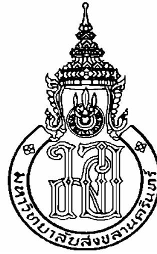

Development of *Bacillus megaterium* Formulations for Suppression of Rice Sheath Blight and Study of Mechanisms of Biocontrol

Amornrat Chumthong

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Thesis Title Development of *Bacillus megaterium* Formulations for Suppression of Rice Sheath Blight and Study of Mechanisms of Biocontrol

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ชื่อวิทยานิพนธ์ การพัฒนาสูตรตำรับของ *Bacillus megaterium* เพื่อควบคุมโรคกาบใบแห้งของข้าว และศึกษากลไกการควบคุมโรคโดยชีววิธี

ผู้เขียน นางสาวอมรรัตน์ ชุมทอง

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บทคัดย่อ

การวิจัยนี้มีวัตถุประสงค์เพื่อพัฒนาสูตรตำรับเชื้อแบคทีเรียปฏิปักษ์ *Bacillus megaterium* ให้อยู่ในรูปแบบผลิตภัณฑ์ที่มีประสิทธิภาพในการควบคุมโรคกาบใบแห้งของข้าว สามารถใช้ได้ง่าย และมีความคงตัว โดยเริ่มจากการทดสอบความทนต่อรังสีอุลตร้าไวโอเลต (UV), อุณหภูมิสูง และความเป็นกรด-ด่าง (pH) ของเอนโดสปอร์ การเพาะเลี้ยงและหาสภาวะเหมาะสมในการผลิตเอนโดสปอร์เชื้อแบคทีเรียปฏิปักษ์ *B. megaterium* พบว่า เอนโดสปอร์ทนต่อรังสี UV (48 ชั่วโมง), อุณหภูมิสูง 80 องศาเซลเซียส (36 ชั่วโมง) และทนต่อ pH 2-10 (15 วัน) การเพาะเลี้ยงเชื้อแบคทีเรียปฏิปักษ์ในถังหมักขนาด 30 ลิตร โดยใช้อาหารที่มีมันฝรั่งและ dextrose เป็นส่วนประกอบ 20 ลิตร เลี้ยงไว้ที่อุณหภูมิ 30-35 องศาเซลเซียส อัตราการกวน 200 รอบ/นาที และให้อากาศ 1 vvm เป็นเวลา 4 วัน เชื้อแบคทีเรียปฏิปักษ์สามารถผลิตเอนโดสปอร์ได้สูงสุด

สูตรตำรับเชื้อแบคทีเรียปฏิปักษ์รูปแบบแกรนูลละลายน้ำเตรียมด้วยวิธี wet granulation ซึ่งประกอบด้วย sodium alginate 10 กรัม, polyvinyl pyrrolidone K-30 5 กรัม, lactose monohydrate 85 กรัม และสารแขวนลอยสปอร์ 21 มิลลิลิตร สูตรตำรับแกรนูลมีคุณสมบัติทางกายภาพและชีวภาพที่เหมาะสม ละลายน้ำได้ดี มีความหนืดพอเหมาะสำหรับการนำไปใช้พ่น มีปริมาณเชื้อแบคทีเรียปฏิปักษ์ในสูตรตำรับ 9 Log. number/กรัม และมีประสิทธิภาพในการยับยั้งเส้นใยของเชื้อราสาเหตุมากกว่า 90 เปอร์เซ็นต์ หลังจากเก็บไว้ที่อุณหภูมิห้อง (26-30 องศาเซลเซียส) เป็นเวลา 24 เดือน จากการทดสอบประสิทธิภาพของสูตรตำรับในสภาพเรือนทดลอง พบว่า กรรมวิธีที่พ่นด้วยสูตรตำรับแกรนูลละลายน้ำ 1, 5 และ 10 วัน หลังปลูกเชื้อราสาเหตุโรค สามารถควบคุมและยับยั้งการเกิดโรคกาบใบแห้งของข้าวได้ดีกว่ากรรมวิธีควบคุมประมาณ 50 เปอร์เซ็นต์

สูตรตำรับเชื้อแบคทีเรียปฏิปักษ์แกรนูลฟู่สำหรับหว่านหรือพ่น เตรียมด้วยวิธี wet granulation โดยใช้ ส่วนผสมของสปอร์ใน lactose monohydrate 60 เปอร์เซ็นต์, polyvinyl pyrrolidone K-30 5 เปอร์เซ็นต์, citric acid 5 เปอร์เซ็นต์, tartaric acid 10 เปอร์เซ็นต์ และ sodium

bicarbonate 20 เปอร์เซ็นต์ สูตรตำรับแกรนูลฟู่ มีคุณสมบัติทางกายภาพที่เหมาะสม แตกกระจายตัวในน้ำได้ดีและรวดเร็วภายในเวลา 30 วินาที หลังจากเก็บสูตรตำรับแกรนูลฟู่ไว้ที่อุณหภูมิห้อง เป็นเวลา 24 เดือน สูตรตำรับยังคงมีเชื้อแบคทีเรียปฏิปักษ์ที่อยู่รอด ประมาณ 8 Log. number/กรัม และสามารถยับยั้งการเจริญของเส้นใยเชื้อรา *R. solani* ได้อย่างมีประสิทธิภาพมากกว่า 90 เปอร์เซ็นต์ ในสภาพเรือนทดลอง พบว่า การหว่านด้วยสูตรตำรับแกรนูลฟู่ 1, 5 และ 10 วัน หลังปลูกเชื้อราสาเหตุโรค ทำให้เกิดโรคกาบใบแห้งน้อยที่สุด (29.42±5.67 เปอร์เซ็นต์) เมื่อเทียบกับชุดควบคุม (51.81±3.02 เปอร์เซ็นต์)

สูตรตำรับเชื้อแบคทีเรียปฏิปักษ์เม็ดฟู่ เตรียมโดยนำสูตรตำรับเชื้อแบคทีเรียปฏิปักษ์แกรนูลฟู่ 96 เปอร์เซ็นต์ ผสมกับสารหล่อลื่น polyethylene glycol 6000 4 เปอร์เซ็นต์ แล้วนำไปตอกเป็นเม็ดด้วยเครื่องตอกยาเม็ดแบบสากเดี่ยว พบว่า เม็ดฟู่แตกกระจายตัวในน้ำได้หมดภายใน 3 นาที จากการนับปริมาณเชื้อแบคทีเรียปฏิปักษ์ในสูตรตำรับเม็ดฟู่หลังจากเก็บไว้ที่อุณหภูมิห้องเป็นเวลา 24 เดือน พบว่า มีเชื้อแบคทีเรียปฏิปักษ์ในสูตรตำรับ 9 Log. number/เม็ด และยังคงมีประสิทธิภาพในการยับยั้งเส้นใยของเชื้อราสาเหตุมากกว่า 90 เปอร์เซ็นต์ ในสภาพเรือนทดลอง พบว่า การหว่านหรือพ่นด้วยเม็ดฟู่ก่อนปลูกเชื้อราสาเหตุโรค 1 วัน ตามด้วยการพ่นด้วยสารละลายเม็ดฟู่ 1, 7 และ 15 วัน หลังจากปลูกเชื้อราสาเหตุโรค สามารถควบคุมและยับยั้งโรคกาบใบแห้งของข้าวได้ดี

การทดสอบในสภาพแปลงทดลองขนาดเล็ก พบว่า การใช้สูตรตำรับคลุกเมล็ดร่วมกับการพ่นด้วยสูตรตำรับแกรนูลละลายน้ำหรือการหว่านด้วยสูตรตำรับแกรนูลฟู่หลังจากปลูกเชื้อราสาเหตุโรคในวันที่ 1, 5 และ 10 สามารถควบคุมและยับยั้งการเจริญของเชื้อราสาเหตุ *R. solani* ได้มากกว่าการใช้สารเคมีฆ่าเชื้อรา Iprodione นอกจากนี้ แปลงที่ใช้สูตรตำรับเชื้อแบคทีเรียปฏิปักษ์เหล่านี้ยังให้ผลผลิตข้าวสูงและมีเมล็ดข้าวดีมากกว่าแปลงที่ใช้สารเคมีและแปลงที่เป็นชุดควบคุม

การศึกษากลไกการควบคุมโรคกาบใบแห้งของข้าวโดยชีววิธีของเชื้อแบคทีเรียปฏิปักษ์ *B. megaterium* พบว่า เชื้อแบคทีเรียปฏิปักษ์สามารถผลิตสารระเหยและสารซึ่งแพร่กระจายได้ที่มีคุณสมบัติยับยั้งหรือทำลายเชื้อรา *R. solani* สารปฏิชีวนะที่กรองได้จากน้ำเลี้ยงเชื้อแบคทีเรียปฏิปักษ์ *B. megaterium* สามารถออกฤทธิ์ยับยั้งเชื้อราสาเหตุโรคได้ หลังจากผ่านความร้อนที่อุณหภูมิ 121 องศาเซลเซียส เป็นเวลา 20 นาที น้ำหนักโมเลกุลของสารปฏิชีวนะ คือ 58 kDa ด้วยวิธี SDS-PAGE มีค่า MIC และ IC_{50} ที่สามารถยับยั้งการเจริญของเส้นใยเชื้อรา *R. solani* คือ 0.50 μg/ml และ 0.34 mg/ml ตามลำดับ และสารปฏิชีวนะดังกล่าวสามารถย่อยแป้งได้ ซึ่งอาจเป็นสารที่จำแนกได้ในกลุ่ม α-amylase ที่ทนความร้อน

Thesis Title	Development of *Bacillus megaterium* Formulations for Suppression of Rice Sheath Blight Disease and Study of Mechanisms of Biocontrol
Author	Miss Amornrat Chumthong
Major Program	Pharmaceutical Sciences
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ABSTRACT

This thesis described a protocol to develop the suitable formulations containing a bacterium, *Bacillus megaterium*, for controlling sheath blight disease of rice. These formulations were produced with special emphasis not only on their efficacy and applicability but also on their stability and long shelf life.

First, the durability of *B. megaterium* endospores against UV irradiation, high temperature and pH was evaluated. The endospores remained active after being treated with UV irradiation (48 h), high temperature at 80 ^{0}C (36 h) and pH 2-10 (15 days). The substrate and optimal growth of *B. megaterium* in 30 l fermentation was determined. Culturing this bacterium in 20 l potato dextrose broth (at 30-35 ^{0}C, 200 rpm, 1 vvm for 4 days) provided the highest number of endospores.

Water-soluble granule formulation containing endospores of *B. megaterium* were subsequently prepared by the wet granulation technique using 10 g sodium alginate, 5 g polyvinyl pyrrolidone K-30, 85 g lactose monohydrate and 21 ml bacterial endospores suspension. This formulation had good physical and biological characteristics such as high-water solubility and optimal viscosity value which was suitable for spray application. The viability of bacteria in water-soluble granules was in the range of 9 Log. number/g and high mycelial growth inhibition of *R. solani* (more than 90%) after 24 months storage at room temperature (26-30 ^{0}C) was obtained in the laboratory test. In the efficacy testing under greenhouse condition, spraying rice plants with the bacterial water-soluble granules 1, 5 and 10 days after pathogen inoculation was more effective than control (only with *R. solani*) of approximately 50%.

Effervescent granules containing endospores of *B. megaterium* were also developed for application either by broadcasting or spraying. The formulation composed of 60%

lactose monohydrate contained bacterial endospores, 5% polyvinyl pyrrolidone K-30 and effervescent base (5% citric acid, 10% tartaric acid and 20% sodium bicarbonate). They were completely disintegrated in water within 30 sec at room temperature. After the effervescent granules were stored at room temperature for 24 months, the number of living bacteria in effervescent granules was approximately 8 Log. number/g. In addition, it had a capacity to inhibit a mycelial growth of *R. solani* effectively (at more than 90% inhibition). In the greenhouse experiment, effervescent granules applied by broadcasting at 1, 5 and 10 days after pathogen inoculation had the lowest % infection of sheath blight disease (29.42±5.67%), comparing with the control treatment (51.81±3.02%).

Bacterial effervescent tablet was prepared by mixing 96% bacterial effervescent granules with 4% polyethylene glycol 6000 (w/w). Tablets were compressed using a single punch tablet machine. They were completely disintegrated in water within 3 min. The viable bacteria in effervescent tablets were counted during storage at room temperature for 24 months. The viability of bacteria remained high (9 Log. number/tablet) and the formulation was effective in inhibiting mycelial growth of *R. solani* (at more than 90% inhibition). In a greenhouse test, effervescent tablets applied by broadcasting or spraying 1 day before pathogen inoculation, followed by spraying 1, 7 and 15 days after pathogen inoculation, suppressed the development of sheath blight disease lesions and severity effectively.

In a small pilot field test, combining seed treatment with either spraying water-soluble granules or broadcasting effervescent granules at day 1, 5 and 10 after pathogen inoculation was more effective than a chemical fungicide (Iprodione) in suppressing rice sheath blight disease. Moreover, rice plants applied by these bacterial antagonist formulations had higher panicle and whole kernel weights than those of chemical fungicide and control.

The *B. megaterium* was found to produce the volatile and diffusible antifungal compounds. The antifungal compounds in the culture were heat stable (testing at 121 ^{0}C for 20 min). The molecular weight of heat stable component was 58 kDa, as estimated by SDS-PAGE, with the MIC and IC_{50} values at 0.50 μg/ml and 0.34 mg/ml respectively. In addition, the heat stable component was capable of hydrolyzing a soluble starch. This antifungal compound may identify as thermostable α-amylase group.

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Amornrat Chumthong

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LIST OF ABBREVIATIONS AND SYMBOLS

PCA	= Plate count agar
PDB	= Potato dextrose broth
PDA	= Potato dextrose agar
rpm	= Revolution per minute
vvm	= Volume per volume per minute
PVP, K-30	= Polyvinyl pyrrolidone K-30
cps	= Centipoises
SEM	= Scanning electron microscope
PEG 6000	= Polyethylene glycol 6000
MWCO	= Molecular weight cutoff
DEAE-Sephacel	= Diethylaminoethyl-Sephacel
SDS-PAGE	= Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
MIC	= Minimum inhibitory concentration
IC_{50}	= Fifty percent inhibition concentration

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Sheath blight of rice (*Oryza sativa* L.), caused by *Rhizoctonia solani* Kühn, is one of the most destructive rice diseases worldwide (Ou, 1985). This fungal disease is one of the major constraints to rice production, with yield losses of between 10 and 36%, depending on the growth stage when the disease occurs (Teng *et a*l., 1990). 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). Use of biological control agents is one alternative that has been proposed and evaluated in numerous pathosystems with varying degrees of success (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; Vasantha 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. The instability of the fresh cells of the effective antagonists also makes its impossible for possible commercialization and long-term adoption for usage. As a result, successfully formulating the biological control organisms is required for the stable and economical development of biofungicides (Lewis, 1991; Lumsden *et al*., 1995; Larkin *et al*., 1998; Schisler *et al*., 2004).

Bacillus megaterium was isolated from paddy rice soil collected from Satun province. This bacterium was screened from the 323 potential bacterial antagonists that were

isolated from paddy rice fields in 14 provinces in the southern part of Thailand (Kanjanamaneesathian *et al*., 1998). Biochemical and physiological properties of *Bacillus* sp. were determined and the bacterium was identified as *B. megaterium* (Pengnoo *et al*., 2000). *B. megaterium* are non-pathogenic bacteria (Rourke, 2004; United Nations, 2005). In addition, experiments on laboratory animals showed that *B. megaterium* displayed no toxicity when the acute oral toxicity, acute dermal toxicity and intravenous injection testing were evaluated by Thailand Institute of Scientific and Technological Research (TISTR, 2008). This bacterium produced heat stable antibiotics which could suppress mycelial growth of *R. solani*, which caused rice sheath blight disease (Pengnoo *et al*., 2000). Preliminary testing of bacterial floating granules for broadcasting application to control sheath blight of rice has been investigated in the greenhouse (Kanjanamaneesathian *et al*., 1998) and in the field conditions (Pengnoo *et al*., 2000). Although these formulations demonstrated the desired characteristics and provided quite satisfactory protection for rice plants from *R. solani* infection in both tests (Kanjanamaneesathian *et al*., 1998; Kusonwiriyawong *et al*., 1999; Pengnoo *et al*., 2000), they had a comparatively short shelf life, and the numbers of bacterial antagonists in the formulations greatly declined during 6 months storage (Pengnoo *et al*., 2000). Kusonwiriyawong *et al*. (1999) reported that floating granule formulations which had smallest particle size performed better in suppressing sheath blight disease than those which had larger particles. It is possible that cells of *B. megaterium* in the formulations which had a large particle size are entrapped inside the particle and cannot function to protect rice plants from *R. solani* infection, especially when antibiotic produced by effective *B. megaterium* is involved in the disease suppression (Pengnoo *et al*., 2000).

Later, novel formulations containing *B. megaterium* spores have been developed and used for sheath blight disease control trials (Wiwattanapatapee *et al*., 2004). In the preliminary tests, these pellet formulations showed good floating properties and gradually released their bacterium load over time. After production, they also contained 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).

However, when the sheath blight fungal attacks and the infection developed rapidly, the slow-released formulation may not be effective in controlling the disease. Thus, more

researches are needed to find ingredients and type of formulations which have better physical characteristics to ensure that the bacteria in the formulations can be dispersed effectively when applied to rice plants in field conditions. With adequate media and conditions suitable for endospores production, it is hoped that survival of *B. megaterium* can exceed 90% during 24 months storage. The formulation should be in a form that can be easily handled and used by the farmers. It must also be able to stabilize the bacteria during production, distribution and storage (Burges and Jones, 1998). In addition, these new formulations which contain viable endospores should also be tested to evaluate its efficacy to control rice sheath blight in greenhouse and field conditions.

