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Efficacy of Novel Formulations of *Bacillus megaterium* in Suppressing Sheath Blight of Rice Caused by *Rhizoctonia solani*

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Abstract: Bacterial antagonists in floating pellet and water-soluble granule formulations, produced using *Bacillus megaterium* (No.16) with pharmaceutical technology, were tested under both greenhouse and field conditions. When used by broadcasting to rice plants, the floating pellet formulation was as good as a fungicide (Iprodione) in suppressing sheath blight in the pot tests under the greenhouse condition. When sprayed onto rice plants, the water-soluble granule formulation was as effective as a fungicide and was more effective than the floating pellet formulation in the field test when percentage of rice tillers with sheath blight symptoms was assessed.

Key words: Biological control, formulation, sheath blight disease

INTRODUCTION

Rice sheath blight, caused by *Rhizoctonia solani* Kühn, is one of the most destructive rice diseases worldwide (Ou, 1985). This fungal disease is second in importance only to rice blast disease, caused by *Magnaporthe grisea* (Anam. *Pyricularia oryzae*) (Rush and Lee, 1992; Thurston, 1995; Dehne and Oerke, 1998). Management of sheath blight disease of rice has been directed toward the integration of cultural practices with chemical control (Chin and Bhandhufalck, 1990; Damicone *et al*., 1993). However, chemical control using effective fungicides has various undesirable effects, such as being phytotoxic to rice plants (Groth *et al*., 1990) and the requirement for critical timing of fungicide application may hinder its usage (Lee and Rush, 1983). Alternatively, using rice plants resistant to sheath blight disease has been considered as a control option (Rush and Lee, 1992).

Biological control has also become a prominent option for controlling various plant diseases (Cook, 1993; Larkin *et al*., 1998). Rice sheath blight is one of the plant diseases which have been controlled using a biological control approach (Mew and Rosales 1986; Vasantha Devi *et al*., 1989; Kanjanamaneesathian *et al*., 1998; Pengnoo *et al*., 2000). However, fresh cells of potential antagonists have been used for sheath blight control testing in most of these studies (Mew and Rosales, 1986; Vasatha Devi *et al*., 1989; Gnanamanickam and Mew, 1990; Gnanamanickam *et al*., 1992). Although effective and suitable for research purposes, fresh cells of antagonists may not be suitable for use in the rice field by the farmers (Pengnoo *et al*., 2000). Preliminary testing of bacterial formulations to control sheath blight of rice has been investigated in pot testing in the greenhouse (Kanjanamaneesathian *et al*., 1998) and in the field conditions (Pengnoo *et al*., 2000). Although these formulations demonstrate the desired characteristics and provide quite satisfactory protection for rice plants from *R. solani* infection in both tests (Kanjanamaneesathian *et al*., 1998; Pengnoo *et al*., 2000), they have a comparatively short shelf life and the numbers of bacterial antagonists in the formulations greatly decline during 6 months storage (Kanjanamaneesathian *et al*., 2000). This undesirable characteristic of the formulation makes it unsuitable for large scale production and commercialization. For this reason, further research and development regarding improved formulations are required.

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Research in the area of improved formulations and delivery systems is critical for further development and implementation of effective biological control (Lewis, 1991; Lumsden *et al*., 1995; Larkin *et al*., 1998). Novel formulations of bacterial antagonists such as floating pellets have been developed and used for sheath blight disease control trials in the greenhouse condition (Wiwattanapatapee *et al*., 2004). In the preliminary tests, these pellet formulations containing *B. megaterium* show good floating properties and gradually release their bacterium load over time. After production, they also contain a high level of *B. megaterium* after storage for 6 months at room temperature. In a greenhouse test, this formulation showed promising results in suppression of the development of sheath blight (Wiwattanapatapee *et al*., 2004).

The objective of this research was to compare the efficacy of floating pellet (by broadcasting) with water-soluble granule (by spraying) in sheath blight disease suppression in both greenhouse and field conditions using novel formulations of *B. megaterium*.

