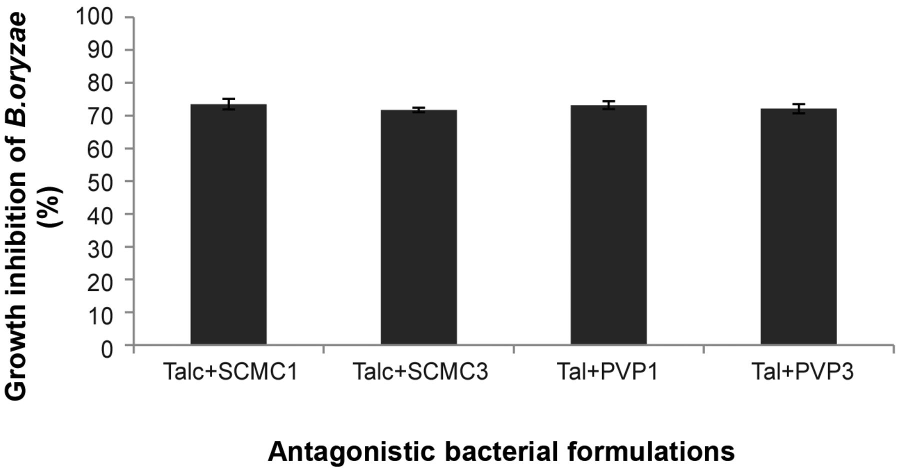

Figure 6 Inhibition of *B. oryzae* growth caused by Talc+SCMC1, Talc+SCMC3, Talc+PVP1 and Talc+PVP3 formulations. Error bars represent the standard errors of the means from 4 replications.

Antagonistic bacteria were developed as dry formulations based on talc and adhesive. The populations of antagonistic bacteria were decreased over time, and they remained at 10^6 CFU/g after 6 months of storage at room temperature (28 ± 2 °C). El−Hassan and Gowen (2006) reported *B. subtilis* survived in glucose, talc powers and peat formulation at 8.6, 7.8, and 3.5 log10 CFU/g, respectively, for 1 year of storage at 25 ± 2°C. The formulation containing talc powder, possibly, maintained the viability of *B. subtilis* spores. The viability of antagonistic bacteria in the formulation is a critical point in the success of biofungicide. Schisler and Van Cauwenberge (2002) reported that trisaccharide melezitose enhanced the survival of *Cryptococcus nodaensis,* the biocontrol agent of Fusarium head blight disease by freeze−dried technique. An *in vitro* test of talc powder formulations of *B. subtilis* inhibited the mycelial growth of *B. oryzae*by over 70.0%. Meng *et al*. (2015) reported dry flowable formulation at 10^9 CFU/mL of *B. subtilis*T429 inhibited the mycelial growth of *Magnaporthegrisea* by 70.0%.

Efficacy of Antagonistic Bacterial Formulations on Naturally Infection of *B. oryzae* on Rice Seed and Seedling

During 0, 3 and 6 months of product storage at room temperature (28 ± 2°C), rice seed discolouration was treated with 1, 2, 3 and 4 g of *B. subtilis* formulations as Talc+SCMC1, Talc+SCMC3, Talc+PVP1 and Talc+PVP3 products compared with 0.5 g of mancozeb per 1 kg of rice seed. Naturally infected *B. oryzae* seeds were investigated using a blotter method. The seeds treated with Talc+PVP1 formulation at 2 g showed the highest decrease in *B. oryzae* infections at 67.3% at 0 months of product storage. At 3 and 6 months of product storage, naturally *B. oryzae* infection on seeds showed the highest decrease at 94.3 and 72.3% after treatment with 1 g and 3 g of Talc+PVP1 formulation, respectively. In addition, rice seeds treated with Talc+PVP1 formulation reduced *B. oryzae* infection at higher than 0.5 g of mancozeb treatment (Table 1).