In this research, *B. megaterium* endospores are used as a bioactive substance in suppressing sheath blight disease. Before the formulation process, it is important to have the information about the physical and biological characteristics of *B. megaterium* endospores. Therefore, the durability of *B. megaterium* endospores in persistence against UV irradiation, high temperature and pH have been evaluated (Chapter 2). Then, the effect of culture medium using agricultural produces on the number and size of *B. megaterium* endospores was studied. Formulation is one of the crucial steps in transforming the cells of the effective antagonists to the finished products which possess the features beneficial for effective plant disease control. In this study, three types of formulations of *B. megaterium* such as water-soluble granules, effervescent granules and effervescent tablets have been developed (Chapter 3-5). Water-soluble granules for spray application are easy to produce, handle and apply, and are less dusty than powder (Paau, 1998). Effervescent formulations can be applied by spraying on plant or by direct broadcasting to water. When broadcasted to the rice plant canopy, the bacterium in the effervescent formulations would be released quickly before the formulation deposits at the bottom of rice field due to gravity force. This would enhance the chance in which the bacterium could remain on the water surface and colonize rice plant at the water-rice plant interface to provide protection against pathogen infection and colonization. In addition, effervescent formulations may be also suitable for use to control other plant diseases such as in the plants grown hydroponically system. The efficacy of *B. megaterium* formulations in suppression of rice sheath blight disease have been tested both under greenhouse and field conditions (Chapter 3-6). Finally, the active compounds produced by *B. megaterium* effective in inhibiting *R. solani* have been characterized (Chapter 7).

Objectives

1. To evaluate the durability of *B. megaterium* endospores under UV irradiation, high temperature and pH
2. To investigate agricultural produces as substrate for producing endospores of *B. megaterium*
3. To develop bacterial formulations in water-soluble granules, effervescent granules and effervescent tablet for used either by spray or broadcast application
4. To evaluate the physical characteristics and viability of the bacteria in the formulations
5. To test the efficacy of the selected bacterial formulations in suppression of rice sheath blight disease in greenhouse and field conditions
6. To characterize the active compounds produced by *B. megaterium* in inhibiting mycelial growth of *R. solani*

1.2 Literature Review

1.2.1 Pathogen of rice sheath blight disease

1.2.1.1 *Rhizoctonia solani* (Kühn)

Rhizoctonia solani (Kühn) has been identified in the subdivision Deuteromycotina and Class Agonomycetes, and divided in the anastomosis group 1 IA (AG-1 IA) (Ogoshi, 1987; Sneh *et al*., 1991; Agrios, 2005). The fungus is present in most soils and, once established in a field, remains there indefinitely. The mycelium, which is fast growth, colorless when young, becoming yellowish brown when older, 8-12 μm in diameter, consists of long cells and produces branches that grow at approximately right angles to the main hypha, are slightly constricted at the junction, and have a cross wall near the junction (Figure 1.1). The mycelium consists of monilioid cells involved in the formation of sclerotia as a hard, weather-resistant structure, and which can remain alive in for several years. Sclerotia are white when young, becoming brown or dark brown. Individual sclerotia measure up to 5 mm but may unite to form a larger mass in culture (Figure 1.2). *R. solani* has the wide rang of hosts, such as cucumbers, tomatoes, potatoes, eggplants, beans, and rice. The rice sheath blight fungus infected 20 species of weeds from 11 families and that the sclerotia or diseased tissues obtained from the weeds produced typical symptoms of rice sheath blight on rice plants (Ou, 1987; IRRI, 1993). The fungus produces enzymes and toxin that can degrade plant tissue, such as pectic enzymes, cellulolytic enzymes, and proteolytic enzymes (Parmeter and Whitney, 1970).

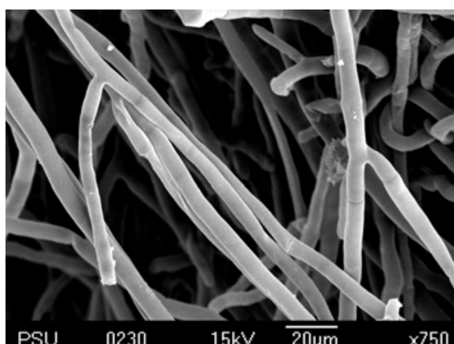

Figure 1.1 The mycelium of *R. solani* showing its branching at a right angle and septa close to the branching point

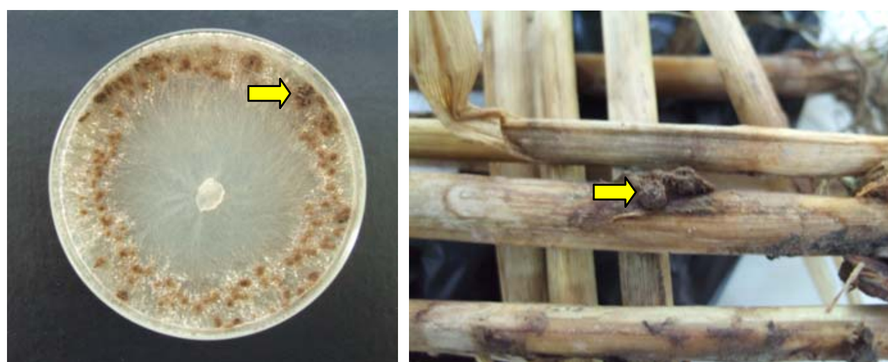

Figure 1.2 Sclerotia of *R. solani* on PDA medium (left) and on the rice plant tissue (right)

1.2.1.2 Disease cycle of rice sheath blight disease

Figure 1.3 outlines the disease cycle of rice sheath blight disease. The disease usually infects the plant at late tillering or early internode elongation growth stages. The fungus penetrates through the cuticle or the stomatal slit. Infection pegs are formed from each lobe of the lobate appressorium of infection cushion. The mycelium grows from the outer surface of the sheath going through the sheath edge and finally through the inner surface. Primary lesions are formed while the mycelium grows rapidly on the surface of the plant tissue and inside its tissue. It proceeds upwards and laterally to initiate formation of the secondary lesions. Later, the symptoms are observed on the upper leaf sheath and on the leaf blade. Disease may spread both vertically and horizontally from one hill to another through leaf-to-leaf or leaf-to-sheath contacts (IRRI, 1993; Chaudhary, 2002).

This fungus survives for a long time in the soil from year to year as sclerotia. Sclerotia will float to the surface of flooded rice fields in the subsequent rice crop. When sclerotia touch the plant, they germinate and the fungi penetrate into the leaf sheath. New sclerotia, which have developed on infected stem surfaces, fall from the plant to complete the life cycle. Sclerotia can remain alive in the soil for several years. They can also cause infection in several weeds (Ou, 1987; IRRI, 1993).

It is commonly assumed that the critical factors for disease development are relative humidity and temperature. Relative humidity ranging from 96 to 100% and temperature ranging from 28-32 ^{0}C has been reported to favor the disease (Reissing *et al*., 1986; IRRI, 1993). High supply of nitrogen fertilizer and growing of high-yielding, high-tillering, nitrogen-responsive improved varieties favor the development of the disease. High leaf wetness and high frequency of tissue contacts among plants also favor the disease. The pathogen can be spread through irrigation water and by movement of soil and infected crop residues during land preparation (IRRI, 1993; Castilla *et al*., 1996; Chaudhary, 2002; Slaton *et al*., 2003; Tang *et al*., 2007).

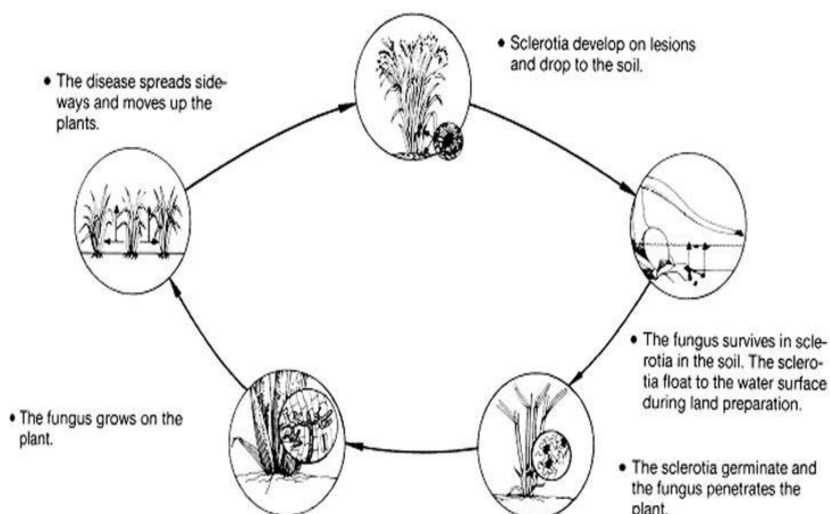

Figure 1.3 The life cycle of rice sheath blight disease (IRRI, 1983)

The severity of sheath blight has increased in recent years due to increased use of highly susceptible varieties, a lack of crop rotation, thicker stands and use of higher nitrogen

rates, and earlier planting dates. Symptoms appear from tillering to milk stage and include: (1) 1-3 cm long oval or ellipsoidal greenish gray spots on the leaf sheath just above the water or soil; in advanced stages the spots can extend all the way to the leaf blade, (2) leaves have irregular lesions with gray-white centers and brown margins or orange banding, (3) lesions can occur on flag leaf and panicles, (4) damaged flag leaves and grain reduces yield (IRRI, 1993; IRRI, 2003) (Figure 1.4).

The disease starts during the maximum growth stage of the rice crop. Under favorable conditions, the disease increases as the plant grows older. The damage caused by the disease depends on the infection of the plants at plant growth stages. Damage ranges from partial infection of the lower leaves with little effect on grain development to premature plant disease. On some varieties, the panicle can be attacked during hot, humid weather. Both yield and grain qualities are reduced when the infection prevents the flow of water and nutrients to the grain. Grain may develop only partially or not at all. Often the grain at the base of the panicle will not fill. Poorly developed grain usually breaks up during milling thus reducing grain quality (IRRI, 1993; Pande, 1994; Chaudhary, 2002; Tang *et al*., 2007).

Figure 1.4 Rice stalk affected by sheath blight disease

1.2.2 *Bacillus* sp.

1.2.2.1 Characteristics of *Bacillus* sp.

The genus *Bacillus* have been classified in the kingdom Prokaryote, division Bacteria, order Cytophagaceae, family Bacillaceae and genus *Bacillus* (Sneath, 1986). *Bacillus* is a common soil microorganism that is often recovered from water, air and decomposing plant residues. It consists of a large number of diverse, rod-shaped, chemoheterotrophic, gram-positive bacteria. These cells are 0.5-2.5 μm wide and 1.2-10 μm long (Reva *et al*., 2004). Some species are strictly aerobic, others are facultative anaerobes or microaerophilic, but all are catalase positive. *Bacillus* species also produce oval or cylindrical endospores that are resistant to adverse environmental conditions and provide a selective advantage for survival and dissemination (Figure 1.5). This group of bacteria is quite diverse, with a range of mole % G+C content from 32 to 69% (Sneath, 1986). The genus *Bacillus* have proved to be particularly useful because of the ease of their mass cultivation, speed of growth, use of cheap substrates (which in many cases are wastes) and the diversity of potential products. Currently, there are 77 recognized species of the genus *Bacillus* (Table 1.1).

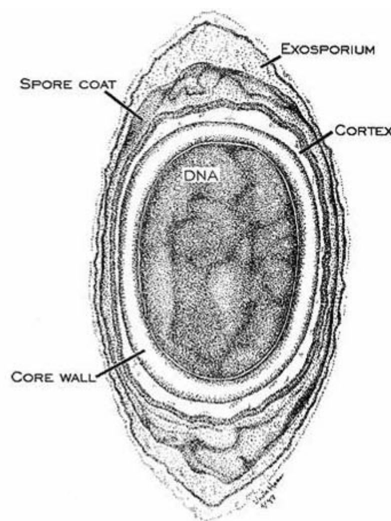

Figure 1.5 Endospore of *Bacillus* sp. (Sneath, 1986)

Table 1.1 Recognized *Bacillus* species, subspecies not included (modified from Sanders *et al*., 2003)

B. agaradhaerens	*B. alcalophilus*	*B. amyloliquefaciens*
B. anthracis	*B. atrophaeus*	*B. azotoformans*
B. badius	*B. benzoevorans*	*B. carboniphilus*
B. cereus	*B. chitinolyticus*	*B. circulans*
B. clarkii	*B. clausii*	*B. coagulans*
B. cohnii	*B. edaphicus*	*B. ehimensis*
B. fastidiosus	*B. firmus*	*B. flexus*
B. fumarioli	*B. fusiformis*	*B. gibsonii*
B. globisporus	*B. halmapalus*	*B. haloalkaliphilus*
B. halodenitrificans	*B. halodurans*	*B. halophilus*
B. horikoshii	*B. horti*	*B. infernos*
B. insolitus	*B. kaustophilus*	*B. laevolacticus*
B. lentus	*B. licheniformis*	*B. marinus*
B. megaterium	*B. methanolicus*	*B. mojavensis*
B. mucilaginosus	*B. mycoides*	*B. naganoensis*
B. niacini	*B. oleronius*	*B. pallidus*
B. pasteurii	*B. pseudalcaliphilus*	*B. pseudofirmus*
B. pseudomycoides	*B. psychrophilus*	*B. psychrosaccharolyticus*
B. pumilus	*B. schlegelii*	*B. silvestris*
B. simplex	*B. siralis*	*B. smithii*
B. sphaericus	*B. sporothermodurans*	*B. stearothermophilus*
B. subtilis	*B. thermoamylovorans*	*B. thermocatenulatus*
B. thermocloaceae	*B. thermodenitrificans*	*B. thermoglucosidasius*
B. thermoleovorans	*B. thermosphaericus*	*B. thuringiensis*
B. tusciae	*B. vallismortis*	*B. vedderi*
B. vulcani	*B. weihenstephanensis*	

The species *B. megaterium* have been classified in the kingdom Prokaryote, division Bacteria, order *Cytophagaceae*, family *Bacillaceae* and genus *Bacillus* (Cowan *et al*., 1974). It is a spore-forming bacterium with a broad habitat range such as soil, seawater, sediments, rice paddies, honey, dried food and milk (Vary, 1994).

B. megaterium is strictly aerobic, the gram-positive bacteria and a bacillus or a rod which is a bacterium with a cylindrical shape (Figure 1.6). Its cell wall contains polysaccharide. *B. megaterium* is about 1.3-2 μm in width and about 3-6 μm in diameter (Brock and Madigam, 1991; Prescott *et al.*, 1993). It uses lateral flagella for movement. It produces one endospore per one cell in 10 hours that are resistant to adverse environmental conditions, providing a selective advantage for survival and dissemination. The endospore contains the important enzymes such as endoproteinase and phospolipase (Brock and Madigam, 1991; Bertagnolli *et al*., 1996).

The highest culturing temperature is about 45-55 ^{0}C. The colony of this bacterium is yellowish white to brown but exhibit dark color when cultured in broth. Most of the antibiotics of this bacterium are produced in stationary phase and some antibiotics are produced in exponential phase (Prescott *et al.*, 1993).

B. megaterium is a non-pathogenic bacterium (Rourke, 2004; United Nations, 2005). It possesses some very useful and unusual enzymes and a high capacity for the production of exoenzymes (Table 1.2). The economic importance of *B. megaterium* includes its production of vitamin B_{12} and penicillin amidases, its ability to express foreign proteins without degradation, and its use in AIDS diagnostics (Vary, 1994). In addition, *B. megaterium* have been shown to act against plant pathogenic fungi such as onion disease caused by *Sclerotium cepivorum* (Wong and Hughes, 1986), soybean disease caused by *R. solani* (Liu and Sinclair, 1991; 1992; 1993) and rice diseases caused by *Fusarium roseum*, *Alternaria alternata* and *R. solani* (Islam and Nandi, 1985).

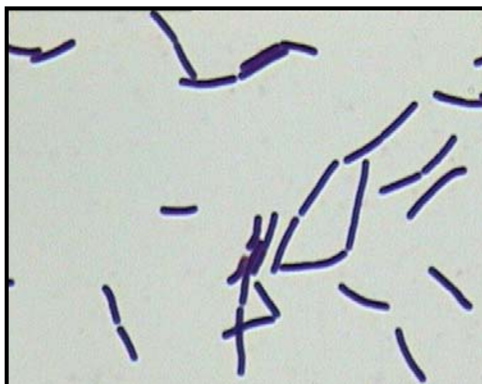

Figure 1.6 *B. megaterium*, gram-positive and a rod-shaped bacterium in chains

Table 1.2 Industrial products of *B. megaterium* and their applications (modified from Vary *et al*., 2007)

Products	Applications
α-Amylases	Can replace pullulanases
β-Amylases	Bread industry
Chitosanasen	Yeast cell wall lysis
Glucose dehydrogenase	Generator of NADH/NADPH, glucose blood test, biosensors
Neutral protease	Leather industry
Oxetanocin production	Inhibits HIV, hepatitis B virus, cytomegalovirus, herpes virus
Penicillin amidase	Construction of synthetic penicillins
Toxic waste cleanup	Herbicides, C-P bond lysis
Vitamin B_{12} production	Aerobic and anaerobic Vitamin B_{12} producer

1.2.2.2 Biological control by *Bacillus* sp.

The excessive use of chemical fungicides has caused environment pollution and harmfully effects on human beings (Athiel *et al*., 1995; Radice *et al*., 1998; Datta and Gopal, 1999; Radice *et al*., 2001; Ochiai *et al*., 2002; Calhelha *et al*., 2006; Çali, 2008). The application of biological control agents, particularly *B. megaterium*, is one alternative that has been proposed and evaluated in numerous pathosystems with varying degrees of success (Liu and Sinclair, 1992;

Bertagnolli *et al*., 1996; Zheng and Sinclair, 2000; Pengnoo *et al*., 2000; Chiou and Wu, 2001; Jock *et al*., 2002; Wiwattanapatapee *et al*., 2004; Jung and Kim, 2005).

