

CHAPTER 2

MATERIALS AND METHODS

Materials

1. Hydrogenated vegetable oil (Lubritab[®], Edward Mendell Co.Inc. Carmol, New York, USA)
2. Hydroxyethyl cellulose 4000 (S. Tong Chemicals Co., Ltd., Thailand)
3. Hydroxypropyl methylcellulose 4000 (Methocel E4M Premium, Rama Production Co., Ltd., Thailand)
4. Iprodione (Rovral[®] Fungicide, Bayer CropSciences, Australia)
5. Lactose monohydrate (Pharmatose[®] 200M, DMV International, Thailand)
6. Magnesium stearate (S. Tong Chemicals Co., Ltd., Thailand)
7. Methylcellulose 4000 (Methocel A4M Premium, Rama Production Co., Ltd., Thailand)
8. Plate count agar (Difco[®], Becton, Dickinson and Company Sparks, MD, USA)
9. Potato dextrose agar
10. Potato dextrose broth
11. Sodium alginate (Srichand united dispensary, Co. Ltd., Bangkok, Thailand)
12. Sodium Carboxymethylcellulose 1500 (Srichand united dispensary, Co. Ltd., Bangkok, Thailand)
13. Sodium starch glycolate (Explotab[®], Edward Mendell Co. Inc. Carmol, New York, USA)
14. Talcum (S. Tong Chemicals Co., Ltd., Thailand)

Equipments

1. A nest of sieves (No. 10, 20, 50, 80, 100 and 120, Endocotts Ltd., UK)
2. Autoclave (Hiclave[®], Hirayama, Japan)
3. Brookfield digital rheometer model DV-III (Brookfield Engineering Labs, USA)
4. Critical point drier (Polaron[®] 7501)
5. Erweka abrasion tester (K.S.L. Engineering Co.,Ltd., Thailand)
6. Erwera tablet hardness tester (K.S.L. Engineering Co.,Ltd., Thailand)
7. Fritsch analysette (Germany)
8. Hand-held sprayer
9. Hot air oven (HA20, K.S.L. Engineering Co.,Ltd., Thailand)
10. Jolting apparatus (Vankel, USA)
11. Laminar air flow hood (Jouan, Thailand)
12. Microscope (Olympus Optical Co., Ltd., Japan)
13. Microwave oven (Turbora[®] TRX249M)
14. pH meter (Mettler-Toledo Co., Ltd., Thailand)
15. Planetary mixer (Kenwood[®] chef, Gallenkamp Griffin, Great Britain)
16. Scanning electron microscope (SEM) (JSM-5800LV, Jelo, Japan)
17. Shaker (Dacatron[®] 7203 LB Science Co.,Ltd., Thailand)
18. Sieve (No.14 and 16)
19. Single punch tableting machine (Yeo heng Co.,Ltd., Thailand)
20. Test tube shaker (Velp[®], Scientifica, Italy)

Experimental methods

1. Optimization of bacterial culturing time

One ml of *B. megaterium* suspension was transferred to the flask containing 100 ml of potato dextrose broth (PDB). The bacteria were cultured in PDB at 37 ± 1 °C. The number of endospores in the flask was counted after culturing at 12 h intervals up to 144 h. One ml of bacterial culture was incubated in water bath at 65°C for 1 h to get rid of the vegetative cells. Endospores were counted using the drop plate method (Zuberer, 1994). The number of endospores was plotted against culturing time and the optimal culturing time was then determined.

2. Preparation of bacterial suspension

B. megaterium was cultured in PDB at 37 ± 1 °C for 72 h and bacterial endospores were separated by centrifugation at 3,000 rpm for 8 min. The endospores were washed three times and re-suspended in sterile distilled water. The endospores were incubated in water bath at 65°C for 1 h to get rid of the vegetative cells. Then the spore suspension was stored in a glass container at 10 ± 0.5 °C.