Table 1 *Bipolaris oryzae* reduction (%) on rice seed after seed treatments, with antagonistic bacterial formulations stored for 0, 3 and 6 months using a blotter method

treatments	Reduction of *B. oryzae* incidence on rice seed (%)		
	Storage time (months)		
	0	3	6
1 g Talc+SCMC1/kg of seed	12.2 ± 0.4^{m}	48.4 ± 0.6^{h}	19.1 ± 0.5^{k}
2 g Talc+SCMC1/kg of seed	23.0 ± 0.5^{k}	5.4 ± 0.3^{l}	31.4 ± 0.3^{j}
3 g Talc+SCMC1/kg of seed	19.1 ± 0.4^{l}	15.1 ± 0.5^{k}	18.2 ± 0.4^{k}
4 g Talc+SCMC1/kg of seed	47.3 ± 0.3^{f}	20.2 ± 0.6^{j}	46.2 ± 0.4^{gh}
1 g Talc+SCMC3/kg of seed	51.1 ± 0.4^{d}	56.2 ± 0.5^{f}	47.2 ± 0.6^{gf}
2 g Talc+SCMC3/kg of seed	53.2 ± 0.4^{c}	48.2 ± 0.6^{h}	37.3 ± 0.3^{i}
3 g Talc+SCMC3/kg of seed	27.3 ± 0.3^{j}	51.1 ± 0.5^{g}	59.1 ± 0.6^{d}
4 g Talc+SCMC3/kg of seed	55.2 ± 0.4^{b}	34.2 ± 0.6^{i}	67.3 ± 0.5^{c}
1 g Talc+PVP1/kg of seed	51.3 ± 0.3^{c}	94.3 ± 0.6^{a}	45.2 ± 0.4^{h}
2 g Talc+PVP1/kg of seed	67.3 ± 0.3^{a}	89.3 ± 0.6^{b}	17.8 ± 0.7^{k}
3 g Talc+PVP1/kg of seed	55.2 ± 0.4^{b}	58.7 ± 0.8^{e}	72.3 ± 0.3^{a}
4 g Talc+PVP1/kg of seed	38.7 ± 0.3^{h}	48.3 ± 0.6^{h}	70.3 ± 0.3^{b}
1 g Talc+PVP3/kg of seed	49.1 ± 0.5^{e}	69.1 ± 0.5^{d}	55.1 ± 0.4^{e}
2 g Talc+PVP3/kg of seed	26.3 ± 0.3^{j}	15.1 ± 0.5^{k}	68.3 ± 0.3^{c}
3 g Talc+PVP3/kg of seed	40.2 ± 0.4^{g}	56.1 ± 0.5^{f}	48.2 ± 0.6^{f}
4 g Talc+PVP3/kg of seed	23.3 ± 0.3^{k}	79.1 ± 0.5^{c}	45.2 ± 0.4^{h}
0.5 g mancozeb/kg of seed	31.3 ± 0.3^{i}	89.3 ± 0.6^{b}	68.3 ± 0.3^{c}

Note: For each column, Values indicated by the same letter are not significantly different according to the DMRT ($P \leq 0.05$)

Rice seeds were treated with each *B. subtilis* formulations and grown on the sterile sand at room temperature (28 ± 2°C) for 14 days. Brown spot disease on the seedlings was assessed. The seedlings treated with 2 and 3 g of Talc+PVP1 and 2 g of a Talc+SCMC3 formulation showed the highest reduced brown spot disease but no significant difference at 87.3, 88.3 and 88.1%, respectively at 0 months of storage. At 3 months of product storage, brown spot disease on the seedlings was the most highly reduced at 92.5% after being treated with 2 g of Talc+PVP3. After 6 months of product storage, the seedlings treated with 3 g of Talc+PVP1 showed the highest decrease at a 77.3% reduction of brown spot disease. Moreover, seedlings treated with Talc+PVP1, Talc+PVP3 and Talc+SCMC3 formulations showed a reduction in brown spot disease with a treatment of higher than 0.5 g of mancozeb (Table 2).