Biological control was defined as "the use of natural or modified organisms, genes or genes products to reduce the effects of undesirable organisms (pests, diseases) and to favor desirable organisms such as crops, trees, animals, and beneficial insects and microorganisms" (NRC, 1987). While the definition of biocontrol suggested by Cook and Baker (1983) is based on "pest suppression with biotic agents, excluding the process of breeding for resistance to pests, sterility techniques and chemical modifying pest behavior".

Many different bacterial strains can be used as an effective biological control agent but *Bacillus* spp. may have received the most attention. This is because *Bacillus* spp., which is omnipresence in soils, possesses special characteristics such as its tolerance to high temperature, extreme pH and drought condition, and chemical and mechanical stress. This bacterium has rapid growth rate in liquid culture and is considered to be an effective and safe biological agent. Several strains of *Bacillus* sp. were effective for controlling various plant pathogens (Table 1.3). For instance, Sadfi *et al*., (2002) demonstrated that *B. licheniformis* and *B. cereus* reduced a dry rot disease of potato tubers caused by *F. roseum* var. *sambucinum*. An antagonistic strain of *B. subtilis* showed the efficacy to control black rot of brassicas (Wulff *et al*., 2002). Collins and Jacobsen (2003) used *B. subtilis* isolate for suppressing the sugar beet cercospora leaf spot. *Bacillus* sp. was shown to inhibit *Streptomyces scabiei* which caused common scab disease in potato, decreasing the infection from 75 to 35% (Han *et al*., 2005). Biological control of *Bacillus* sp. has been tested against *F. verticillioides* in the maize agroecosytem (Cavaglieri *et al*., 2005; Pereira *et al*., 2007). Examples of post-harvest control of Botrytis on fruit and vegetable, using antagonistic *Bacillus* sp. had also been demonstrated (Mari *et al*., 1996; Walker *et al*., 1998).

B. megaterium has also been reported to possess the capacity to control various diseases (Islam and Nandi, 1985; Liu and Sinclair, 1992; Bertagnolli *et al*., 1996; Pengnoo *et al*., 2000; Chiou and Wu, 2001; Jock *et al*., 2002; Wiwattanapatapee *et al*., 2004; Jung and Kim, 2005) (Table 1.3).

Table 1.3 Examples of the application of *Bacillus* sp. for plant disease control

Antagonists	Pathogens	Crops	References
B. subtilis	*R. solani*	Lettuce	Pusey, 1989
B. subtilis	*Phytophthora cactorum*	Apple	Utkhed and Smith, 1991
B. subtilis	*Fusarium moniliform*	Corn	Hebber *et al.,* 1992
B. subtilis	*F. oxysporum*	Cotton	Zhang *et al.,* 1996
B. subtilis	*R. solani*	Tomato	Asaka and Shoda, 1996
B. subtilis	*Pytium* sp., *P. ultimun*	Sunflower	Liang *et al.,* 1996
B. subtilis	*P. irregulare, P. ultimun*	Wheat	Kim *et al.,* 1997
B. subtilis	*R. solani*	Rice, Bean, Wheat, Soybean	Lazzaretti and Bettiol, 1997
B. subtilis	*R. solani*	Potato	Schmiedeknech *et al.,* 1998
B. cereus, B. pumilus	*R. solani*	Radish	Gosoni *et al*., 1998
B. subtilis	*R. solani*	Wheat	Ryder *et al.,* 1999
B. subtilis	*P. nicotianae*	Cucumber	Dileep-Kumar, 1999
B. megaterium	*R. solani*	Soybean	Zheng and Sinclair, 2000
B. megaterium, B. pumilus	*R. solani*	Rice	Pengnoo *et al.,* 2000
B. subtilis	*R. solani*	Rice	Arunyanart *et al.,* 2001
B. subtilis	*R. solani, F. oxysporum*	Kidney bean	Estevez de Jensen *et al*., 2002
B. amyloliquefaciens	*R. solani*	Vegetable	Yu *et al*., 2002
B. subtilis	*Monilinia vaccinii*	Blueberry	Ngugi *et al*., 2005
B. firmus	*R. solani*	Bambara groundnut	Pengnoo *et al.,* 2006

1.2.3 Production of antagonistic bacterial agent

A critical factor that must be considered when selecting a biological control agent for commercial development is the availability of a cost-effective production and stabilization technology that yields an optimally effective form of the antagonist. More studies on

the practical aspects of mass production and formulation need to be undertaken to make new biological control products stable, effective, safer and more cost effective (Fravel *et al*., 1999).

Agosin and Aguilera (1998) stated that the major characteristics to market a biofungicide are as followed: (1) the abundant and cost-effective production of microbial propagules, (2) the ability to survive downstream process, (3) the stability and adequate shelf life of the final product upon storage, preferably without refrigeration, (4) the tolerance to environmental variations in temperature, desiccation, irradiation and relative humidity in order to survive and establish active populations in the soil and (5) the consistent efficacy under the field conditions at commercially feasible rates.

One of the most important reasons for the limited commercial application of biofungicides is the high cost of production, due to the high cost of substrate, low biomass productivity, and limited economies of scale (Rhodes, 1996). The practical efficacy of a biological control agent greatly depends on the quality of the inoculants, itself a function of the production and formulation processes (Whipps, 1997).

Liquid and solid fermentation are commonly used for producing inoculums of biological control agents.

Liquid fermentation involves the inoculation of the microorganisms onto liquid medium in the flask and 1-30 l fermentors (in case of laboratory scale) or in the 100-1,000 l fermentors (in case of commercial scale). Large scale production of bacteria using liquid fermentation technology has been well developed and used in food and beverage industries such as starch, bread, cheese, beer and wine (Demain, 1981; Pandey *et al*., 2000; Wait *et al*., 2001; Nieto-Arribas *et al*., 2009). For instance, liquid fermentation technology has been used to produce drinking beverage containing *Lactobacillus* sp. (Nsofor *et al*., 1996; Daly and Davis, 1998; Crittenden *et al*., 2003). This may not be the case for the production of bacterial biological control agents as the demand of the product in Thailand does not justify the investment for large scale production. Research regarding the production of bacterial biological control agents is thus confined in the laboratory in which an effective bacterium is cultured in various substrates in flask and small fermentors (Besson, 1987; Sandrin *et al*., 1990; Oyama and Kubota, 1993; Leifert *et al*., 1995; Makkar and Cameotra, 1997).

In liquid fermentation, specific parameters for aeration, temperature and pH control, carbon and nitrogen sources must be developed for each organism. Supplementation of additional carbon sources showed no further enhancement in enzyme production while supplementation of nitrogen sources such as peptone and tryptone in the fermentation medium showed a marked increase in production (Sandhya *et al*., 2004). Many biological control agents are easily produced in liquid culture in lab scale, but when produced in large scale, they do not produce the expected quantity or quality of propagules, essentially for the low oxygen availability in fermentors (Phae and Shoda, 1991; Slininger *et al*., 1996; Sen and Swaminathan, 1997).

Two main obstacles for producing the biological control agents using liquid fermentation are the separation of the biomass from the culture media and the foam forming during cultivation. In the separation process, it is necessary to reduce the volume of liquid or obtain a final dry formulation. Before drying, the microorganism propagules are separated by filtration or centrifugation. Drying can also be accomplished by freeze drying, atomization, or bed-fluid drying, preserving the inoculums for a long time with high viability (Beudeker *et al*., 1989).

Foaming in a fermentor is generally cumbersome and the use of an antifoam agent is in most cases unavoidable. The feature of this method is closely related to the chemical nature of the product, which contains both hydrophobic and hydrophilic parts in its structure. The advantage of this method also lies in the minimal use of an antifoam agent, which is sometimes causes physiological and economical disadvantage in fermentation. The sample obtained in this procedure was used for a plant test and the effectiveness of it was confirmed (Asaka and Shoda, 1996). In the study with *B. subtilis*, Phae and Shoda (1991) found that lipopeptide antibiotics were detected only in the foam formed during cultivation. This indicates that continuous separation and condensation of the product is possible only by collecting the foam under properly controlled foaming conditions.

Solid fermentation using solid organic substrates is one possibility for producing the biomass of biological control agents.

Solid fermentation has the following features. (1) Traditional oriental foods are produced by solid fermentation (e.g. enzymes, organic acids, koji-making, etc.), (2) Most of the microorganisms used are fungi but some bacteria are successfully cultivated with this technology,

(3) Solid substrates require only addition of water or other nutrients, (4) Vessels in solid fermentation are small relative to the product yield, (5) Low moisture content reduces the problem of contamination, (6) Extraction processes are simpler, (7) Amount of solvents used for extraction is much smaller, (8) Spore inoculation does not need a seed tank, (9) Products can be used for direct applications such as animal feed and fertilizer etc.

The separation procedure of the solid material to yield antibiotic is much simpler in solid fermentation than in liquid fermentation, and smaller amounts of solvent are needed in solid fermentation. This is mainly because the water content of solid material is much lower than that of the liquid medium, and because the accumulated antibiotic concentration in solid fermentation is higher than that in liquid fermentation. In these respects, solid fermentation is more promising for the mass production of *B. subtilis* as a biological control agent. Table 1.4 outlined the advantage of culturing *B. subtilis* with solid fermentation in the production of iturin A. As iturin A has five homologues, the production of fractions with a longer side chain which show stronger antibiotic activity was greater in solid fermentation, indicating that solid fermentation has an advantage over liquid fermentation at least in regard to this point (Ohno *et al*., 1993).

Table 1.4 Comparison of the antibiotic, iturin A production between in solid fermentation and in liquid fermentation (Ohno *et al*., 1993)

Parameter	Solid fermentation	Liquid fermentation
Scale	3 (kg)	5 (l)
Cultivation time (day)	2	5
Iturin productivity (mg/g wet culture/day)	0.68	0.038
Total iturin (mg) per container	5,500	900
Culture volume (l) per 1 g iturin	1.5	7.2

1.2.4 Formulations of *Bacillus* sp.

A formulated microbial product is defined as a product composed of biomass of a biological control agent and ingredients to improve the survival and effectiveness of the product. Formulations of microbial biomass can be liquid or dry formulations.

1.2.4.1 Liquid formulations

Liquid formulation products are known as flowable or aqueous suspensions consisting of biomass suspensions in water, oils, or combinations of both (emulsions).

In general, oil suspension formulation was better than dormant aqueous suspensions in that organisms suspended in oil with low moisture were less prone to premature re-growth until reactivation with moisture, and thus the products were less likely to be overgrown by contaminant during storage and transport (Paau, 1998). Alternatively, the organisms can be suspended in oil at high concentration in various degrees of dehydration and remain viable (Johnston, 1962). This formulation delivers organisms in a physiologically dormant state and does not encourage the growth of contaminants during storage or transport.

Emulsion was considered to be good for the survival and performance of biological control agent. Chiou and Wu (2003) demonstrated that *B. amyloliquefaciens* B190 could survive in their designed emulsion for 9 months at least. The survival of *B. amyloliquefaciens* B190 was longer in distilled water than in emulsion. Tween 80 was an adjuvant that could improve *B. amyloliquefaciens* B190 to distribute evenly on surface of plant. Whereas mineral oil was able to reduce the lethal effect on *B. amyloliquefaciens* B190 cause by irradiation.

The main advantage of water-in-oil (W/O) emulsion over granular formulations is that the oil traps water around the organism and slows water evaporation once applied. This is particularly beneficial for organisms that are sensitive to desiccation. Some examples of successful application of W/O emulsions for formulation of microorganisms for improved delivery include formulation of the mycoherbicides *Alternaria cassiae*, *A. crassa* and *Colletotrichum truncatum* (Quimby *et al*., 1989; Amsellem *et al*., 1990; Boyette, 1994; Egley and Boyette, 1995) and formulation of *Fusarium lateritium* for control of Eutypa dieback in grapevine (Ho *et al*., 2005). A W/O emulsion of *Ascochyta pteridis* was produced using soybean and

paraffin oils, and paraffin wax (Womack and Burge, 1993; Womack *et al*., 1996). This emulsion was stable and displayed low fungi toxicity.

1.2.4.2 Solid formulations

Solid formulations are generally preferred over liquid formulations because they provide extended shelf life and ease in transportation and storage. Solid formulations can also be made into liquid-or water-based suspension as required for drench, spray or root-dip applications (Lumsden *et al*., 1995). Solid formulation products include wettable powders, dusts, granules, pellets, beads and microcapsules. Organisms can be formulated into dry concentrate or wet powders for easy storage, transport and application. Depending on their component ingredients, these powders can be applied to the soil. They can be done by (1) applying directly to soil with no further manipulations; (2) suspending in water or other carriers for spray applications; or (3) dusting onto seeds to deliver the organisms. The two general types of powders are easily distinguished by their moisture content (Paau, 1998).

1.2.4.2.1 Wettable powders and dusts

Wettable powders consist of dry inactive and active ingredients (biomass) intended to be applied as a suspension in liquid. Wettable powder was easy for storage and application. Chiou and Wu (2003) produced wettable powder of biofungicide containing *B. amyloliquefaciens* B190 for control lily gray mould. In this study, calcium hydroxide and sodium carbonate were compatible with *B. amyloliquefaciens* B190, they had ability to reduce the disease severity. Moreover, Lee *et al*. (2006) have reported that *B. licheniformis* N1 as wettable powder formulation was effective against tomato gray mold in addition to grow promotion activity on tomato plants. Wettable powder of *B. licheniformis* N1 were generated and evaluated for the activity to control strawberry gray mold disease caused by *Botrytis cinerea* (Kim *et al*., 2007). This formulation contained corn starch, olive oil, sucrose and bacterial fermentation culture. The disease control value of formulation on strawberry leaves was 81% under production conditions, as compared with the 61.5% conferred by a chemical fungicide, Iprodione.

Dusts are powder-like and consist of dry inactive and active ingredients to be applied dry, generally to seeds or foliage. Powder formulations containing microorganism to

control rice sheath blight disease were prepared (Vidhyasekaran and Muthamilan, 1995; Nandakumar *et al.*, 2001 and Radja Commare *et al.*, 2002). Nandakumar *et al*. (2001) reported that the *Pseudomonas* application as a talc-based formulation through seed, root, soil and foliar application either alone or in combination (seed+root+soil+foliar) effectively reduced sheath blight disease incidence, promoted plant growth and ultimately increased yields under greenhouse or field conditions. Radja Commare *et al.* (2002) reported that the talc-based formulation of *P. fluorescens* and its mixture (with and without chitin) were tested against sheath blight in rice. The application of talc-formulation through seed, root, soil and foliar spray significantly reduced the sheath blight both under greenhouse and field conditions. A talc-powder formulation of *B. firmus* as seed treatment was developed to combat leaf blight disease of bambara groundnut. Nevertheless, this formulation did not cause abnormality of seedling shape and had no effect on the germination of bambara groundnut seeds (Pengnoo *et al*., 2006).

In addition, experimental dust formulations of *Bacillu*s sp. that have effectively reduced plant disease have included clays (Osburn *et al*., 1995); peat and chitin (Manjula and Podile, 2001; Sid Ahmed *et al*., 2003); methylcellulose (Racke and Sikora, 1992); Ca-alginate, alginate manucol (Schmidt *et al*., 2001); carboxymethylcellulose, vegetable oil and polyvinyl pyrrolidone (Kanjanamaneesathian *et al*., 2000) and peptone (Ferreira *et al*., 1991; Schmidt *et al*., 2001); nutrient medium (Smith *et al*., 1993) and glucose, talc powders and peat (El-Hassan and Gowen, 2006).

1.2.4.2.2 Granules and pellets

Granules can be described as a free flowing, aggregated product composed of dry inactive and active ingredients. Granular formulations have advantages of reduced transportation costs and easy, well-understood application. They can be applied directly to the target plant, in furrow, or in the case of water dispersible granules, mixed into water where the suspension of biomass and inactive ingredients are applied to targets as a spray (Schisler *et al*., 2004). Granule product is an inert carrier containing the organisms. Carrier substances are clay minerals, starch polymers, dry fertilizers and ground plant residues (Ross and Lembi, 1985). Choice of carrier is dependent on absorption (more important for formulating slurries of organisms), hardness, bulk density and product disintegration rate in water (Polon, 1973). The

product can be coated with various materials to control the rate of release. However, the rate of microbial release is also dependent on the size of the particle.

Typically there are three types of granules: (I) the organisms are attached to the outer surface of a granular carrier in a rotating drum by a sticker; (II) the organisms are sprayed onto a rotating granular carrier without a sticker; (III) the organisms are incorporated into a carrier paste or powder which sets as a matrix, size being controlled by passing the product through a sieve. Type (III) is the most common formulation which has been used to formulate nitrifying microorganisms. The formulation is called a capsule when the carrier forms a protective coat around a core aggregate of microorganisms (Jones and Burges, 1998). Granules are generally easy to handle and apply, and are less dusty than powders. They are, however, more bulky and have higher material, storage and transport cost. Both dry and moist granules are suitable for broadcast and in furrow applications. Some granules can be fabricated in such a way that they will disintegrate the instant they reach sufficient moisture. Such granules, called water dispersible granules, are suitable for spray applications (Paau, 1998).

Schmiedeknecht *et al*. (1998) have produced *B. subtilis* water dispersible granules to control potato disease. While, Marten *et al*. (1999) have found that *B. subtilis* B2g as granules, spore suspension or seed treatment showed promising result in suppression of fungal disease of sunflower, cabbage and cucumber. Antagonist *B. megaterium* had been used in formulation study as floating granules (Kanjanamaneesathian *et al*., 1998; Kusonwiriyawong *et al*., 1999; Pengnoo *et al*., 2000) and its efficacy to suppress sheath blight disease of the product had been tested in both greenhouse and field conditions. Although these formulations demonstrated the desired characteristics and provided quite satisfactory protection for rice plants from *R. solani* infection in both tests, they had a comparatively short shelf life, and the numbers of bacterial antagonists in the formulations greatly declined during storage. In addition, the effective dose and frequency of application of these formulations in controlling sheath blight disease has not been investigated. This undesirable characteristic of the formulation makes it unsuitable for large scale production and commercialization.