3. Preparation of *B. megaterium* formulations

3.1 Preparation of granules containing *B. megaterium* for spray application (GS)

Granules containing *B. megaterium* for spray application were prepared by wet-granulation using different proportions of bacterial suspension, lactose and one of these following binders; sodium alginate (Alg), hydroxypropyl methylcellulose 4000 (HPMC 4000), sodium carboxymethylcellulose 1500 (SCMC 1500), methylcellulose 4000 (MC 4000) or hydroxyethyl cellulose 4000 (HEC 4000) as listed in Table 1. All ingredients were mixed in a planetary mixer until they

became a damp mass. Then this mass was passed through a sieve No.16 and dried in a hot air oven at 60°C for 2 h.

3.2 Preparation of tablets containing *B. megaterium* for spray application (TS)

Tablets containing *B. megaterium* for spray application were prepared by wet-granulation using different proportions of bacterial suspension, lactose, SCMC 1500, sodium starch glycolate, talcum and magnesium stearate as listed in Table 2. Bacterial suspension, lactose and SCMC1500 were mixed in a planetary mixer until they became a damp mass. Then this mass was passed through a sieve No.16 and dried in a hot air oven at 60°C for 2 h. These granules were then passed through a sieve No.14 and mixed with sodium starch glycolate, talcum and magnesium stearate for 3 min. Tablets were compressed using a single punch tableting machine tooled with 3/8 inch round flat faced punch.

3.3 Preparation of granules containing *B. megaterium* for broadcast application (GB)

Granules containing *B. megaterium* for broadcast application were prepared by wet-granulation using different proportions of bacterial suspension, lactose, Alg and hydrogenated vegetable oil (HVO) as listed in Table 3. All ingredients were mixed in a planetary mixer until they became a damp mass. Then this mass was passed through a sieve No.16 and dried in a hot air oven at 55°C for 4 h. After that the dried granules were screened through another sieve No.14 to a suitable size.

3.4 Preparation of tablets containing *B. megaterium* for broadcast application (TB)

Tablets containing *B. megaterium* for broadcast application, using different proportions of lactose, Alg and HVO as listed in Table 4, were prepared by machine-molding tablet triturate. All ingredients were mixed in a planetary mixer until

they became a damp mass. This mixture was then packed into the cavities of a triturate mold and the tablets were carefully pushed out. The tablets were dried in hot air oven at 55°C for 4 h.

Table 1. Compositions of ingredients in different granule formulations for spray application

| Ingredients (g) | Formulations | | | | |
|---------------------------|--------------|---------|---------|-------|--------|
| | GS-Alg | GS-HPMC | GS-SCMC | GS-MC | GS-HEC |
| Bacterial suspension (ml) | 30 | 30 | 30 | 30 | 30 |
| Alg | 20 | - | - | - | - |
| HPMC 4000 | - | 20 | - | - | - |
| SCMC 1500 | - | - | 20 | - | - |
| MC 4000 | - | - | - | 20 | - |
| HEC 4000 | - | - | - | - | 20 |
| Lactose | 80 | 80 | 80 | 80 | 80 |

Table 2. Compositions of ingredients in different tablet formulations for spray application

| Ingredients (g) | Formulations | | |
|---------------------------|--------------|-------|-------|
| | TS-5 | TS-10 | TS-15 |
| Bacterial suspension (ml) | 30 | 30 | 30 |
| SCMC 1500 | 5 | 10 | 15 |
| Lactose | 95 | 90 | 85 |
| sodium starch glycolate | 4 | 4 | 4 |
| Talcum | 3 | 3 | 3 |
| Magnesium Stearate | 1 | 1 | 1 |

Table 3. Compositions of ingredients in different granule formulations for broadcast application

| Ingredients (g) | Formulations | | |
|---------------------------|--------------|-------|-------|
| | GB-19 | GB-29 | GB-39 |
| Bacterial suspension (ml) | 30 | 30 | 30 |
| Alg | 2 | 2 | 2 |
| HVO | 19 | 29 | 39 |
| Lactose | 79 | 69 | 59 |