MATERIALS AND METHODS

Formulations and *B. megaterium* (No. 16) used in the experiment: Two novel formulations were produced using pharmaceutical technology for use in both greenhouse and filed trials to control sheath blight disease in this experiment. A floating pellet formulation was prepared using an extrusion-spheronization process as described by Wiwattanapatapee *et al*. (2004) and applied by broadcasting to rice plants in both greenhouse and field tests. The floating pellet formulation (at 15 g in each pot) used in these tests contained 10^8 cfu g^{-1} of *B. megaterium* (No. 16) and had sustained release characteristic (Wiwattanapatapee *et al*., 2004).

A water-soluble granule formulation was prepared using the wet granulation method and applied by spraying on the rice plants in both greenhouse and field tests. This formulation was composed of lactose (Veghel, The Netherlands), polyvinyl pyrrolidone (supplied by Vidhyasom, Thailand), sodium alginate (Sigma-Aldrich, USA) and bacterial suspension. All ingredients were mixed with the bacterial suspension in a planetary mixer until they became a damp mass. This mass was then passed through a sieve (1.6×1.6 mm pore size) and dried in an incubator at 65°C for 2 h.

In the greenhouse test, the water-soluble granule formulation containing bacteria at 10^9 cfu g^{-1} was dissolved in tap water (at 3 g 100 mL^{-1}) and sprayed on the rice plants with a hand-held sprayer. In the field test, this same formulation was dissolved in tap water (at 10 g 100 mL^{-1}) and sprayed on the rice plants with a knapsack sprayer.

One isolate of *B. megaterium* was used as the active ingredient in the formulation. This bacterium was isolated and selected for use in this experiment based upon its ability to inhibit mycelial growth and sclerotial germination of *R. solani* and suppress sheath blight lesion on rice tissue *in vitro* (Kanjanamaneesathian *et al*., 1998). This bacterium was identified using biochemical and physiological tests as described by Pengnoo *et al*. (2000). The mode of action of *B. megaterium* in inhibiting *R. solani* was through the production of an unidentified heat stable antibiotic substance (Pengnoo *et al*., 2000).

Experimental design and treatment in the greenhouse test: The efficacy of the formulations against sheath blight was investigated under greenhouse condition, Prince of Songkla University, Surat Thani campus, Surat Thani in 2003. There were six treatments, which consisted of rice plants (cv. Khao Dawk Mali 105) inoculated with either floating pellets containing *B. megaterium*, floating pellets (blank) (for broadcasting), water-soluble granules containing *B. megaterium*, water-soluble granule (blank) (for spraying), or fungicide (Iprodione). Each treatment consisted of three replications (four rice seedlings for one replication). Rice plants inoculated only with sterile rice seeds infested with *R. solani* were used as a control treatment. The experiment was arranged in a Complete Randomized Design (CRD).

Pot preparation: Paddy rice field soil [clay texture (32.4% sand, 18.9% silt, 48.7% clay), pH 7.1, 3.0% organic matter, 0.2% total N, 23.7% mg kg^{-1} available P and 0.6 mg kg^{-1} available K] was used in the pot test. These soil samples were loaded in plastic pots (21 cm in diameter and 18 cm in height) and the pot was filled with tap water until the soil was soaked. The water level was maintained above the soil level. After 72 h, the soils were agitated manually to break up aggregates and excess water was drained. Soil level in the plastic pots was adjusted to a height of 13 cm so that 5 cm depth of water was retained in each plastic pot.

Pathogen inoculation: Twenty grams of sterile rice seeds infested with *R. solani* were placed in the centre of each plastic pot 25 days after sowing, 1 day prior to formulation application. The sterile rice seed was dispersed with a sterile spatula so that the pathogen inoculum made contact with all rice plants. The water level in the plastic pot was maintained at the same level throughout the experiment.

Formulation application: Either floating pellet or water-soluble granule formulations were applied to the rice seedlings in the plastic pots. Floating pellet formulation at 15 g (contained the bacterium at 10^8 cfu g^{-1}) was placed at the centre of each plastic pot, on top of the inoculum of *R. solani*.