Physically, pellets are bigger than powder and granules and they have spherical shape. Like powders and granules, these products contain an inert carrier holding the organisms (Ross and Lembi, 1985). Floating pellets containing endospores of bacterial biological control

agent, *B. megaterium*, were firstly prepared using extrusion-spheronization process (Wiwattanapatapee *et al*., 2004). The formulations composed of hydrogenated vegetable oil, lactose, microcrystalline cellulose, and cross-linked sodium carboxyl methylcellulose. After production, they also contained a high level of *B. megaterium* after storage for 6 months at room temperature. (Wiwattanapatapee *et al*., 2004). Due to the hydrophobic property of these formulations, the bacteria were slowly liberated into the environment. However, when the sheath blight fungal attacks and the infection developed rapidly, the slow-released formulation may not be effective in controlling the disease. Thus, more researches are needed to find ingredients and type of formulations which have better physical characteristics to ensure that the bacteria in the formulations can be dispersed effectively when applied to rice plants in field conditions.

1.2.4.2.3 Microcapsules and gel beads

Preparation of talcum powder or starch granules involves simple technology and is more time and cost effective than the production of alginate beads (Green *et al.,* 1998; Lumsden *et al.,* 1995). Although alginate beads have the disadvantage of yielding a bacterial biological control agent with a shorter shelf life and reduced efficacy for inhibiting mycelial growth of *Rhizoctonia in vitro* than the granular or powder formulation, they are more uniform in size and more suitable for use as an amendment of soil or potting medium during mechanical seeding of greenhouse trays where delivery of consistent dosage of biological control agent is important. In addition, the polymer gel beads are able to protect bacterial antagonist from the environments.

Entrapment of biocontrol organisms in calcium alginate gel bead has been used widely (Connick, 1988). Alginates are biopolymers that are stable when dry (Mugnier and Jung, 1985). Most commercial alginates are derived from kelp but other organisms can also produce alginates. Alginates produced by *Azotobacter vinelandii* can substitute the one that produced by kelp in the preparation of biocontrol products for plant disease control (DeLucca *et al.*, 1990). However, further developments have reduced the cost of producing alginate substantially. Practically, alginate production is carried out by suspending propagules of the biocontrol agent in 1-5% sodium alginate and 10-20% bulking agent (Fravel *et al.*, 1985; Lewis and Papavizas,

1985). The suspension is added drop wise into a gellant, usually 0.25 M $CaCl_2$ (calcium chloride) or 0.1 M $CaC_{12}H_{22}O_{14}$ (calcium gluconate) to form the gel beads.

The term microencapsulation is also associated with formulation of microbial biomass and refers to surrounding small amounts of active ingredient with a protective inert layer. The layer can consist of any number of polymers and the encapsulation itself is generally achieved either chemically (Winder *et al*., 2003) or by air-drying (Côté *et al*., 2001) or spray-drying (Tamez-Guerra *et al*., 2000) liquid suspensions containing active and microencapsulating ingredients.

Elçin (1995) prepared *B. sphaericus* 2362 calcium alginate microcapsule for use in mosquito control. The effects of acidic pH and spore loading on the larvicidal activity of free and encapsulated *B. sphaericus* 2362 were tested against *Culex* sp. mosquito larvae by bioassays performed in the laboratory. Results showed that encapsulation was also effective on *B. sphaeric*us 2362 spore stability. Although ultraviolet (UV) light reduced the viability of *B. sphaericus* 2362 spores in shorter times, *B. sphaericus* 2362 calcium alginate microcapsules were more resistant to continuous UV exposure, increased persistence against high temperature and several chemical materials.

Moreover, carrageenan microcapsules of the mosquito pathogen *B. sphaericus* 2362 were prepared and tested for larvicidal activity against *Culex* sp. larvae in the laboratory (Elçin and Oktemer, 1995). In the present study, the toxicity of the bacterium was stable after encapsulation procedure, showing that the applied technique did not adversely affect toxin activity. This activity against *Culex* sp. could still be observed after a period of 70 days at pH 3 and 4, although no viable spores remained. This finding confirms that the active toxin can be released from the microcapsules even in the absence of viable spores, since it is evident that the microcapsules were washed thoroughly with sterile saline before bioassays. The advantages of this method are unaffected larvicidal activity, an important increase in spore stability, easy preparation and low cost of the biodegradable matrix.

Zayed (1997) reported that alginate beads-immobilized cells of *B. megaterium* exhibited much higher efficiency in increasing the availability of phosphorus than free cells. This suggests that alginate protected *B. megaterium* cells against their specific bacteriophages,

completely inhibited the phosphate-dissolving activity of bacteria in pure liquid culture and markedly decreased their number in rhizosphere of maize plants.

For the commercialization of biological control products, the major difficulty is to reach the market and to be competitive with the chemical fungicides. This difficulty can be overcome by devising the product which a consistent efficacy and having reasonable shelf life. Recently, commercial formulations of *Bacillu*s-based biological control products have already existed in the form of flowable or aqueous suspensions and wettable powders (Table 1.5).

Some *Bacillus* sp. formulations have been produced to control plant diseases. However, there are very few reports concerning the use of *B. megaterium* commercially. The development of *B. megaterium* as bacterial antagonist formulation commercially should thus be investigated to control plant diseases.

Table 1.5 Commercial *Bacillus* sp. based biological control products (modified from Schisler *et al*., 2004)

Bacterial strain	Primary target	Formulation type*	Product name
B. subtilis QST 713	Fungi and bacteria on vegetables and fruit	WP	Serenade
B. subtilis B246	Fungi on avocado	liquid	Avogreen
B. licheniformis SB3086	Fungi on turf	Flowable	Ecoguard
B. subtilis GB03	Fungi on cotton and soybeans	WP (Conc.), flowable	Kodiak
B. pumilis GB34	Fungi on soybeans	WP (Conc.)	Yield Shield
B. amyloliquefasciens and *B. subtilis* GB122	Fungi on bedding plants	Dry flake	Bio Yield
B. subtilis MBI600	Fungi on cotton and soybeans	WP (Conc.)	Subtilex
B. subtilis MBI600 and *Rhizobium*	Fungi on soybeans	Flowable	Hi Stick
B. subtilis GB03, *B. lichenformis, B. megaterium*	Fungi on oranamental and turf	Flowable	Companion
B. pumilus QST2808	Asian soybean rust	Flowable	Ballad
B. pumilus	Fungi on cucurbit, grape and vegetables	WP	Sonata
B. subtilis FZB24	Fungi on potato, corn, vegetable and ornamentals	Flowable	Rhizo-Plus
B. subtilis	Fungi on durian and rice	WP	Larminar
B. subtilis	Fungi on durian and rice	WP	Rotor

* WP (Conc.) = wettable powder concentrate

1.2.5 Mechanisms of biological control by *Bacillus* sp.

The use of chemical compounds has failed to control plant diseases due to resistance, environment pollution and damage to human health. Because of these disadvantages, the use of microorganisms for pathogen control and for plant growth promotion is becoming more common. However, the success of biocontrol and yield increase depends on the nature of the antagonistic properties and on the mechanisms of action of the organism.

The modes of action of the biological control agent by *Bacillus* sp. can be categorized as follows:

1.2.5.1 Competition

Competition occurs between micro-organisms when space or nutrients (i.e. carbon, nitrogen and iron) are limiting, and its role in the biological control of plant pathogens has been studied for many years, with special emphasis on bacterial biological control agents (Handelsman and Stabb, 1996). An important attribute of a successful rhizosphere biological control agent would be the ability to remain at high population density on the root surface, providing protection of the whole root for the duration of its life. Colonization of plant roots or rhizosphere by bacteria has been widely studied, but very few studies have been done with *B. megaterium.*

Liu and Sinclair, (1991; 1992; 1993) demonstrated that *B. megaterium* strain B153-2-2 populations were established and survived after introduction into the soil and suggested that B153-2-2 was competitive in soybean field soil.

1.2.5.2 Antibiosis

The mechanism of antibiosis has been shown to be one of the most important (Thomashow and Weller, 1996). Over a hundred antibiotics from the genus *Bacillus* have been identified and there is strong potential and scope for use of *Bacillus* sp. in biological control systems (Zuber *et al*., 1993; Klich *et al*., 1994; Cook *et al*., 1995). Members of the genus *Bacillus* produce a variety of antibacterial and antifungal peptide antibiotics such as iturin A, surfactin, kanosamine and zwittermycin A (Asaka and Shoda, 1996; Milner *et al*., 1996; Silo-Suh *et al*.,

1998; Yu *et al*., 2002; Raaijmakers *et al*., 2002; Romero *et al*., 2007). Many Bacilli are able to produce several different antibiotics that have a broad range and sometimes overlap in their function (Leifert *et al*, 1995; Raaijmakers *et al*, 2002; Yu *et al*, 2002; Risøen *et al*, 2004). The antibiotics from *Bacillus* sp. are nearly always peptide in nature and generally of low molecular weight. In several cases D-rater than L-amino acids are present and the structure can be cyclic or have a cyclic component (Katz and Demain, 1977).

Antibiotic production by strains of *Bacillus* has been shown to be important to successful biological control of several crop diseases. For example, the antifungal antibiotic iturin A from *B. subtilis* FR-2 have been to use as biological control agent of cucumber fungal pathogen and rice blast and sheath blight (Pusey, 1989). The antibiotic kanosamine (Milner *et al*., 1996) and zwittermicin (Silo-Suh *et al*., 1998) produced by the biological control agent *B. cereus* UW85, appears to be important in biological control of *Phytophthora* root rot of alfalfa. *B. subtilis* RB14, which showed antibiotic activities against several phytopathatogens in vitro by producing the antibiotics iturin A and surfactin, was subjected to a pot test to investigate its ability to suppress damping-off of tomato seedlings caused by *R. solani* (Asaka and Shoda, 1996). Antifungal antibiotic from *B. firmus* TRV 9-5-2 that are resistant to high temperature (121 ^{0}C for 20 min) in suppressing leaf blight disease of bambara groundnut (*Vigna subterranea*) (Pengnoo *et al*., 2006). This may be the first study which demonstrates that antibiotics from *B. megaterium* are responsible for suppressing rice sheath blight disease (Kanjanamaneesathian *et al*., 1998; Pengnoo *et al*., 2000).

Many *Bacillus* spp. including strains of *B. megaterium*, are capable of producing a range of different enzymes, antibiotics and toxins, many of which can be antagonistic to plant pathogens (El-Banna, 2005; Chakraborty *et al*., 2006). The biocontrol ability of *B. subtilis* has been extensively researched with a number of lipopeptide antibiotics identified as contributing to the antifungal capabilities of the bacterium (Ongena *et al*., 2004, 2007; Romero *et al*., 2007).

The role of extracellular enzymes in pathogenesis as well as in the biocontrol of plant pathogens has received increase attention. Extracellular enzyme synthesis in *Bacillus* spp. has been the subject of extensive research with the identification of more than 40 enzymes (Mackay *et al*., 1986; Priest, 1977; Simonen and Palva, 1993). Leelasuphakul *et al*. (2006) have reported that *B. subtilis* NSRS 89-24 contain an extracellular enzyme with β-1, 3-glucanase

activity and an antibiotic both of which inhibit the growth of two rice pathogens *Pyricularia grisea* and *R. solani*. Bertagnolli *et al*. (1996) showed that the levels of 11 extracellular enzyme activities found in *B. megaterium* B153-2-2 culture filtrates (Table 1.6). Moreover, Priest (1977) was reviewed the extracellular enzyme synthesis in the genus *Bacillus* which have showed that *B. megaterium* secreted the carbohydrases, proteases, penicillinases and nucleases and phosphatases (Table 1.7).

Table 1.6 The level of extracellular enzyme activities in cell-free culture filtrates of *B. megaterium* B153-2-2 (Bertagnolli *et al*., 1996)

Extracellular enzyme assayed	Level of extracellular enzyme activities
DNase	4
RNase	5
Lipase	9
α-amylase	4
Cellulase	1
Chitinase	1
Pectinase	2
Pectin lyase	8
β-Glucanase	4
Protease	8
Urease	5

Table 1.7 Extracellular enzymes of *B. megaterium* (Priest, 1977)

Enzyme	Comments
1. Carbohydrases	
β-amylase	Exohydrolysis of the α-1,4-glucosidic linkages in polysaccharides yielding β-maltose
cyclodextrin glucanotransferase	Synthesizes cyclo-(Schardinger) dextrins from starch
dextranase	Cell-bound enzymes catalyzing the exohydrolysis of dextran to glucose
2. Proteases	
metal protease	Enzymes require Ca^{2+} for stability and Zn^{2+} for activity; pH optimum at or near neutrality
3. Penicillinases	
β-lactamase	Hydrolysis of the amide bond in the β-lactam ring of penicillins and cephalosporins
penicillin amides	Hydrolysis of the peptide linkage of penicillin
4. Nucleases and phosphatases	
5-nucleotidase	Cell-bound enzyme in these species

Kildea *et al*. (2008) report that *B. megaterium* strain MKB135 is facilitated by multiple modes of action which did significantly reduce septoria tritici blotch (STB) of wheat seedling and adult plants under outdoor conditions, both in 2004 and 2006. The fact that the culture filtrate of *B. megaterium* strain MKB135 did reduce STB on wheat seedlings, albeit to a lesser extent than both the bacterial and cell wall treatments may indicate the presence of such antifungal metabolites. Bertagnolli *et al*. (1996) identified *B. megaterium* endoproteinases capable of reducing *R. solani* growth in vitro. More recently, Jung and Kim (2005) identified a *B. megaterium* antibiotic (KL39) with antifungal properties against *Phytophthora capsici* Leonian in *in vitro* assays and greenhouse experiments. Under controlled environmental conditions, a cell wall fraction from *B. megaterium* strain MKB135 was as efficient at reducing STB disease seedlings as were the whole bacterial cells. Peptidoglycan and teichoic acid are the two major

components of *B. megaterium* cell wall; other components include proteins and lipoteichoic acid (Ivatt and Gilvarg, 1978; Giles and Reynolds, 1979; Johnstone *et al*., 1982). The relative importance of cell wall constituents in controlling STB disease remains to be determined.

1.2.5.3 Plant growth promoting rhizobacteria (PGPR)

Plant growth promoting rhizobacteria (PGPR) can increase plant growth and vitality through production of phytohormones like auxins, gibberellins, abscisic acid, ethylene and cytokinins (Varma *et al*., 2004). These hormones are involved in many aspects of plant life such as root elongation, cell elongation and proliferation (Varma *et al*., 2004).

Lopez-Bucio *et al*. (2007) have identified a *B. megaterium* strain that promoted growth and development of bean (*Phaseolus vulgaris*) and *Arabidopsis thaliana* plants. The results have shown that plant-growth promotion and root-architectural alterations by *B. megaterim* involve auxin and ethylene independent mechanisms.

Rajkumar and Freitas (2008) reported that inoculation of *B. megaterium* Bm4C on the Indian mustard (*Brassica juncea*) promoted plant growth and protected the plant from nickel (Ni) toxicity. The increase in plant growth by Bm4C may be attributed to the maximum production of IAA and solubilisation of phosphate.

Increased growth, nutrient uptake and yield parameters of chickpea (*Cicer aritenium* L.) were demonstrated when phosphate solubilizing *B. megaterium* was inoculated along with the biological control fungus *Trichoderma* spp. and *Rhizobium* (Rudresh *et al*., 2005).

B. megaterium plays are important role in growing plant with available forms of phosphorus producing organic acids and CO_2 which increase the soil acidity and consequently convert the insoluble forms of phosphorus into soluble ones (Alexander, 1977; Saber *et al*., 1977). The use of *B. megaterium* as a biofertilizer in the alkaline soil increases the availability of soil phosphorus (Abdel-Ati and Hammad, 1996). In addition, *B. megaterium* can also improve root development and increase the rate of water and mineral uptake (Alexander, 1977).

In addition, *Bacillus* sp. not only protected the plants and reduced the disease but also, in some cases, increased the yield of plants. These results are in agreement with studies by Ghonim (1999) and Roberti and Selmi (1999), both of which demonstrated that seed treatments with *B. subtilis* were significantly more suppressive of *Fusarium* wilt of tomato caused

by *F. oxysporum* f. sp. lycopersici. Utkhede *et al*. (1999) demonstrated that the strain BACT-O of *B. subtilis* has the potential to increase the growth and yield of cucumber plants inoculated with *Pythium aphanidermatum* in soil under glasshouse conditions.

Moreover, some strains of *Bacillus* sp. suppressed the effectiveness of antagonists in plant disease control and also enhanced plant growth promotion (Utkhede and Smith, 1992; Asaka and Shoda, 1996; Peterson *et al*., 1996; Srinivasan *et al*., 1997; Marten *et al*., 1999; Emmert and Handelsman, 1999; Bais *et al*., 2004; Pengnoo *et al*., 2006). Jetiyanon *et al*. (2008) reported that the film coating of seeds with spores of *B. cereus* RS87 demonstrated early plant growth enhancement as well as seeds using their vegetative cells. IAA release from strain RS87 may be one of the mechanisms for plant growth enhancement.