Table 4. Compositions of ingredients in different tablet formulations for broadcast application

| Ingredients (g) | Formulations | | | |
|---------------------------|--------------|-------|-------|-------|
| | TB-19 | TB-29 | TB-39 | TB-49 |
| Bacterial suspension (ml) | 30 | 30 | 30 | 30 |
| Alg | 2 | 2 | 2 | 2 |
| HVO | 19 | 29 | 39 | 49 |
| Lactose | 79 | 69 | 59 | 49 |

4. Enumeration of viable bacteria in the formulations

The enumeration was carried out to determine the number of viable bacteria in 1 g of the formulation. One g of each bacterial formulation was dissolved in 99 ml of sterile distilled water in a glass container at room temperature (26 - 30° C). Viable bacteria in the formulations were counted using the drop plate method (Zuberer, 1994). The viable bacteria were cultured in plate count agar (PCA) at room temperature for 18 – 24 h after which colony forming units (CFU) were counted. The value (CFU/g) of viable bacteria was the average of 6 replications (6 drops) per dilution.

5. Evaluation of physical properties of *B. megaterium* formulations

5.1 Evaluation of physical properties of bacterial granules for spray application (GS)

5.1.1 Particle size of granules

The particle size was studied by a standard sieve analysis method. A nest of sieves included sieve No. 10, 20, 40, 60, 80, 100 and 120. One hundred g of each formulation were placed on a sieve No. 10 and shaken by a Fritsch Analysette

for 15 min. The bacterial formulation retained on each sieve was weighed and the diameter of the particles was calculated [Equation (1)]. Three replicates were performed for each formulation.

$$d_{wn} = \frac{\sum nd^4}{\sum nd^3} \quad (1)$$

In equation (1), d_{wn} is the weight-moment or volume-weighted mean, n is the number of particles in a size range whose midpoint and d is one of the equivalent diameters (Martin, 1993).

5.1.2 Disintegration time of bacterial granules

One g of bacterial granules from each formulation was placed in 99 ml of distilled water in a 250-ml beaker at room temperature (26 - 30°C). The granules were stirred at 200 rpm until they dissolved. Disintegration time of the granules was recorded and three replicates were performed for each formulation.

5.1.3 pH of 1% w/w and 5% w/w solution of bacterial granules

Either 1 or 5 g of bacterial granules from each formulation was dissolved in 99 ml, or 95 ml of distilled water, respectively, at room temperature (26 - 30°C). The pH value of 1% w/w and 5% w/w solution were measured by pH meter. Three replicates were performed for each formulation.

5.1.4 Viscosity of 1% w/w and 5% w/w solution of bacterial granules

Either 1 or 5 g of bacterial granules from each formulation was dissolved in 99 ml, or 95 ml of distilled water, respectively, at room temperature (26 - 30°C). The viscosity value of 1% w/w and 5% w/w solution were measured by Brookfield Digital Rheometer Model DV-III. Three replicates were performed for each formulation.

5.2 Evaluation of physical properties of bacterial tablets for spray application (TS)

5.2.1 Average weight, thickness, hardness and friability of tablets

Tablets (n = 20) were randomly sampled from each formulation. The weight of each tablet was measured by an analytical balance and the average of weight was calculated for each formulation. The thickness and hardness of each tablet were measured by Erweka tablet hardness tester and the average of thickness and hardness were calculated for each formulation. Tablet friability was conducted on 6.5 g of tablets using Erweka abrasion tester. The drum was rotated at 25 rpm for 4 min. Loss of tablet weight with respect to the initial value was then calculated as percent friability [Equation (2)]. Three replicates were performed for each formulation.

$$\% \text{ friability} = (M_i - M_f / M_i) \times 100\% \quad (2)$$

In equation (2), M_i is the initial weight and M_f is the terminal weight.

5.2.2 Disintegration time of bacterial tablets

Disintegration time to prepare 1% w/w solution of bacterial tablets in water was measured using the same method as described in section 5.1.2. Three replicates were performed for each formulation.