Water-soluble granule formulation at 150 mL (at 3 g 100 mL^{-1}, contained the bacterium at 10^9cfu g^{-1}) was sprayed on the rice seedlings in the plastic pots using a handheld sprayer in both the first and the second experiment.

Rice seedlings, sprayed with fungicide at 150 mL (at 1 g L^{-1}), were used as a benchmark to compare the efficacy of the formulations. Rice seedlings in each plastic pot inoculated with only 20 g of sterile rice seeds infested with *R. solani* were used as a control treatment.

Disease assessment: Sheath blight disease assessment in the greenhouse tests was carried out at 7 days after formulation application. Disease was assessed by counting the number of rice seedlings which showed sheath blight symptoms.

Scanning electron microscope (SEM) observation of the bacterial endospore on plant surface: Leaf sheath and leaf blade of the rice plant were randomly sampled after spraying with water-soluble granule formulations in the pot test. The plant samples were mounted on the stub. These specimens were then coated with gold particles and observed with LV-SEM 5800 (JEOL, Japan). Micrographs of bacterial endospores on the surface of leaf sheath and leaf blade were taken.

Experimental design and treatments in the field test: The efficacy of the formulations against sheath blight was investigated under field conditions at Muang District, Phetchaburi Province, Thailand in 2003. This site was chosen for the field trial because it is located in the central region of Thailand where sheath blight disease is a threat to the farmers. There were eight treatments in the field trial. Treatment consisted of a rice plant (cv. RD-23) inoculated with either floating pellet (at 10, 20, or 30 g for each rice hill) or water-soluble granule [at 100, 200 or 300 mL (at 10 g 100 mL^{-1}) for each rice hill in each subplot] or a chemical fungicide [Iprodione at 3 g 3 L^{-1} (approximately at 0.25 g for each rice hill)], one day after pathogen inoculation. Rice plants inoculated only with *R. solani* were used as a control treatment. Each treatment consisted of six replications, with twelve rice hills for one replication. The experiment was arranged in a Randomized Complete Block Design (RCBD).

Rice field preparation: The rice plot had clay texture (32.4% sand, 18.9% silt and 48.7% clay), pH 7.1, 3.0% organic matter, 0.2% total N, 23.7% mg kg^{-1} available P and 0.6 mg kg^{-1} available K. It was flooded with water and ploughed until any soil aggregates were broken up. Excess water was drained 2 days later and the field partitioned into 6 blocks. Each block was further partitioned into 8 subplots (2×2 m) by 30 cm width earth embankment to prevent water movement among the subplots. The rice field was flooded again and the water level was maintained by opening or closing a small gate on each subplot embankment.

Rice seedling and rice plant preparation: Rice seedlings (cv. RD-23) were raised in the seedling bed of a farmer's field in Muang District, Phetchaburi Province, Thailand. Rice (cv. RD-23) was selected for this field trial because it is very susceptible to sheath blight disease. After 14 days, these rice seedlings were transplanted into the rice field with 20 cm spacing between and within rows in each 2×2 m subplot. Eight rows and eight columns of rice seedlings were planted in each subplot, with two rice seedlings planted at each hill site. The two rows and two columns of rice plants planted adjacent to the each embankments in each sub plot were used only as guard rows. The four rice plants in the innermost rows and columns were used for disease assessment, while the eight other adjacent rice plants (in the cross configuration surrounding these four innermost rice plants) were used for yield assessment.

Pathogen inoculation: Only twelve rice hills in the centre of each subplot were inoculated with the pathogen in the efficacy test in the field trial. Twenty grams of sterile rice seeds infested with *R. solani* were placed in the centre of each rice hill, at 45 days after transplanting rice seedlings to the field. The water level in the subplot was maintained at the same level throughout the experiment.

Formulation application: Twelve rice hills were applied with either floating pellet or water-soluble granule formulations, 1 day after inoculating the pathogen. Floating pellet formulation (either at 10, 20, or 30 g) was placed at the centre of each rice hill, on top of the inoculum of *R. solani*.