1.2.5.4 Induce systemic resistance (ISR)

Induce systemic resistance (ISR) defined as "the process of active resistance dependent on the host plant's physical or chemical barriers, activated by biotic or antibiotic agent (inducing agent)" (Kloepper *et al*., 1992). Previous studies have shown that cell wall extracts of gram-negative were able to ISR within a range of plants (Van Peer and Schippers, 1992; Leeman *et al*., 1995; Duijff *et al*., 1997; Van Wees *et al*., 1997). Although we know of no gram-positive cell wall extracts initiating ISR, there are numerous examples of *Bacillus* spp. capable of triggering ISR on a range of different plants (Kloepper *et al*., 2004). Mechanisms of ISR resulting from treatment with *Bacillus* spp. include increased activity of pathogenesis-related proteins such as chitinase and β-1, 3-glucanase, enhanced hydrogen peroxide production and the elicitation of resistance associated with the salicylic pathway (Bargabus *et al*., 2003, 2004; Ryu *et al*., 2003, 2004; Zhang *et al*., 2002). The ability of *B. megaterium* to induce resistance to sheath blight disease has not previously been documented. However, Chakraborty *et al*. (2006) showed that *B. megaterium* strain TRS-4 can promote the growth of tea plants and induce resistance to brown root rot disease caused by *Fomes lamaoensis* (Murr.) Sacc. and Trott., and Lopez-Bucio *et al*. (2007) found that *B. megaterium* strain UMCV1 promoted growth and altered the root structure of Arabidopsis [*Arabidopsis thaliana* (L.) Heynh.] plants, through possibly novel unidentified mechanisms.

Ryu *et al*. (2003, 2004) and Farag *et al*. (2006) showed that volatiles released by *B. subtilis* strain GB03 and *B. amyloliquefasciens* strain IN937a (in particular 2, 3-butanediol) were able to promote plant growth and to induce systemic resistance in Arabidopsis against *Erwinia carotovara* subsp. *carotovora*. Antifungal properties of bacterial volatiles have also been demonstrated by Fernando *et al*. (2005) and Kai *et al*. (2007). Recently Vespermann *et al*. (2007) showed that bacteria volatiles have both the ability to suppress or enhance the growth of a range of fungi including important agricultural pathogens such as *Fusarium culmorum* Sacc., *R. solani* and *Phoma betae* Frank. Compounds including aldehydes, alcohols and sulphides were identified by Fernando *et al*. (2005) and Kai *et al*. (2007) among the volatiles and it may be these that have possible antifungal properties. Kildea *et al*. (2008), the design of the dual culture liquid assay meant that fungal growth inhibition observed in these experiments was due to antifungal volatiles released by *B. megaterium* MKB135. As the assay was based on the germination and subsequent growth of *Mycosphaerella graminicola* over 48 h, the volatiles may have inhibited or retarded germination.

CHAPTER 2

EVALUATION OF THE DURABILITY AND EFFECT OF CULTURE MEDIUM ON THE PRODUCTION OF *BACILLUS MEGATERIUM* ENDOSPORES

2.1 Introduction

B. megaterium is a gram positive, rod shaped endospore-forming bacteria. It is considered aerobic, but it is also capable of growing under anaerobic conditions when necessary. Endospores are highly resistant to environmental stresses such as high temperature (some endospores can be boiled for several hours and retain their viability), strong acids, disinfectants, UV irradiation, desiccation, and many toxic chemicals (Setlow, 1988, 1995; Setlow and Setlow, 1993). Although cryptobiotic, they retain viability indefinitely such that under appropriate environmental conditions, they germinate into vegetative cells. Endospores are formed by vegetative cells in response to environmental signals that indicate a limiting factor for vegetative growth, such as exhaustion of an essential nutrient. They germinate and become vegetative cells when the environmental stress is relieved.

B. megaterium produces endospore in 10 h after incubation. When nutrient has depleted, vegetative cell is separated and it produces the septum (4 h after incubation). The septum still grows and the immature endospore is inside (5.5 h after incubation). Then there are the cortex between the tissues, the protein covering cortex and the collection of calcium and dipicolinic acid (6.5 to 8 h after incubation). There is the mature endospore. The enzyme destroys sporangium to release endospore in the complete conditions (Prescott *et al.*, 1993)

For these reasons, the endospores of *B. megaterium* are the suitable inoculum which should be used for producing an effective formulation. With endospores as an active ingredient, the formulation may be expected to possess required features needed for commercializing the finishing products. Improved efficacy in comparison with the existing products and a comparatively longer shelf life are two most crucial characteristics. However, this

inoculum must be obtained from culturing the bacterium using cheap substrates without compromising the quality and quantity of the inoculum.

In term of the practical production of the bacterial inoculum, an agricultural produces, such as sweet potato (*Impomoea batatasil* (L.) Poir), cassava root (*Manihot esculenta* Crantz), rice, brown rice and sticky rice (*Oryza sativa* L.) and Job's tear (*Coix lachryma* jobi L.), should be utilized to replace potato (*Solanum taberosum*) as a substrate to culture *B. megaterium*.

This study is necessary because potato is comparatively expensive than other produces and may not be suitable for using as a substrate to produce bacterial antagonist for commercial purpose. In this investigation, both number of bacterial cells and size of endospore of this bacterium were assessed after culturing and 90 days after storing at 10 ^{0}C. After that, scale-up of bacterial endospores from shake flasks to 30 l fermentation was prepared using selected substrates as a completely minimal culture medium.

The aims of this chapter were (1) to evaluate the durability of *B. megaterium* endospores in persistence against UV irradiation, high temperature and pH and (2) to investigate agricultural produces which are suitable to use for producing endospores of *B. megaterium*.

2.2 Materials and Methods

2.2.1 Evaluation of the durability of *B. megaterium* endospores

2.2.1.1 Persistence of endospores against UV irradiation

Fresh cell and endospore suspension of *B. megaterium* (8.67 and 8.71 Log. number/ml, respectively) were transferred into sterile tubes and irradiated at room temperature with a 20-W General Electric UV lamp from 20 cm distance inside a UV cabinet for 48 h. Samples were taken in intervals (0, 3, 6, 12, 24, 36 and 48 h) and viable bacteria were counted on plate count agar (PCA) using drop plate technique (Zuberer, 1994).

The mycelial growth inhibition of the bacterium was tested using dual culture method. A 1% v/v solution of bacterial suspension was prepared in sterile distilled water. One ml solution was sampled and mixed with melted potato dextrose agar (PDA) at 1:10 (v/v) ratio in Petri dishes (9.0 cm diameter) then an agar plug of *R. solani* was placed at the center of the plate to determine its effect in inhibiting mycelial growth of *R. solani*. Treatment consisted of *R. solani* cultured on PDA incorporated with solution of bacterial suspension, while a culture of *R. solani* on PDA with sterile water incorporated was used as a control. Each treatment consisted of six replications. Mycelial inhibition of *R. solani* was assessed as % mycelial inhibition 36 h after culturing *R. solani* [Equation (2.1)] (Gamliel *et al*., 1989).

$$\% \text{ mycelial inhibition} = 100 - [(r^2/R^2) \times 100] \qquad (2.1)$$

In equation (2.1), r is colony radius of *R. solani* on PDA incorporated with 1% v/v solution of bacterial suspension and R is colony radius of *R. solani* on PDA incorporated with sterile water.

2.2.1.2 Persistence of endospores at high temperature

Fresh cell and endospore suspension of *B. megaterium* (11.57 and 11.60 Log. number/ml, respectively) were incubated in water bath at 80 ^{0}C for 48 h. Samples were taken in intervals (0, 3, 6, 12, 24, 36 and 48 h) and viable bacteria were counted using drop plate technique.

Percentage mycelial growth inhibition of these solutions was tested using dual culture method (Equation 2.1).

2.2.1.3 Persistence of endospores against pH

Fresh cell and endospore suspension of *B. megaterium* (7.66 and 7.59 Log. number/ml, respectively) were incubated in buffers of different pH (2-10) at room temperature (26-30 ^{0}C) for 15 days. Samples were taken in intervals (0.5, 1, 2, 7, and 15 days) and viable bacteria were counted using drop plate technique

2.2.2 Bacterium cultivation in shake flasks

2.2.2.1 Medium preparation for bacterium cultivation

Potato dextrose broth (PDB) was prepared using 200 g of potato, 20 g of dextrose and 1,000 ml of distilled water. Potato was boiled in 500 ml of distilled water. After this, the supernatant was obtained and 20 g of dextrose was added. Distilled water was added to this mixture until the volume was at 1,000 ml. This broth was autoclaved at 121 ^{0}C for 20 min and ready for use for bacterium cultivation when cooled. Broth prepared using other substrates was also made in a similar manner (except boiling time) but the same amount of sweet potato, cassava root, rice, brown rice, sticky rice or Job's tear were used to substitute potato (Figure 2.1). Time required for heating each substrate before the solid part was discarded and the broth is ready for cultivating the bacterium was also measured. Estimate cost of preparing broth media using these agricultural produces as nutrient sources was also calculated.

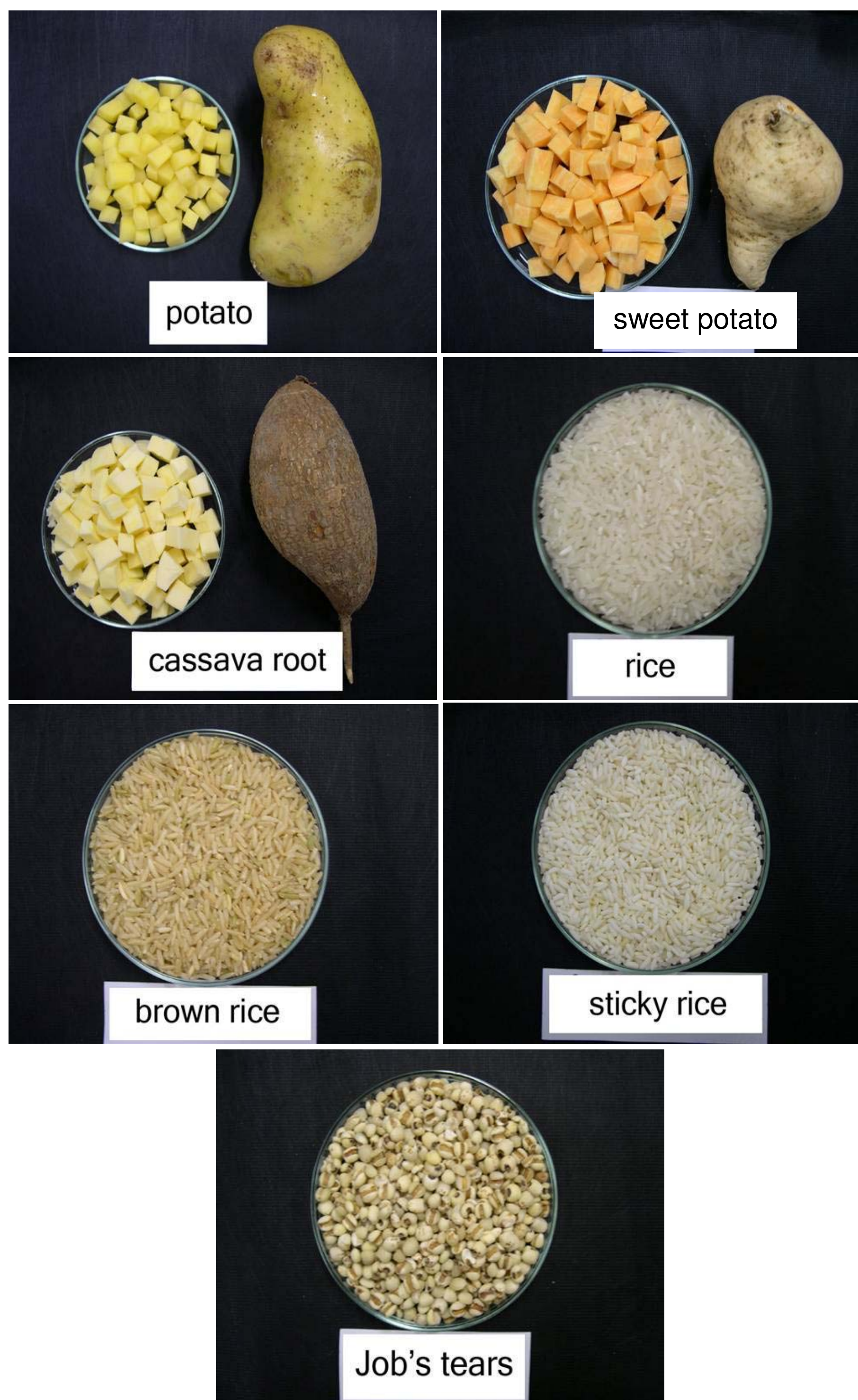

Figure 2.1 Agricultural produces as nutrient sources for bacterial endospores cultivation

2.2.2.2 Cultivation of bacterium in broth media

Single colony of *B. megaterium* cultured on PDA for 24 h was transferred to flasks containing 100 ml of each broth media prepared as described above. These flasks were incubated at 26-32 ^{0}C for 4 days on the rotary shaker at 150 rpm. After 4 days of incubation, bacterial cells were separated by centrifuged at 3,000 rpm for 10 min, washed 3 times and re-suspended in sterile distilled water (1:2 v/v). The cell suspensions were heated in water bath at 80 ^{0}C for 20 min to kill vegetative cells of the bacterium. The endospores suspended in the reduced volume of sterile distilled water were stored in the refrigerator for enumeration.

2.2.2.3 Enumeration of *B. megaterium* endospores after cultivation

Endospores of *B. megaterium* were enumerated after 4 days of cultivation and after 90 days storage at 10 ^{0}C. The enumeration was carried out on PCA using drop plate technique (Zuberer, 1994). The plates were incubated at 37 ^{0}C for 1 day after which colony-forming units were counted. The value of viable bacterium (Log. number/ml) was the average (±S.D.) of four replications per dilutions.

2.2.2.4 Measurement of *B. megaterium* endospores

Endospores of *B. megaterium* suspended in the broth media prepared from each substrate were mounted on slide. The measurement of bacterial endospores was carried out using ocular micrometer mounted stereo-zoom microscope. Both width and length (μm) of the bacterial endospore was assessed using 50 endospores taken from each broth media. Data of the width and length of the endospores were subjected to one way analysis of variance and compared with Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$ and $P \leq 0.01$using SAS computer package program.

2.2.3 Bacteria cultivation in 30 l fermentation

2.2.3.1 Medium preparation for bacteria cultivation

Four kg of potatoes were washed, peeled and diced. Potatoes were boiled in 10 l of distilled water until they were soft enough to be eaten but not overcooked. Then, the

supernatant was filtrated and mixed with 400 g of dextrose. Distilled water was added to this mixture until the volume was at 20 l. This broth was transferred to the fermentor (dimension 30 l) (Figure 2.2), autoclaved at 121 ^{0}C for 20 min and ready for use for bacteria cultivation when cooled. Broth prepared using other substrates was also made in a similar manner but the same amount of sweet potato and cassava root were used to substitute potato.

Figure 2.2 The fermentor (dimension 30 l)

2.2.3.2 Cultivation of bacteria in broth media

Two liters of *B. megaterium* suspension cultured on PDB for 24 h was transferred to the fermentor containing 20 l of each broth media prepared as described above. This fermentor was incubated at 30-35 ^{0}C, 200 rpm and 1 vvm for 7 days. The bacterial endospores obtained at day 1, 2, 3, 4, 5, 6 and 7 were incubated in water bath at 80 ^{0}C for 20 min to get rid of the vegetative cells. Then, endospores of *B. megaterium* were enumerated on plate count agar (PCA) using drop plate technique (Zuberer, 1994). The plates were incubated at 37 ^{0}C for 1 day after which colony-forming units were counted. The value of viable bacteria (Log. number/ml) was the average (±S.D.) of four replications per dilutions.

2.3 Results

2.3.1 Evaluation of the durability of *B. meaterium* endospores

2.3.1.1 Persistence of endospores against UV irradiation

The effect of UV irradiation on the resistance of *B. megaterium* in fresh cell and endospore suspension can be seen in Figure 2.3 and 2.4. The number of viable bacteria of fresh cells decreased to about 3 Log. number/ml after 12 h of UV exposure, and after 24 h of exposure no viable bacteria could be detected. Percentage mycelial inhibition of fresh cells was rapidly decreased after 3 h of UV exposure and after 6 h of exposure had not ability to inhibit mycelial growth of *R. solani*. The resistance of *B. megaterium* against UV is also increased by endospores. After 48 h of UV exposure, about 3 Log. number/ml of living endospores were detected. The bacterial endospores had ability to inhibit mycelial growth of *R. solani* although the tests were carried out after 36 h of UV exposure.