5.2.3 pH of 1% w/w, 5% w/w and 10% w/w solution of bacterial tablets

The pH value of 1% w/w, 5% w/w and 10% w/w solution of bacterial tablets in water were measured by pH meter using the same method as mentioned in section 5.1.3. Three replicates were performed for each formulation.

5.2.4 Viscosity of 1% w/w, 5% w/w and 10% w/w solution of bacterial tablets

The viscosity value of 1% w/w, 5% w/w and 10% w/w solution of bacterial tablets in water were measured by Brookfield Digital Rheometer Model DV-III using the same method as mentioned in section 5.1.4. Three replicates were performed for each formulation.

5.3 Evaluation of physical properties of bacterial granules for broadcast application (GB)

5.3.1 Particle size of granules

The particle size was studied by a standard sieve analysis method as described in section 5.1.1.

5.3.2 Density of granules

Bulk densities of the formulations were measured using a Jolting Apparatus. A 100-ml cylinder was weighed. Then the bacterial formulation was dispensed into the cylinder until it reached 50 ml and the cylinder containing granules was weighed. The granule weight was calculated [Equation (3)]. The cylinder containing granules was tapped 3 times by a Jolting apparatus, after that the formulation volume and bulk volume were recorded. The bulk density was then calculated [Equation (4)]. The distilled water was added into another 100-ml cylinder until it reached 100 ml, then this water was filled into the cylinder containing granules until it reached 100 ml. The remaining water volume in the cylinder containing water without granules was read. True volume and true density were then calculated [Equation (5) and (6)]. Three replicates were performed for each formulation.

$$M = M_2 - M_1 \quad (3)$$

$$\text{bulk density} = M / V_b \quad (4)$$

$$V_p = 100 - V \quad (5)$$

$$\text{true density} = M / V_p \quad (6)$$

In equation (3), (4), (5) and (6), M_1 is the weight of the cylinder, M_2 is the weight of the cylinder containing granules, M is the granule weight, V_b is the bulk volume, V is the volume of the remainder water in the cylinder containing water without granules and V_p is the true volume (Martin, 1993).

5.4 Evaluation of physical properties of bacterial tablets for broadcast application (TB)

5.4.1 Average weight, thickness, hardness and friability of tablets

The weight of each tablet was measured using the same method as described in section 5.2.1 and the average of weight was calculated for each formulation. The thickness, the hardness and percent friability of each tablet were measured as described in section 5.2.1. Three replicates were performed for each formulation.

5.4.2 Evaluation of floating ability of tablets

Tablets ($n = 10$) from each formulation were randomly sampled and dispersed to 100 ml of distilled water in a glass container at room temperature (26 - 30°C). The number of tablets remained on the water surface were recorded at 15 min interval up to 2 h. The calculated percentage of floating tablets was plotted against time. Four replicates were performed for each formulation.

5.4.3 Evaluation of bacterial release

For bacterial release study, 1 g of bacterial tablets from each formulation were left floating on 99 ml of sterile distilled water in a glass container at room temperature (26 - 30°C) for 15 min, 30 min, 45 min and 1 h. The tablets were

then removed and 1 ml of medium was sampled. The number of bacterial release was counted using the drop plate method (Zuberer, 1994). The value (CFU/ml) of viable bacteria was the average of 6 replications (6 drops) per dilution. The percentage of bacterial release was then calculated [Equation (7)].

$$\% \text{ Bacterial release} = n \times 100 / N \quad (7)$$

In equation (7), n is the number of viable bacteria in medium at each time point and N is the initial number of viable bacteria in formulation.

6. Scanning electron microscope (SEM) observation of the selected formulations and endospores on plant surface

6.1 SEM observation of the selected formulations

One g of the selected bacterial formulations from each formulation was randomly sampled from the plastic container. A few samples of these formulations were mounted on the stub. This specimen was then coated with gold particles and observed with SEM. Micrographs of bacterial endospores on the surface and cross-section of the formulations were taken.