The water-soluble granule formulation [at 100, 200 or 300 mL (at 10 g 100 mL^{-1}) for each rice hill in each subplot] was sprayed to the rice hills using a knapsack sprayer. Rice plant sprayed with fungicide (Iprodione 3 g 3 L^{-1}; at approximately 0.25 g for each rice hill) were used as a benchmark to compare the efficacy of the formulations.

Rice plants inoculated with only 20 g of sterile rice seeds infested with *R. solani* were used as a control treatment.

Disease and yield assessment: For sheath blight disease assessment, only the 4 innermost rice hills were uprooted and sheath blight symptoms were assessed 7 days after formulation application. Roots of the uprooted rice plants were washed to eliminate excessive soils. These roots were later cut and discarded and the above-ground portions of the rice plants were used for sheath blight disease assessment. Disease was assessed by counting the number of tillers which showed sheath blight disease symptoms. The entire length of the lesion on each rice tiller which had sheath blight symptoms was also measured. Fresh and dry weight of the inoculated rice plants were also assessed after disease measurement in the field test.

For rice yield assessment, rice plants were harvested from the remaining eight hills at the end of the experiment, 110 days after transplanting. Panicles were cut at the base of the uppermost inter-node and the weight of these panicles was assessed.

Statistical analysis: Data from both greenhouse and field tests were subjected to standard analysis of variance procedures for a completely randomized design using the Statistical Package for Social Science (SPSS/PC+) computer software package. One-way analysis of variance was carried out on the percent of rice seedlings (in the greenhouse trials) and rice tillers (in the field trials) which showed sheath blight symptoms. One-way analysis of variance was also done on the length of the lesions on each rice tiller (from the field trial) which had sheath blight symptoms. Data was compared with Duncan's Multiple Rang Test (DMRT) at $p<0.05$.

RESULTS

Fungicide (Iprodione) was best in suppressing sheath blight disease in the pot test. A floating pellet of *B. megaterium* was as effective as Iprodione in sheath blight suppression. Both floating pellet and water-soluble granule formulations containing *B. megaterium* were effective in sheath blight suppression. Blank formulation (either floating pellets or water-soluble granules) could not control sheath blight disease in the pot test in the greenhouse condition (Table 1).

Upon observing the surface of leaf sheath and leaf blade after spraying with granule formulations, the SEM micrographs showed a number of endospores of *B. megaterium* on both rice plant tissues (Fig. 1).

The water-soluble granule formulation, containing *B. megaterium* (at 300 mL) was as effective as the fungicide (Iprodione) in sheath blight suppression in the rice field when % tillers with sheath blight symptom was assessed (Table 2). However, the fungicide (Iprodione) was better than water-soluble granule formulation (at 300 mL) when lesion lengths were compared (Table 2).

At the end of the experiment (110 days after trans planting), rice plants applied with either floating pellet (at 20 g) or water-soluble granule (at 200 mL)

Table 1: Efficacy of novel formulations in suppressing the development of sheath blight disease in the pot test

Treatments	Tillers with sheath blight symptom* (%)
Floating pellet	
With *B. megaterium***	18.4 cd*****
Blank	62.7ab
Water-soluble granule	
With *B. megaterium****	38.9bc
Blank	55.9ab
Fungicide (Iprodione)*****	0.0d
Control (only *R. solani*)	92.7a

*Percentage of infected rice tillers = all infected rice tillers/total rice tillers× 100, **Contained the bacterium at 10^8cfu g^{-1} [broadcasted at 15 g], ***Contained the bacterium at 10^9cfu g^{-1} [sprayed at 150 mL (at 3 g 100 mL^{-1})], ****Fungicide at 1 g L^{-1} [sprayed at 150 mL], *****Means followed by the same letter are not significantly different at the 5% level by Duncan's Multiple Range Test