Table 2 Brown spot disease reduction (%) on rice seedlings after seed treatments with antagonistic bacterial formulations stored for 0, 3 and 6 months and examined by using a sand method

Treatments	Reduction of disease incidence on rice seedlings (%)		
	Storage time (months)		
	0	3	6
1 g Talc+SCMC1 /kg rice seed	55.5 ± 0.8^{e}	54.1 ± 0.5^{h}	63.2 ± 0.4^{c}
2 g Talc+SCMC1/kg of seed	33.4 ± 0.6^{g}	55.1 ± 0.5^{h}	38.2 ± 0.4^{e}
3 g Talc+SCMC1/kg of seed	55.2 ± 0.6^{e}	75.0 ± 0.5^{e}	13.2 ± 0.4^{f}
4 g Talc+SCMC1/kg of seed	65.2 ± 0.7^{cd}	91.2 ± 0.6^{ab}	26.0 ± 0.5^{f}
1 g Talc+SCMC3/kg of seed	44.1 ± 0.6^{f}	58.4 ± 0.8^{fg}	63.1 ± 0.4^{c}
2 g Talc+SCMC3/kg of seed	88.1 ± 0.6^{a}	58.7 ± 1.1^{fg}	63.5 ± 0.7^{c}
3 g Talc+SCMC3/kg of seed	77.2 ± 0.4^{b}	59.1 ± 1.2^{fg}	63.8 ± 0.7^{c}
4 g Talc+SCMC3/kg of seed	67.2 ± 0.6^{c}	59.4 ± 1.4^{f}	72.6 ± 1.2^{b}
1 g Talc+PVP1/kg of seed	44.1 ± 0.3^{f}	50.0 ± 0.5^{g}	74.0 ± 0.5^{b}
2 g Talc+PVP1/kg of seed	87.3 ± 0.8^{a}	88.1 ± 0.4^{bc}	76.0 ± 0.5^{a}
3 g Talc+PVP1/kg of seed	88.3 ± 0.6^{a}	75.1 ± 0.5^{fg}	77.3 ± 1.2^{a}
4 g Talc+PVP1/kg of seed	66.2 ± 0.6^{c}	49.9 ± 1.2^{g}	13.2 ± 0.4^{g}
1 g Talc+PVP3/kg of seed	66.8 ± 0.5^{c}	58.4 ± 0.8^{fg}	26.0 ± 0.5^{f}
2 g Talc+PVP3/kg of seed	56.5 ± 0.7^{c}	92.5 ± 1.3^{a}	38.8 ± 0.7^{e}
3 g Talc+PVP3/kg of seed	66.2 ± 0.6^{c}	87.1 ± 0.6^{c}	38.1 ± 0.4^{e}
4 g Talc+PVP3/kg of seed	78.3 ± 0.4^{b}	72.3 ± 1.4^{e}	51.0 ± 0.5^{d}
0.5 g mancozeb/kg of seed	64.2 ± 0.6^{d}	80.0 ± 1.2^{d}	26.0 ± 0.5^{f}

Note: For each column, Values indicated by the same letter are not significantly different according to the DMRT ($P \leq 0.05$)

In this study, the development of *B. subtilis* as dust formulation was applied as a seed treatment. The formulation reduced *B. oryzae* infection in rice seed and brown spot disease of the seedlings similar to the study by El−Hassan and Gowen (2006), who reported that lentil seed treatment with glucose, talc powders, and peat formulations of *B. subtilis* decreased wilt disease caused by *Fusarium oxysporum* f. sp. *lentis* by 93.3, 43.0, 63.0%, respectively, on a susceptible lentil variety. *Bacillus* species were developed as a commercial biocontrol product such as Serenade, EcoGuard, Kodiak, Yield Shield, Bio yield and Subtilex to control plant disease (Schisler *et al*., 2004).

From the experiment, antagonistic bacteria formulation showed good result to inhibit *B. oryzae* on seed. For more effective of formulation should be develop coating method of formulation on seed and apply to other plant.

CONCLUSION

This study suggests that *B. subtilis* was isolated from rice seeds, inhibited mycelia growth of *B. oryzae*. It has potential to develop as bioproduct for controlling seed−borne diseases. Treated seed with *B. subtilis* formulation in talc power reduce infection of *B. oryzae* on seed and rice seedling. Moreover, treated seed with bacterial antagonist decrease the use of foliar fungicides applications in rice production.

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