6.2 SEM observation of endospores on plant surface

One g of the selected formulations for spray application was dissolved in 99 ml of sterile distilled water at room temperature (26 - 30°C). Then leaf sheath or leaf blade sampled were immersed in the bacterial solution for 1 h. They were then fixed in 0.25% glutaraldehyde solution for 2 h. The samples were then washed by an increasing concentration (step by step) of alcohol and dried in Critical Point Drier. The samples were mounted on the stub and coated with gold particles and observed with SEM. Micrographs of bacterial endospores on the surface of the leaf sheath or leaf blade were taken.

7. Testing the efficacy of selected granule and tablet formulations containing *B. megaterium* under greenhouse conditions

7.1 Experimental design and treatments

There were thirteen treatments as listed in Table 5. Each treatment consisted of five replications (twelve rice seedlings (cv. Hom Mali 105) per replication). Rice plants inoculated only with *R. solani* were used as a control treatment. The experiment was arranged in a Complete Randomised Design (CRD).

7.2 Pot preparation

Paddy rice field soil from Trang [Sandy loam texture (64.59% sand, 15.83% silt, 19.57% clay), pH 7.16, 1.59% organic matter, 0.07% total N, 3.00 mg/kg available P and 0.08 mg/kg available K] was used in the pot test. This soil sample was 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 soil was agitated manually to break up aggregates and excess water was drained. Soil level in the plastic pots was adjusted to a height of 14.5 cm so that 3.5 cm depth of water was retained in each plastic pot.

7.3 Pathogen inoculation

Fifteen g of sterile rice seeds infested with *R. solani* were placed in the centre of each plastic pot 80 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.

Table 5. Different types of treatment used under greenhouse condition

| Number | Treatment |
|--------|--|
| (1) | Control |
| (2) | Iprodione |
| (3) | <i>B. megaterium</i> endospores suspension |
| (4) | Formulation GS-Alg (Granules for spray application) |
| (5) | Blank Formulation GS-Alg (Formulation GS-Alg without bacterial suspension) |
| (6) | Formulation GS-SCMC (Granules for spray application) |
| (7) | Blank Formulation GS-SCMC (Formulation GS-SCMC without bacterial suspension) |
| (8) | Formulation TS-15 (Tablets for spray application) |
| (9) | Blank Formulation TS-15 (Formulation TS-15 without bacterial suspension) |
| (10) | Formulation GB-19 (Granules for broadcast application) |
| (11) | Blank Formulation GB-19 (Formulation GB-19 without bacterial suspension) |
| (12) | Formulation TB-39 (Tablets for broadcast application) |
| (13) | Blank Formulation TB-39 (Formulation TB-39 without bacterial suspension) |

7.4 Formulation application

Rice seedling sprayed with fungicide (Iprodione) was used as control to compare the efficacy of the formulations. Iprodione (10000 ppm), *B. megaterium* endospores suspension (0.1% v/v), formulation GS-Alg (1% w/w), blank formulation GS-Alg (1% w/w), formulation GS-SCMC (1% w/w), blank formulation GS-SCMC (1% w/w), formulation TS-15 (10% w/w) and blank formulation TS-15 (10% w/w) were sprayed on the rice seedlings in the plastic pots at 150 ml/pot using a hand-held

sprayer. Formulation GB-19 (10 g), blank formulation GB-19 (10 g), formulation TB-39 (10g) and blank formulation TB-39 (10g) were placed in the centre of each plastic pot. The bacteria number was at 10^9 CFU/pot except formulation TS-15 which the number of bacteria was at 10^7 CFU/pot.