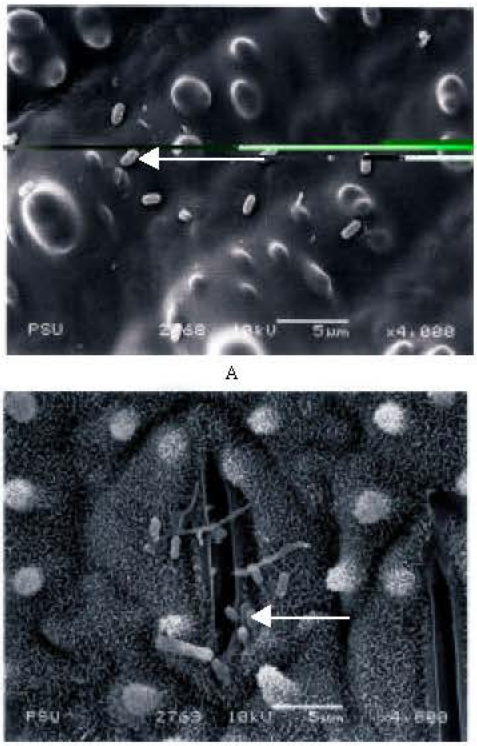

Fig. 1: SEM micrographs of bacterial endospores (arrow) (A) on the surface of leaf sheath and (B) on leaf blade of rice plants from the pot test

Table 2: Efficacy of novel formulations in suppressing the development of sheath blight disease in the field

Treatments*	Tillers with sheath blight symptoms** (%)	Lesion length (cm)
Floating pellet (g)		
10	82.3b	16.6b***
20	84.5ab	16.2b
30	79.9b	15.1c
Water-soluble granule		
100 mL	80.1b	13.8d
200 mL	70.6c	13.6d
300 mL	50.6d	12.9d
Iprodione	55.7d	8.5e
Control (only *R. solani*)	92.5a	18.7a

*All types of formulations contained *B. megaterium*, ** Percentage of infected rice tillers = all infected rice tillers/total rice tillers×100, *** Means followed by the same letter are not significantly different at the 5% level by Duncan's Multiple Range Test

Table 3: Efficacy of novel formulations on fresh and dry weight of rice plants and weight of rice panicles

Treatments	Fresh weight (g)*	Dry weight (g)**	Panicle weight (g)***
Floating pellet (g)			
10	73.8ab	58.0ab	182.8ab****
20	85.4a	66.1a	218.9a
30	57.9b	43.3b	158.5b
Water-soluble granule			
100 mL	73.3ab	56.2ab	190.8ab
200 mL	84.6a	64.6a	217.3a
300 mL	69.8ab	55.4ab	190.6ab
Iprodione	74.6ab	60.2ab	202.6ab
Control (*R. solani*)	77.5ab	55.9ab	186.2ab

*,**Fresh and dry weight are the average of four rice hills which were inoculated with novel formulations and *R. solani* inoculum, ***Panicle weight is the average of eight rice hills which were inoculated with novel formulations and *R. solani* inoculum, ****Means followed by the same letter are not significantly different at the 5% level by Duncan's Multiple Range Test

formulations, both containing *B. megaterium*, had quite high fresh, dry and panicle weights. These, however, was not significantly different from those with 10 or 30 g of floating pellets, 100 or 300 mL of water-soluble granules, fungicide (Iprodione) or control (inoculated only with *R. solani*) (Table 3).

DISCUSSION

Sheath blight disease of rice has been controlled with either fresh cells (Mew and Rosales, 1986; Vasatha Devi *et al*., 1989; Gnanamanickam and Mew, 1990; Gnanamanickam *et al*., 1992) or formulations of antagonists (Kanjanamaneesathian *et al*., 1998; Pengnoo *et al*., 2000; Wiwattanapatapee *et al*., 2004). Mcintyre and Press (1991) stated that research to devise effective formulations is essential because farmers will be more likely to accept biological control measures when they are familiar with their handling properties and can use conventional equipment to apply them.