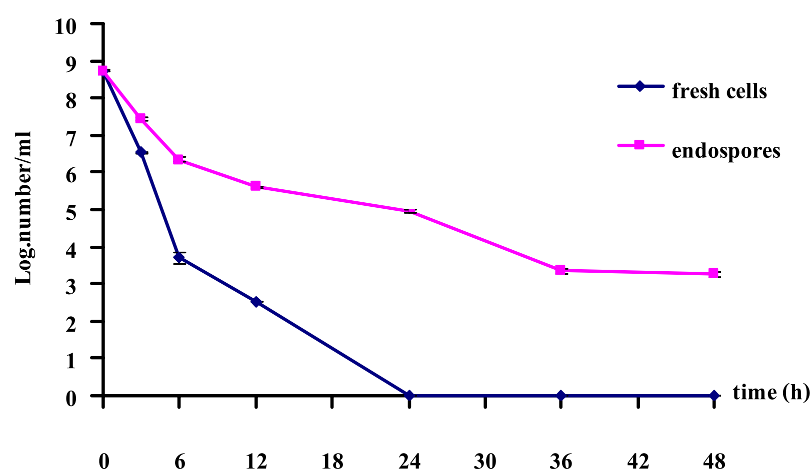

Figure 2.3 Effect of UV irradiation on stability of fresh cells and endospores of *B. megaterium*

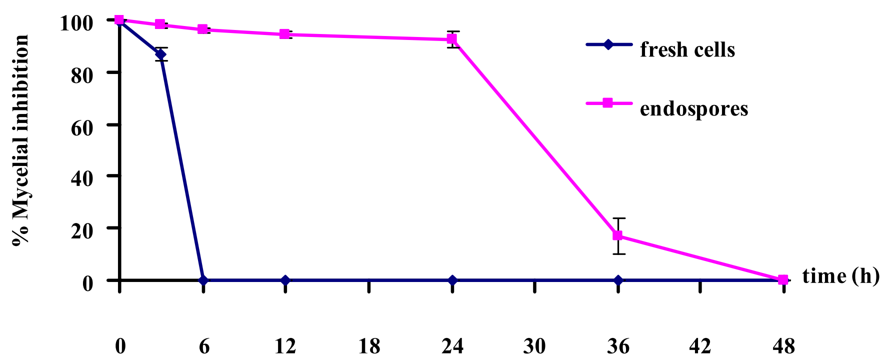

Figure 2.4 Percentage of mycelial inhibition of fresh cells and endospores after 48 h of UV exposure

2.3.1.2 Persistence of endospores at high temperature

In order to examine the viability and efficacy of the bacterium at high temperature, fresh cells and endospores were incubated at 80 ^{0}C. As can be seen from Figure 2.5 and 2.6, the number of viable bacteria of fresh cells decreased to about 3 Log. number/ml after 12 h of high temperature incubation, and after 24 h of incubation no viable bacteria could be detected. Percentage mycelial inhibition of fresh cells was rapidly decreased after 6 h of 80 ^{0}C incubation and after 24 h of incubation had not ability to inhibit mycelial growth of *R. solani*. The resistance of *B. megaterium* against high temperature is also increased by endospores. After 48 h of 80 ^{0}C incubation, about 4 Log. number/ml of living endospores were detected. The bacterial endospores had ability to inhibit mycelial growth of *R. solani* although the tests were carried out after 36 h of 80 ^{0}C incubation.

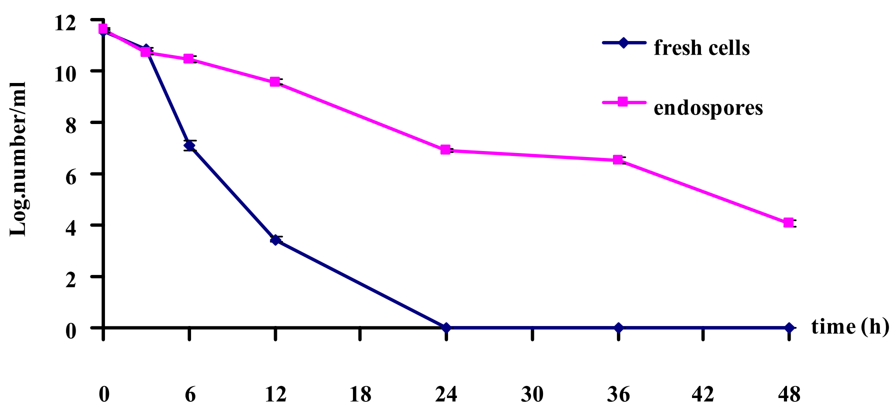

Figure 2.5 Effect of high temperature on viability of fresh cells and endospores of *B. megaterium*

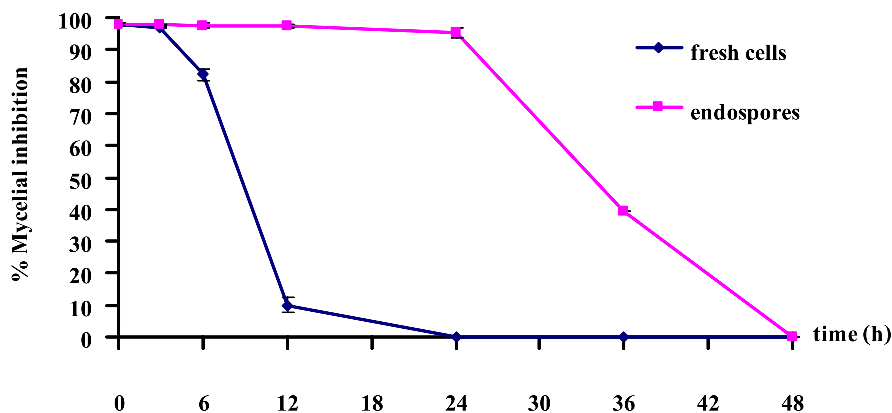

Figure 2.6 Percentage of mycelial inhibition of fresh cells and endospores after 48 h of high temperature incubation

Survival capability of the bacteria under UV exposure and high temperature is important because it would increase the potentiality of using this particular strain of bacteria for formulation study and use it successfully to control disease in the field.

2.3.1.3 Persistence of endospores against pH

The results shown that endospores viability of *B. megaterium* remained high after incubated in buffers of different pH (2-10) at room temperature (26-30 ^{0}C) for 15 days; this was due to the suitable structure of the endospore complex, which could resist in different pH environment. In contrast, fresh cells were sensitive to acidic pH (2-5) and alkaline pH (9-10). However, neutral pH (6-8) regions, no reduction of fresh cells viability was observed. (Figure 2.7, 2.8 and 2.9)

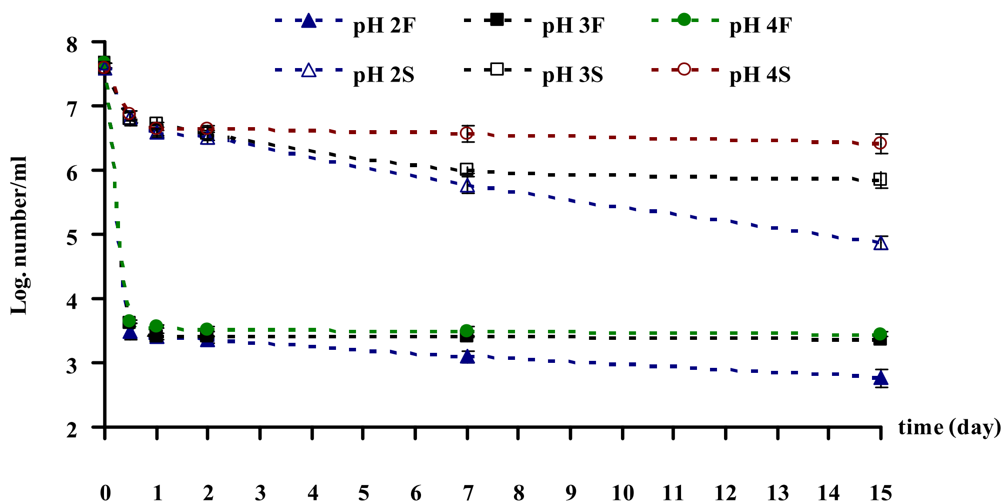

Figure 2.7 Effect of different pH (2-4) on stability of fresh cells (F) and endospores (S) of *B. megaterium*

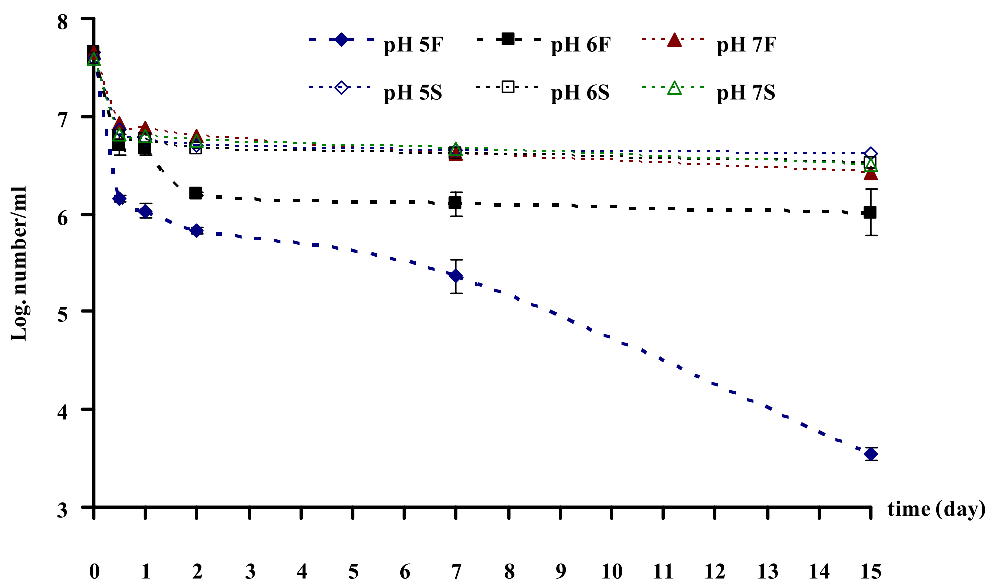

Figure 2.8 Effect of different pH (5-7) on stability of fresh cells (F) and endospores (S) of *B. megaterium*

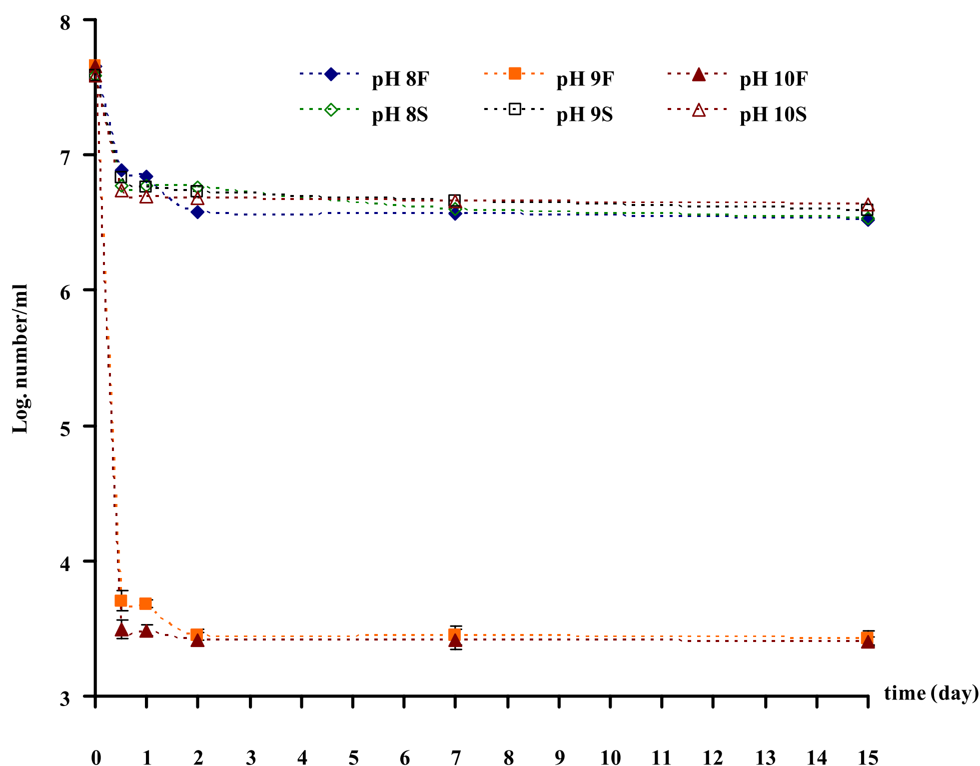

Figure 2.9 Effect of different pH (8-10) on stability of fresh cells (F) and endospores (S) of *B. megaterium*

2.3.2 Bacteria cultivation in shake flasks

2.3.2.1 Estimate cost of broth medium preparation

Time required for heating each substrate was recorded. It was found that the least boiling time of sticky rice was 10.00±2.00 min/l. Boiling time of Job's tear was the highest (33.00±1.00 min/l) (Table 2.1). Estimate cost for preparing broth media using various substrates was calculated. Broth medium prepared from cassava root was cheapest, followed by that prepared from rice, sticky rice and sweet potato, while broth medium prepared from Job's tear was most expensive, followed by that prepared from brown rice, and potato (Table 2.1).

Table 2.1 Boiling time and estimate cost of preparing broth media using various agricultural produces

Types of substrate	Time required for heating substrate (min/l)	Total cost (baht/l)
Potato	17.00±2.00	49.2
Sweet potato	20.00±1.73	28.0
Cassava root	16.67±1.15	19.9
Rice	12.00±1.00	23.0
Brown rice	16.00±1.00	59.4
Sticky rice	10.00±2.00	24.4
Job's tear	33.00±1.00	70.4

2.3.2.2 Enumeration of *B. megaterium* endospores after cultivation

B. megaterium cultured in broth prepared from brown rice had highest number of bacterium after 4 days of cultivation, followed by that cultured in broth prepared from potato, cassava root, sticky rice, rice, Job's tear, and sweet potato (Table 2.2). After 90 days storage at 10 ^{0}C, however, *B. megaterium* cultured in broth prepared from brown rice had declined more than the bacterium cultured in broth prepared from other substrates. But the number of *B. megaterium* cultured in broth prepared from brown rice still remained higher than the bacterium cultured in broth prepared from other materials. A similar trend in the decrease of bacterial number also occurred in bacterium cultured in broth prepared from potato (Table 2.2). Number of bacterium cultured in other materials, such as sweet potato, cassava root, rice, sticky rice and job's tear, remained stable or slightly increased after 90 days storage (Table 2.2).

Table 2.2 Number of endospores of *B. megaterium* cultured in broth prepared from various agricultural produces 4 days after cultivation and 90 days after storage

Types of substrate	Number of *B. megaterium* (Log. number/ml)	
	4 days	90 days
Potato	12.13±0.29 b	9.40±0.33 b
Sweet potato	9.17±0.25 d	10.07±0.42 ab
Cassava root	10.70±0.57 c	10.43±0.56 ab
Rice	9.58±0.49 d	9.50±0.24 b
Brown rice	13.57±0.33 a	11.60±0.99 a
Sticky rice	9.67±0.12 cd	10.17±0.25 ab
Job's tear	9.27±0.25 d	9.40±0.29 b
F-test	**	**
C.V. (%)	4.17	6.15

** Means in each column with difference letters are significantly different at $P \leq 0.01$ by DMRT

2.3.2.3 Measurement of *B. megaterium* endospores

Width of bacterium cultured in broth prepared from sticky rice was greater than that of bacterium cultured in broth prepared from cassava root and brown rice (Table 2.3). Width of bacterium cultured in broth prepared from potato, sweet potato, rice, sticky rice and Job's tear had no statistical difference. Types of substrates used for broth preparation had no effect to the length of endospores of *B. megaterium* (Table 2.3, Figure 2.10).

Table 2.3 Size of endospores of *B. megaterium* cultured in broth prepared from various agricultural produces

Types of substrate	Width (μm)	Length (μm)
Potato	0.85±0.13 ab	1.97±0.09
Sweet potato	0.87±0.17 ab	1.95±0.15
Cassava root	0.76±0.15 b	1.99±0.07
Rice	0.85±0.14 ab	1.97±0.08
Brown rice	0.81±0.18 b	1.97±0.15
Sticky rice	0.95±0.06 a	1.97±0.06
Job's tear	0.85±0.18 ab	1.95±0.19
F-test	*	ns
C.V. (%)	17.71	6.39

* Means in each column with difference letters are significantly different at P≤0.05 by DMRT

ns = means in the same column are not statistical significantly different

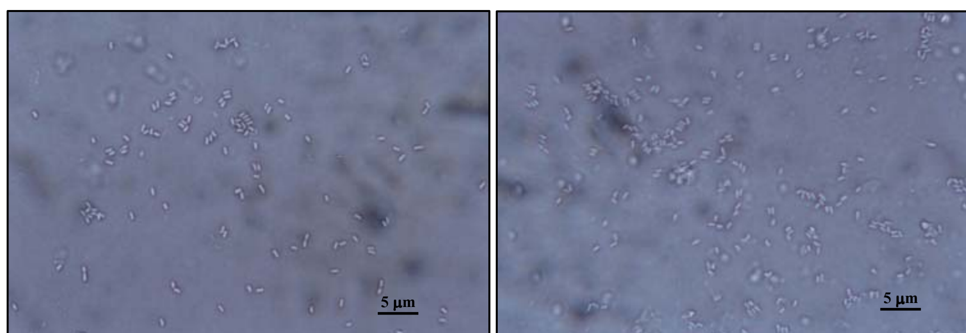

Figure 2.10 Micrographs of *B. megaterium* endospores in sweet potato (left) and cassava root (right) broth medium

From the data, tropical tuber crops are an important source of carbohydrates including sweet potato and cassava root were selected for medium preparation for bacterial endospore cultivation in 30 l fermentation compared with potato. Because of they had high

number of bacterial endospores after cultivation in small-scale fermentation and were cheap for commercial purpose.

2.3.3 Optimization of bacterial endospore culturing media and time in 30 l fermentation

B. megaterium endospores cultured in broth prepared from potato had the highest number of bacterium after 4 days of cultivation, followed by that cultured in broth prepared from cassava root and sweet potato (Figure 2.11). However, cassava root was more preferable than other materials because the number of *B. megaterium* cultured was high (9 Log. number/ml) after 3 days of cultivation. In addition, the cost of cassava root broth was quite lower than that of potato and sweet potato (Table 2.1).