7.5 Disease assessment

Sheath blight assessment in the greenhouse tests was carried out 10 days after formulation application. Roots of rice plants were washed to eliminate excessive soil. 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 symptoms. Percentage of tillers with sheath blight symptoms was calculated [Equation (8)]. The entire length of the lesion on each rice tiller which had sheath blight symptoms was also measured. Fresh weight of the inoculated rice plants were weighed after disease measurement. Then the inoculated rice plants dried in a hot air oven at 70°C for 72 h. Dry weight of the inoculated rice plants was also assessed.

$$\% \text{ of tillers with sheath blight symptoms} = I \times 100 / T \quad (8)$$

In equation (8), I is the number of infected rice tillers and T is the number of total rice tillers.

7.6 Statistical analysis

Data were subjected to standard analysis of variance procedures for a completely randomized design using the Statistical Analysis System (SAS) computer software package. One-way analysis of variance was carried out on the percentage of tillers where sheath blight symptoms occurred on outer leaf sheaths. The one-way analysis of variance was also done on the length of the lesions on each

rice tiller which had sheath blight symptoms. Data was compared with Duncan's Multiple Range Test (DMRT) at $p < 0.01$ or $p < 0.05$.

8. Evaluation of *B. megaterium* adhesion on surface of rice tissues

8.1 Formulation application

B. megaterium endospores suspension (0.1% v/v), formulation GS-Alg (1% w/w), formulation GS-SCMC (1% w/w) and formulation TS-15 (10% w/w) were sprayed on the rice seedlings in the plastic pots at 150 ml/pot using a hand-held sprayer. The number of bacteria was at 10^9 CFU/pot except formulation TS-15 which the number of bacteria was at 10^7 CFU/pot.

8.2 Enumeration of viable bacteria on surface of rice tissues

After spraying the selected formulations 4, 7 and 14 days, 1 g of leaf sheath or leaf blade from rice plants was placed into 99 ml of sterile distilled water in a glass container at room temperature (26 - 30°C) and the container was shaken for 5 min. Then 1 ml of the suspension was sampled and the number of bacteria on the leaf sheath or leaf blade was counted using the drop plate method (Zuberer, 1994). The viable bacteria were cultured in PCA at room temperature (26 - 30°C) for 18 – 24 h after which CFU were counted. The value (CFU/g of leaf sheath or leaf blade) of viable bacteria was the average of 6 replications (6 drops) per dilution.

9. Testing the inhibition of *R. solani* mycelial growth

The inhibition of *R. solani* mycelial growth tests were carried out after the formulations were stored at room temperature (26 - 30°C) for 3 and 6 months. One g of the selected bacterial formulations was dissolved in sterile distilled water 99 ml in a glass container. One ml of the bacterial suspension was mixed with melted potato dextrose agar (PDA) at 1:10 (v/v) in Petri dishes (9.0 cm diameter). The agar block of *R. solani* was cultured on PDA incorporated with suspension of

the bacterial formulations to test the inhibition of *R. solani* mycelial growth at room temperature (26 - 30°C). Each treatment consisted of 6 replications. Culture of *R. solani* on PDA incorporated with sterile water was used as a control. Mycelial inhibition of *R. solani* was assessed as % mycelial inhibition 36 h after culturing *R. solani* [Equation (9)] (Gamliel *et al.*, 1989).

$$\% \text{ mycelial inhibition} = 100 - [(r^2/R^2) \times 100] \quad (9)$$

In equation (9), *r* is colony radius of *R. solani* on PDA incorporated with 1% w/w solution of the selected bacterial formulations and *R* is colony radius of *R. solani* on PDA incorporated with sterile water.

10. Viability of bacteria in the selected formulations at room temperature

Viable bacteria in the selected formulations were counted using the drop plate method (Zuberer, 1994). The viability tests were carried out after the formulations were produced and during storage at room temperature (26 - 30°C) in plastic containers for 6 months. The number of viable bacteria in the formulations was counted after production at one-month intervals up to 6 months. One g of each bacterial formulation was dissolved in 99 ml of sterile distilled water in a glass container. The viable bacteria were cultured in PCA at room temperature (26 - 30°C) for 18 – 24 h after which CFU were counted. The value (CFU/g) of viable bacteria was the average of 6 replications (6 drops) per dilution.