In the greenhouse test, the bacterium in the floating pellet formulation (at 1.5×10^9 cfu for each pot) is 3 times less than that of the water-soluble granule formulation (at 4.5×10^9cfu for each pot). When the floating pellet formulation was applied directly onto the pathogen inoculum 1 day later, the bacterium may have inhibited mycelial growth and subsequently reduce infection and disease incidence. Even at lower dose, the floating pellet formulation is as good as the water-soluble granule in suppressing sheath blight disease. This is possibly due to the effect of an unidentified heat-stable antibiotic substance produced by *B. megaterium* (Pengnoo *et al*., 2000). When applied this floating pellet formulation in a closed system, the concentration of antibiotic substance from the bacterium is high enough and effectively suppresses sheath blight development.

Thus, it is interesting to identify the antibiotic produced by this isolate of *B. megaterium*. This may lead to the discovery of new fungicides with a microbial origin, such as in the case of the Polyoxins and Validamycin A (Yamaguchi, 1998). Fungicides of microbial origin would be an ideal alternative for plant disease control because they are susceptible to microbial degradation and have quite low toxicity to mammals, particularly in the case of Validamycin A (Yamaguchi, 1998). In this regard, they are safer and are considered more environmentally friendly than synthetic fungicides. More recently, it has been reported that strain of *B. megaterium* can promote growth and development of bean (*Phaseolus vulgalis*). The plant growth promoting capability is possibly through an Auxin-and Ethylene-Independent signaling mechanisms when *Arabidopsis thaliana* is used as a model (López-Bucio *et al*., 2007). The effect of our strain of *B. megaterium* on promoting plant growth in rice should also be investigated.

In the field test in which the number of bacterial antagonist is equal in both formulations, water-soluble granule formulation (at 300 mL for each rice hill) is as effective as the fungicide and more effective than floating pellet formulations in sheath blight suppression (Table 2). In the field, the floating pellet formulation may become less effective because this type of formulation, when applied in the relatively open system, may be dispersed from the point of application possibly due to the effect of water movement. Thus, it is likely that the effect of antibiotic produced by *B. megaterium* may be reduced and contributed to the failure of the floating pellet formulation to control sheath blight in the field test (Table 2).

Sheath blight disease is usually most severe during the tillering stage and disease spread (both horizontally and vertically) occurs very rapidly when the rice canopy is thick and relative humidity is very high (Reissig *et al*., 1986). When sprayed, the bacteria in the water-soluble

granule formulation is deposited on various rice plant tissues such as leaf sheath and leaf blade (Fig. 1) and this bacteria may reduce infection and deter disease spread both horizontally and vertically. This may explain the effectiveness of water-soluble granule formulations in reducing both% rice tillers with sheath blight symptoms and lesion length (Table 2).

However, 110 days after transplanting, rice plants sprayed with water-soluble granule formulation were not significantly different from those applied with floating pellet formulations, fungicide (Iprodione), or the control, particularly with respect to panicle weight (Table 3). Thus, it may be necessary to apply water-soluble granule formulation several times during the reproductive and grain filling stages to obtain better yield.

The high inoculum load of *R. solani* (at 20 g for each rice hill) used in this study may also have contributed to the severity of sheath blight disease, particularly when the disease was assessed at the maturation stage (Table 3). It has cancelled out all the effect of both fungicide and bacterial formulation treatment. Nevertheless, this high inoculum load of pathogen, which is artificially inoculated in a uniform pattern, is not likely to occur in rice field with sheath blight disease. Thus, it can be postulated that lower dose of bacterial formulation may be required to effectively control sheath blight disease in the field. Furthermore, the fact that sheath blight severity (Table 2) and panicle weight (Table 3) of rice plant treated with 30 g of floating pellet is contradictory may indicate that the philosophy of the more the better does not necessarily hold true (Stewart *et al*., 1999). It is possible that the ingredients in the floating pellet formulation, when applied at high rate at 30 g, may be preferentially utilized by other soil microbes and plant pathogens which subsequently infect stem of the rice plants and cause yield reduction.

As a result, more studies in rice fields; which have sheath blight disease problem, are required to determine the effective dose and frequency of application of this water-soluble granule formulation in suppressing sheath blight disease. This data is crucial as it is useful for the farmers who will opt to replace chemical fungicides with biological control product for suppressing sheath blight disease of rice.

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