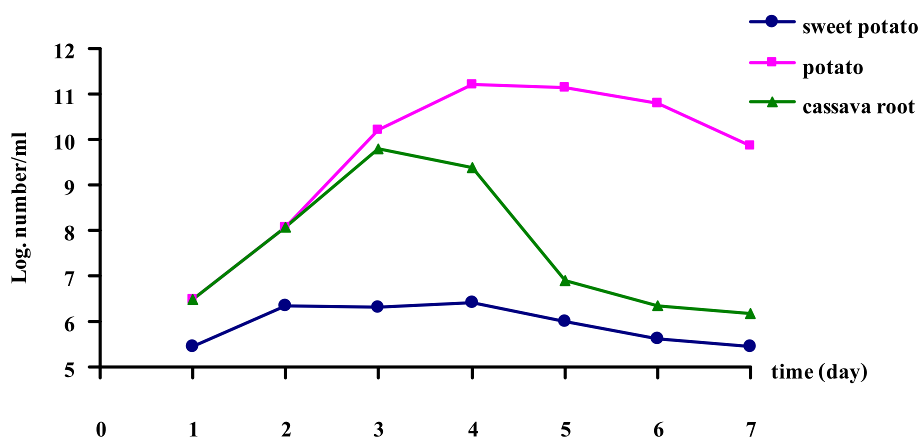

Figure 2.11 The number of viable bacterial endospores at different media and time of incubation

2.4 Discussions

In this study, endospores of *B. megaterium* remained active after being treated with UV light (48 h) and high temperature at 80 ^{0}C (36 h), while fresh cells became inactive with those treatments. This finding would enhance the potentiality of using the endospores of this bacterium for formulation study and use it successfully in the field under harsh environments. Bacterial endospores persisted under different pH (2-10) for 15 days in which the bacterium may survive and multiply in diverse pH rice field soils (Brady and Weil, 2002) and this may contribute to the reduction of rice sheath blight disease incidence.

Due to endospores of *B. megaterium* were resisted to these environmental stress, as well as their long-term survival under adverse conditions. The resistant structure of the bacterial endospores should also be used as an initial inoculum in the formulation process. This is because both quantity (number of bacterial cells) and quality (type of bacterial cells) used in the formulation process may influence the quality of finishing product. When, endospores of bacterial antagonist were incorporated into the materials that have desired characteristics. The result is the finishing bacterial formulation that contains suitable number of living bacterium. Furthermore, the bacterial endospores formulation is remain effective to suppress disease after passing the formulation process, and can be stored in a period up to 24 months without special treatment. After application of bacterial endospores formulation, endospores will be germinate into vegetative cells and colonize the rhizosphere to protect the plant from pathogen attack for a long time. This, however, requires a preliminary study to identify a substrate suitable for cultivating the bacterium with the aim to harvest the endospores for the formulation study.

Development of a suitable medium using inexpensive, readily available agricultural by-products with the appropriate nutrient balance is one of the major steps after effective biological control agent has been selected. These by-products include molasses and brewer's yeast (Lumsden and Lewis, 1988).

In Thailand, molasses had been used for producing biomass of *B. subtilis* that was later used to control rice blast and rice sheath blight diseases. Molasses as carbon source in conjunction with ammonium sulphate as nitrogen source was suitable for use in culturing *B. subtilis* strain NSRS 89-24. This bacterial strain still remained capable of producing antibiotics

effective in inhibiting both *Pyricularia grisea* and *R. solani* (Jutidamrongphan, personal communication). However, although molasses is generally consistent in composition, there may be problems in biomass formation if there are major disparities in batches of this substrate (Lumsden and Lewis, 1988). This characteristic may have a negative effect of utilizing this by-product for bacterial cultivation. For this reason, agricultural produces may be a better choice because nutrients extracted from these substrates are less likely to vary drastically between batches.

In this study, we found that all substrates supported growth of *B. megaterium* very well. However, cassava root was more preferable than other materials because the cost of producing cassava broth was quite lower than that of other materials (Table 2.1). The number of *B. megaterium* cultured in broth prepared from cassava root was quite high after both 4 days of cultivation and 90 days storage (Table 2.2). This level of inoculum should be sufficient for use in the formulation process. Size of endospores of *B. megaterium* cultured in broth prepared from cassava root was as large as that cultured in broth prepared from potato, sweet potato, rice, brown rice, and Job's tear (Table 2.3).

After scale-up of bacterial endospores from shake flasks to 30 l fermentation, cultured broth prepared from potato (at 30-35 ^{0}C, 200 rpm, 1 vvm for 4 days) had higher number of *B. megaterium* endospores than cultured in broth prepared from cassava root and sweet potato. For successful production of biological control agent, sufficient biomass containing adequate amounts of effective propagules must be obtained. For fungal biological control agents, the products were effective in disease control when formulation was prepared using chlamydoendospore, a resistant and survival propagules of the fungus (Lumsden and Lewis, 1988). Chlamydoendospores also allowed greater proliferation of the fungal biological control agents in soil than the formulations containing conidia (Lewis and Papavizas, 1984). Endospores of the bacterium are better than vegetative cells in being used for the formulation preparation because endospores are the site where antibiotics have been produced. The end result is that the finishing product contains not only effective bacterial propagules, but also has the potential to store for a reasonable period after formulation.

In conclusion, *B. megaterium* endospores were persistence against UV irradiation, high temperature and pH (2-10). For commercial purpose, cassava root has potential

for use as a substrate for broth preparation for bacterial endospore cultivation, because the cost was a lot less than other materials. In this study, potato was used for *B. megaterim* endospore cultivation for bacterial formulation preparation. Because it was sold all year round that easy to use for broth preparation, while cassava root had some season in the south of Thailand.

CHAPTER 3

PREPARATION AND EVALUATION OF *BACILLUS MEGATERIUM* WATER-SOLUBLE GRANULES

3.1 Introduction

The major difficulty to utilize biological control agents is the inconsistency and unreliable efficacy, and comparatively short shelf life of the living entities used in the formulation. Nevertheless, these problems can be overcome by the employment of appropriate technology to transform the microorganisms to suitable formulations with have desired characteristics. Spadaro and Gullino (2005) highlighted the desired formulations as having better efficacy, increased shelf life and safety and easier to handling with lower production costs. The formulation should also be in a form which is compatible with other agricultural practices.

For rice sheath blight disease, bacterial formulations have been developed and used to combat this disease in some developing countries (Vasudevan *et al*., 2002; Mew *et al*., 2004). In China, for instance, a liquid formulation has been extensively tested to control sheath blight disease in farm rice field conditions (Mew *et al*., 2004). Although, this type of formulation can be produced in large quantity using a simple fermentation and formulation processes, it may be difficult to store and may have a relatively short shelf life. Thus, an improved formulations and delivery systems, which are crucial for implementation and commercialization of effective biological control, are needed (Lewis, 1991; Lumsden *et al*., 1995; Larkin *et al*., 1998).

A variety of solid formulations, such as floatable granules and floatable pellets have been developed for sheath blight disease control in laboratory and greenhouse conditions (Kanjanamaneesathian *et al*., 1998; Kanjanamaneesathian *et al*., 2000; Pengnoo *et al*., 2000; Wiwattanapatapee *et al*., 2004). However, the prototype of these original formulations had undesirable characteristic, such as having a short shelf-life, the numbers of bacterial antagonists in the formulations greatly declined during storage and could be applied only by broadcasting (Kanjanamaneesathian *et al*., 1998; Pengnoo *et al*., 2000; Wiwattanapatapee *et al*., 2004). For this

reason, a water-soluble granule formulation which improved characteristics is developed to replace the prototype and use for testing against sheath blight disease of rice.

Generally, granule formulations are prepared as the agglomerates of the smaller particles. They are generally irregularly shaped and behave as a single larger particle. They are usually in the 4 to 18 sieve size range although granules of various mesh sizes may be prepared depending upon their application (Ansel *et al*., 1995).

The granules are prepared by moistening the desired powder or blended powder mixture and passing the moistened mass through a screen of the mesh size that will produce the desired size granules. The larger particles thus formed are then dried by air or under hot air oven heat, while they are occasionally moved about on the drying trays to prevent the adhesion of the granules. Granules may also be prepared without the use of moisture by passing compressed masses of powdered material through a granulating machine (Ansel *et al*., 1995).

Granules are generally more stable physically and chemically than are the corresponding powders from which they were prepared. Granules are less likely to cake or harden upon standing than are powders. Because their surface area is less than a comparable volume of powders, granules are usually more stable to the effects of the atmosphere. Because granules are more easily wetted by a solvent than are certain powders which tend to float on the solvent's surface, granules are frequently preferred for making of solutions (Allen *et al*., 2004).

The core component of the granule formulation is the inert carriers such as clay minerals, polymer, dry fertilizers and ground plant residues (Ross and Lembi, 1985). Choice of carrier substance is dependent on absorption, hardness, bulk density and product disintegration rate in water (Polon, 1973). Both dry and moist granules are suitable for broadcast and in furrow applications. Some granules can be water dispersible, are suitable for spray applications (Paau, 1998). Granules tend to pose a lower dermal hazard in comparison to liquid formulations and they do not burn vegetation as many oil-based formulations. They are more advantage than powder formulation, with several important exceptions. During the mixing and loading process, granules pour more easily from the container and, because of their larger particle size, reduce inhalation hazard to the applicator (Paau, 1998). In addition, the finished products are therefore price competitive, since the ingredients of the formulation are cheap and easy to obtain.

This chapter work describes (1) a process to produce a water-soluble granular formulation using relatively simple technology and a small number of ingredients (2) the efficacy of this novel water-soluble granule formulation in suppressing sheath blight disease development under laboratory and greenhouse conditions.

3.2 Materials and Methods

3.2.1 Materials

Lactose monohydrate (Pharmatose® 200M) was obtained from DMV International Distributor (Bangkok, Thailand), polyvinyl pyrrolidone K-30 (PVP, K-30) was supplied by Vidhyasom (Bangkok, Thailand). Sodium alginate was from Srichand united dispensary (Bangkok, Thailand). Iprodione (Rovral® Fungicide) was purchased from Bayer CropSciences (Victoria, Australia). Plate count agar (PCA) (Difco®, Becto) was from Dickinson and Company Sparks MD (New Jersey, USA). Potato dextrose agar (PDA) was prepared using 200 g of potato, 20 g of dextrose, 17 g of agar and 1 l of distilled water. *B. megaterium* and *R. solani* were isolated from paddy soil and rice sheath blight tissue from Satun province, respectively (Kanjanamaneesathian *et al*., 1998).

3.2.2 Preparation of bacterial endospore suspensions

B. megaterium was used as a biocontrol agent based upon its ability to inhibit mycelial growth and sclerotial germination of *R. solani* (Kanjanamaneesathian *et al*., 1998). The bacterium was cultured (9 Log. number/ml) in a 30 l the fermentor containing 20 l of potato dextrose broth (PDB) at 30-35 ^{0}C, stirring at 200 rpm and 1 vvm for 4 days, and bacterial cells at the stationary growth stage were separated by centrifugation at 3,000 rpm for 10 min. The isolated cells were washed 3 times and re-suspended in sterile distilled water (1:2 v/v). The cells were incubated in a water bath at 80 ^{0}C for 20 min to kill the vegetative cells. Then, endospores of *B. megaterium* were enumerated on plate count agar (PCA) using drop plate technique (Zuberer, 1994). The plates were incubated at 37 ^{0}C for 1 day after which colony forming units were counted. The value of viable bacterium (Log. number/ml) was the mean (±S.D.) of four replications per dilution.

3.2.3 Preparation of water-soluble granule formulations (patent pending, No. 7 1 1394)

Water-soluble granules containing the endospores were prepared by wet-granulation using the mixture of different proportions of bacterial endospore suspension (at 13.93±0.08 Log. number/ml), sodium alginate (5-20%), PVP K-30 (5-10%) and lactose monohydrate (70-90%) as listed in Table 3.1. All ingredients were mixed in a planetary mixer (KitchenAid, USA) until they became a damp mass. This mass was passed through a granulator (sieve No.14) and dried in a hot air oven at 60 ^{0}C for 2 h. The dried granules were screened through another sieve No.16. Water-soluble granules without bacterial endospores were prepared in an identical way and referred to as control granules.

Table 3.1 Compositions of ingredients in different water-soluble granule formulations

Ingredients	Formulations (100 g)							
	SG1	SG2	SG3	SG4	SG5	SG6	SG7	SG8
Sodium alginate (g)	5	10	15	20	5	10	15	20
PVP K-30 (g)	5	5	5	5	10	10	10	10
Lactose (g)	90	85	80	75	85	80	75	70
Distilled water (ml)	18	21	24	28	15	18	20	24

3.2.4 Evaluation of the physical properties of water-soluble granules

3.2.4.1 Density

Bulk and tapped density of the formulation were measured using a Jolting apparatus (Vankel, New York, USA). Bulk density was performed by pouring 100 g of granules into a 100 ml graduated cylinder. The volume occupied to the nearest ml was noted and the bulk density deduced in g/ml. Tapped density was measured by tapping graduated cylinder until the granules attained a constant tapped volume and the density value was calculated in g/ml. Three replicates were performed for each test, and the data given as mean (±S.D.).

3.2.4.2 Friability

Granules (10 g) were placed in a Roche friabilator. The samples were rotated at 25 rpm for 16 min, each rotation caused the bacterial granules to fall a distance of 15 cm inside the drum. Afterward, the samples were sieved through a 40 mesh screen, and the weight of sample remaining above the screen was determined and the percentage friability index calculated [(Equation (3.1)]. Three replicates were performed for each test, and the data given as mean (±S.D.).

Friability index = [(granule wt above 40 mesh)/total wt] x 100 (3.1)

3.2.4.3 Dissolution time, pH and viscosity

Bacterial granules (3 g) were suspended in distilled water and volume make up to 100 ml, at room temperature (26-30 ^{0}C). The granule suspension was stirred at 200 rpm until complete dissolution was achieved, the dissolution time recorded, and the pH value of the solution measured. The viscosity value of a 3% w/v solution of the granules was measured using a Brookfield Digital Rheometer Model DV-III. Three replicates were performed for each test, and the data given as mean (±S.D.).

3.2.4.4 Moisture content

Ten g of water-soluble granules were weighted and dried in hot air oven at 60 ^{0}C until they attained a constant weight. The percent moisture content of water-soluble granules was measured. Three replicates were performed.

3.2.5 Enumeration of viable bacteria in water-soluble granules

Viable bacteria in the formulation were counted using the drop plate method (Zuberer, 1994). The viability tests were carried out after granule production and at one-month intervals during storage at room temperature (26-30 ^{0}C) in plastic containers for 24 months. A 1% w/v solution of the granule formulation was prepared in sterile distilled water. The viable bacteria were cultured in PCA at room temperature for 18-24 h, after which colony forming unit were

counted. The value (Log. number/g) of viable bacteria was the average of six replications (six drops) per dilution.

3.2.6 Testing the inhibition of *R. solani* mycelial growth by water-soluble granules containing the endospores of *B. megaterium*

The mycelial growth inhibition of the water-soluble granules was tested after production and during storage at room temperature in plastic containers over a period of 24 months. A 1% w/v solution of bacterial granules was prepared in sterile distilled water. This solution (1 ml) was sampled and mixed with melted potato dextrose agar (PDA) at 1:10 (v/v) ratio in Petri dishes (9.0 cm diameter) then an agar plug of *R. solani* was placed at the center of the plate to determine its effect in inhibiting mycelial growth of *R. solani*. Treatment consisted of *R. solani* cultured on PDA incorporated with solution of bacterial formulation, while a culture of *R. solani* on PDA with sterile water incorporated was used as a control. Each treatment consisted of six replications. Mycelial inhibition of *R. solani* was assessed as % mycelial inhibition 36 h after culturing *R. solani* [Equation (3.2)] (Gamliel *et al*., 1989).

$$\% \text{ mycelial inhibition} = 100 - [(r^2/R^2) \times 100] \quad (3.2)$$

In equation (3.2), r is colony radius of *R. solani* on PDA incorporated with 1% w/v aqueous solution of bacterial formulation and R is colony radius of *R. solani* on PDA incorporated with sterile water.

3.2.7 Evaluation of viable bacteria on surface of rice tissues and testing the inhibition of *R. solani* mycelial growth

Dissolved granule formulation (3% w/v, 9 Log. number/ml) and non-formulated *B. megaterium* endospores (9 Log. number/ml) were sprayed on the three rice seedlings in plastic pots, at 100 ml/pot using a hand-held sprayer. Leaf sheath or leaf blade rice plant samples (1 g) were taken at 1, 4, 7 and 14 days after spraying the formulation and the non-formulated endospores. Leaf tissues were placed into 99 ml sterile distilled water and incubated in water bath at 80 ^{0}C for 20 min to get rid of the other microorganisms. The number of bacteria on the leaf

sheath or leaf blade was assessed using the drop plate method (Zuberer, 1994). The value (Log. number/g of leaf sheath or leaf blade) of viable bacteria was the means (±S.D.) of six separate plants. Percentage mycelial growth inhibition of these solutions was tested using dual culture method (Equation 3.2).

3.2.8 Scanning electron microscope (SEM) observation of endospores on water-soluble granules and plant surface

3.2.8.1 SEM observation of the formulated water-soluble granules containing *B. megaterium* endospores

Samples of bacterial dry granule formulation were mounted on the stub, coated with gold particles and then observed with SEM, and micrographs of the bacterial endospores on the surface taken.

3.2.8.2 SEM observation of endospores on plant surface

Dissolved formulations (3% w/v) were prepared and sprayed on the rice seedlings in plastic pots, at 100 ml/pot, using a hand-held sprayer. At 1 h after spraying, samples of leaf sheath or leaf blade from the rice plants was cut and 1 g portions were fixed in 2.5% glutaraldehyde solution for 2 h. The samples were then washed by an increasing concentration (step by step) of alcohol (0-100%) and dried in a Critical Point Drier. The resulting samples were mounted on the stub, coated with gold particles, and photographed with SEM.

3.2.9 Testing the efficacy of selected water-soluble granule formulation containing *B. megaterium* under greenhouse conditions

3.2.9.1 Experimental design and treatments

Each treatment consisted of eight replications (three rice seedlings (cv. RD-23) per replication). Rice plants inoculated only with *R. solani* were used as a control treatment. The experiment was arranged in a Complete Randomized Design (CRD). The details of the treatments for the greenhouse test are shown in Table 3.2.

Table 3.2 Different types of treatment used for testing under greenhouse conditions

Treatment	Description
Control SG2	Sprayed with 3% w/v solution of the water-soluble granules without bacterial endospores 1, 5 and 10 days after pathogen inoculation (100 ml/replication)
SG2	Sprayed with 3% w/v solution of the bacterial water-soluble granules 1, 5 and 10 days after pathogen inoculation (100 ml/ application) (10.05 Log. number/replication)
Fresh cells	Sprayed with fresh cells 1 day after pathogen inoculation (100 ml/ replication) (10.11 Log. number/replication)
Chemical fungicide	Sprayed with 0.15% w/v Iprodione solution 1 day after pathogen inoculation (100 ml/replication)
Control	Rice plants inoculated only with *R. solani* (no treatment)

3.2.9.2 Pot preparation

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.

3.2.9.3 Pathogen inoculation

Ten g of sterile rice seeds infested with *R. solani* were placed in the centre of each plastic pot 60 days after sowing, 1 day prior to formulation application. The sterile rice seed was dispersed with a sterile spatula so that the pathogen inoculums made contact with all rice plants. The water level in the plastic pot was maintained at the same level throughout the experiment.

3.2.9.4 Disease assessment

Sheath blight assessment in the greenhouse tests was carried out 30 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 lesion which showed sheath blight symptoms. Percent Relative Lesion Height (% RLH) was calculated [Equation (3.3)] (Anh *et al.*, 1986). The entire length of the lesion on each rice tiller which had sheath blight symptoms was also measured.

$$\% \text{ RLH} = Li/Lt \times 100 \qquad (3.3)$$

In equation (3.3), Li is the length of the lesion and Lt is the length of the rice tillers.

3.2.1 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 $p \leq 0.01$ or $p \leq 0.05$.

3.3 Results

3.3.1 Preparation and development of water-soluble granule formulations

Eight water-soluble granules were successfully prepared using different proportions of sodium alginate (5-20%), PVP K-30 (5-10%) and lactose monohydrate (70-90%). All formulations were white in color. Evaluation of physical properties of all formulations was shown in Table 3.3. The pH values of 3% solution of all formulations were slightly acid (5.98-6.19). They were completely dissolved in water within 4-14 min at room temperature. The viscosity values of dissolved formulations were approximately 4-9 times more than distilled water. The friability indexes of all formulations were more than 90% (Table 3.3) and there was significant difference among various formulations.

Table 3.3 pH, dissolution time, viscosity and friability index of water-soluble granule formulations

Formulations	pH	Dissolution time (min)	Viscosity (cp)	Friability index (%)
SG1	6.19±0.17 b	3.14±0.07 f	4.96±0.07 f	96.60±0.36 a
SG2	6.16±0.08 b	4.88±0.04 d	6.60±0.00 e	96.63±0.25 a
SG3	6.07±0.10 bc	8.44±0.16 c	7.68±0.00 c	95.67±0.78 a
SG4	6.07±0.05 bc	13.33±0.53 a	9.04±0.07 b	95.97±1.12 a
SG5	6.00±0.06 c	3.20±0.03 f	4.36±0.07 g	93.20±0.00 b
SG6	5.98±0.02 c	4.42±0.05 e	6.72±0.00 e	93.97±0.38 b
SG7	5.99±0.04 c	9.42±0.03 b	7.16±0.07 d	96.27±0.06 a
SG8	5.98±0.08 c	8.35±0.36 c	9.56±0.07 a	93.83±0.57 b
Sterile distilled water	6.33±0.18 a	-	1.52±0.00 h	-
F-test	**	**	**	**
C.V. (%)	0.81	1.99	1.53	0.59

** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p \leq 0.01$

From the data, formulation SG2 was selected for preparation of water-soluble granule formulation containing *B. megaterium* for spray application because the physical properties were suitable for spray application; pH was in the rage 6.0-7.0, the dissolution time was within 10 min and friability index was high. Furthermore, SG2 are high friability index, short dissolution time and could easy to produce compared to other formulations.

3.3.2 Preparation of water-soluble granules containing *B. megaterium*

The water-soluble granule formulation SG2 containing *B. megaterium* were successfully developed (Figure 3.1). Granule formulation containing various proportions of 10 g sodium alginate, 5 g PVP K-30, 85 g lactose monohydrate and 21 ml bacterial endospores suspension were prepared. The bulk density and tapped density of granules were 0.46±0.01(n=3) and 0.51±0.01 g/ml (n=3), respectively. The friability index was 96.30±0.10% (n=3). The granules (3% w/v) were completely dissolved in water within 6.97±0.37 min (n=3) at room temperature. The pH and viscosity values of dissolved formulations were 5.93±0.01 (n=3) and 5.32±0.07 cps (n=3), respectively. The moisture content was 0.25±0.02%.

Figure 3.1 Bacterial water-soluble granule formulation SG2

3.3.3 Enumeration of viable bacteria in water-soluble granules

The bacterial population in the dry granule formulation remained high after production. The granules contained viable bacterial number of 9.57±0.75 Log. number/g (n=3)

and the bacterial count was still in the range of 9 Log. number/g after storage at room temperature for 6, 12, 18 and 24 months (9.31±0.65, 9.18±0.25, 9.16±0.50 and 9.22±0.65 Log. number/g, respectively).

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

The aqueous solution (1% w/v) of the bacterial granule formulation had the ability to almost totally inhibit mycelial growth of *R. solani* even when the tests were carried out with sample taken after storage at room temperature for 6, 12, 18 and 24 months (% mycelial inhibition were 98.28±0.58, 99.68±0.17, 99.95±0.65 and 99.64±0.14%, n=3, respectively) (Figure 3.2).

Figure 3.2 Inhibition of *R. solani* mycelial growth test on PDA, incorporated with water-soluble granule SG2 solution containing bacteria (left) compared with control (right)

3.3.5 Evaluation of viable bacteria on the surface of rice tissues and testing the inhibition of *R. solani* mycelial growth

The bacterial population on leaf sheath and leaf blade was counted using the drop plate method at day 1, 4, 7 and 14 after spraying the formulation. The number of bacteria on surface of both rice tissues (6 Log. number/g of plant) was 1 log unit higher than the number counted of non-formulated bacteria (5 Log. number/g of plant) (Table 3.4). The number of bacteria on a leaf sheath was also higher than that on the leaf blade when rice plants were sprayed with both types of inoculum. However, the discrepancy of the number of bacteria (between leaf sheath and leaf blade samples) was more prominent when the non-formulated bacterial suspension was applied to rice plants. Bacteria were also more numerous in leaf sheath samples when rice plants were sprayed with a formulated bacterial suspension. The number of bacteria on both rice tissues declined over time regardless of the types of bacterial suspension used to apply on rice plants. Nonetheless, the dramatic decrease of the bacterial population was obvious in rice plants which had been sprayed with the non-formulated bacterial suspension (Table 3.4), indicating that the ingredients in the granular formulation are aiding the adhesion of the endospores to the rice tissues.

In addition, *B. megaterium* antagonist from leaf sheath or leaf blade solution had the high ability to inhibit *R. solani* mycelial growth (% mycelial inhibition was more than 87%, n=3) at 14 days after spraying with formulation (Table 3.5).

Table 3.4 Viability of fresh cells and cells of *B. megaterium* in water-soluble granule formulation on leaf sheath and leaf blade of rice plant

Formulation (A)	Log. number/g plant								
	(B) 1		4		7		14		Means (A)
	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	
Fresh cells	5.95±0.14	5.21±0.14	5.81±0.14	5.01±0.12	5.74±0.07	4.48±0.02	5.39±0.09	4.38±0.11	5.25±0.57 b
SG2	7.03±0.06	6.80±0.01	6.63±0.05	6.48±0.02	6.64±0.02	6.40±0.09	6.36±0.05	5.73±0.02	6.51±0.37 a
Means (B)	6.25±0.75 a		5.98±0.67 b		5.81±0.87 b		5.46±0.75 c		
Means (C)									
Leaf sheath	6.11±0.55 a								
Leaf blade	5.56±0.89 b								
F-test	**								
C.V. (%)	3.84								

** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p \leq 0.01$

Note: A=Formulation (Fresh cell and SG2)

B=Days after spraying formulation (1, 4, 7 and 14 days)

C=Plant tissues were detected (leaf sheath and leaf blade)

Table 3.5 Inhibition of mycelial growth of *R. solani* of fresh cells and water-soluble granules after sprayed on leaf sheath and leaf blade of rice plant

Formulation (A)	% mycelial inhibition								
	(B) 1		4		7		14		Means (A)
	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	
Fresh cells	89.81±1.60	86.75±2.50	87.33±1.58	84.51±2.63	86.24±1.94	82.14±1.88	85.70±1.68	81.53±1.11	85.50±3.06 b
SG2	91.93±2.70	91.14±2.05	91.62±1.28	89.71±2.86	91.62±1.28	91.14±2.05	91.14±2.05	87.33±1.58	90.71±2.25 a
Means (B)	89.91±2.82 a		88.29±3.35 ab		87.79±4.34 ab		86.42±3.86 b		
Means (C)									
Leaf sheath	89.42±2.91 a								
Leaf blade	86.78±4.05 b								
F-test	**								
C.V. (%)	2.13								

** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p \leq 0.01$

Note: A=Formulation (Fresh cell and SG2)

B=Days after spraying formulation (1, 4, 7 and 14 days)

C=Plant tissues were detected (leaf sheath and leaf blade)

3.3.6 SEM observation of bacterial endospores on water-soluble granule and plant surface

The SEM micrograph of the endospores of *B. megaterium* on the surface of granules is shown in Figure 3.3a. Numerous bacterial endospores were found on the surface of the granule, even though the majority of the endospores are encapsulated within the granule formulation. However, all the bacterial cells are easily released when the granules are dissolved into water.

These endospores of *B. megaterium* were also observed on the surface of leaf sheath after spraying with bacterial formulation (Figure 3.3b). The bacterial endospores were also detected on the surface of leaf blade which possessed hairy texture as shown in Figure 3.3c.

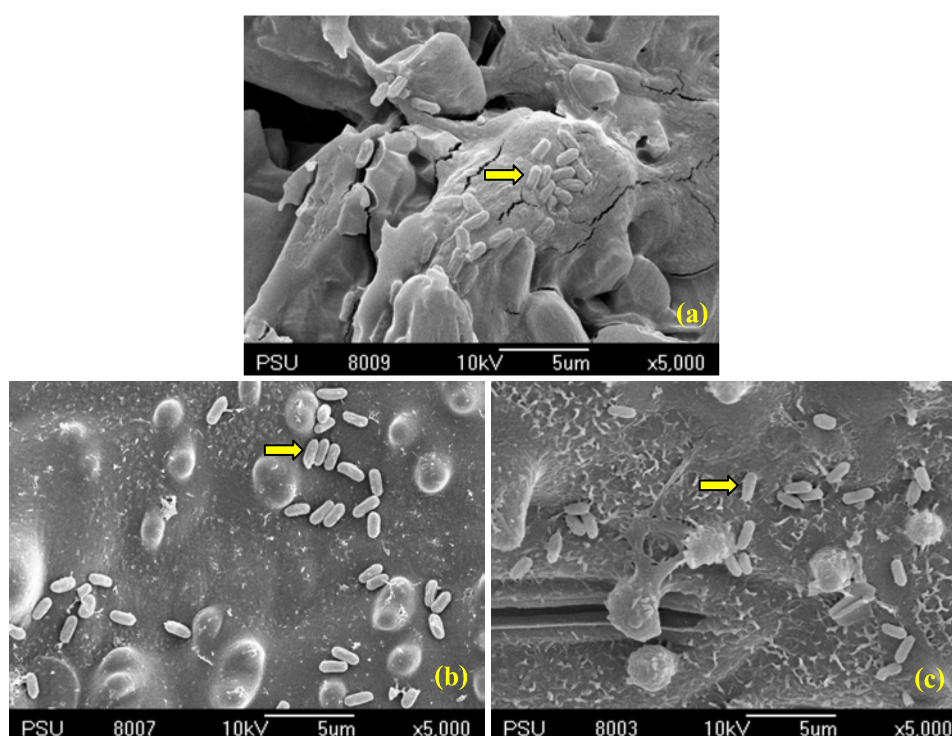

Figure 3.3 Micrographs of bacterial endospores on the surface of water-soluble granule SG2 (a), leaf sheath (b) and leaf blade (c)

3.3.7 Testing the efficacy of water-soluble granules containing *B. megaterium* under greenhouse conditions

In the greenhouse tests, spraying an aqueous solution (3% w/v) of the water-soluble granule formulation SG2 containing endospores of *B. megaterium* 1, 5 and 10 day after pathogen inoculation (10.05 Log. number/ replication) was effective in sheath blight suppression (Table 3.6). Similar aqueous solution of control formulation (without *B. megaterium*) did not control sheath blight in the pot test. However, the chemical fungicide (0.15% Iprodione) was better than the water-soluble granule formulation when number of lesion/tiller, lesion length and % severity are compared (Table 3.6).

Table 3.6 Efficacy of an aqueous solution (3% w/v) of the water-soluble granule formulation SG2 in suppressing the development of sheath blight disease in plants grown in pots under the greenhouse conditions

Treatment	Number of lesion/tiller	Lesion length (cm)/tiller	Severity (% RLH)
Control SG2	10.39±2.81 a	24.52±3.01 b	53.51±2.27 a
SG2	2.11±0.94 c	12.66±4.28 c	28.20±2.60 b
Fresh cells	5.99±1.71 b	24.80±5.17 b	46.37±1.70 a
Chemical fungicide	1.02±0.45 c	4.67±2.14 d	7.84±1.98 c
Control	11.00±4.10 a	27.44±3.84 a	50.89±2.79 a
F-test	*	*	*
C.V. (%)	29.28	20.37	27.88

* Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p \leq 0.05$ and the mean values are followed by a value of Standard Deviation (in the parenthesis), n=8

3.4 Discussions

This work reports an improved formulation of water-soluble granular form to suppress sheath blight disease. The application of pharmaceutical technology to produce these antagonistic bacterial formulations is intent to develop finished products that have improved quality for possible commercialization. Formulations of bacterial antagonists had been tested against wilt disease of pigeonpea (Vidhyasekaran *et al*., 1997), damping-off of tomato (Sabaratnam and Traquair, 2002), fruit rot of chilli (Bharathi *et al*., 2004), post harvest fruit diseases of mango (Govender and Korsten, 2006), stem end rot of avocado's flower (Demoz and Korsten, 2006), damping-off and wilt of sesame (Ryu *et al*., 2006), and gray mold disease of tomato (Lee *et al*., 2006). Schmiedeknecht *et al*. (1998) have produced *B. subtilis* granules to control potato disease. While, Marten *et al*. (1999) have found that *B. subtilis* B2g as granules showed promising result in suppression of fungal disease of sunflower, cabbage and cucumber. Formulations of *Pseudomonas fluorescens* had been evaluated to control sheath blight disease in rice (Rabindran and Vidhyasekaran, 1996; Radja Commare *et al*., 2002). More recently, *B. subtilis* had been reported for controlling sheath blight disease of rice in Jiangsu, China (Mew, 2004). Rojanarat (2005) had formulated bacterial granule formulation GS-Alg which had reduced rice sheath blight disease by 53.33% infection in the greenhouse condition. While rice plants sprayed with water-soluble granule formulation SG2 containing *B. megaterium* had approximately 28.20% of disease incidence (Table 3.5). It is perhaps that the number of viable bacteria of formulation SG2 on surface of leaf sheath and leaf blade (approximately 5 and 4 Log. number/g of plant, respectively) (Table 3.3) were higher than the number counted of formulation GS-Alg (approximately 4 and 2 Log. number/g of plant, respectively) at 14 days after spraying these formulations.

In terms of physical properties, the water-soluble granules of the current formulation, flow freely and do not form dust or fines during handling compared to the powder. They disperse instantaneously in water under slight agitation without forming lumps. The dissolved formulation SG2 has the viscosity of 5.32±0.07 cps, which does not cause

blockage when sprayed with a hand-held sprayer. Since the viscosity of the dissolved formulation was approximately 5 times higher than that of water, as well as Rojanarat (2005) reported that the viscosity of dissolved formulation GS-Alg was higher than of water. It is possible that the ingredient as sodium alginate used in both producing granules may increase the adhesiveness of the droplets of the formulations.

The deposition of bacteria on plant tissues is an important event that is required before the bacteria can multiply and maintain their population on the target tissues. Any factors affecting the deposition of bacteria onto target plant tissues would affect the initial population of the bacteria, and this will subsequently affect the number of bacteria on plant tissue at later points of time. A higher number of the bacterial population was detected from rice tissues sprayed with formulated bacterial suspension (Table 3.4, Figure 3.3b, 3.3c) compared to application of the equivalent number of non-formulated endospores. This may be attributed to the bioadhesive property of sodium alginate, which acts as a viscosifier in the formulation. The population of bacteria obtained from leaf sheath of rice was higher than that obtained from leaf blade, irrespective of the types of bacterial inoculum used to spray the rice plants (Table 3.4). The droplets of the spray have more chance to make impaction on the rice stems where the leaf sheath is located, and therefore remain on this tissue more than on the leaf blade. This is because when an object travels in the air and encounters any barrier, the chance of the object making impaction on the barrier is dependent upon the diameter of the barrier, assuming that the objects had the same mass. This means a droplet which has the same size containing either fresh cells or formulated bacterium has more chance to make impaction on leaf sheath and thereby adheres on this tissue (Deacon, 2006). Bacterial cells were also detected on rice plant tissues with the Scanning Electron Microscope (Figure 3.3b and 3.3c).

In terms of efficacy testing, the chemical fungicide performed better than the water-soluble formulations in sheath blight suppression (Table 3.6). The constant high humidity conditions which are generated in the greenhouse setting favors sheath blight disease development. This condition is intentionally set up to gauge the performance of the formulation. The result in the greenhouse setting showed that the water-soluble formulation is

as effective as the fungicide in reducing sheath blight incidence when numbers of lesion/tiller are assessed (Table 3.6). Nonetheless, the fungicide is more effective than the formulation in suppressing sheath blight severity when average lesion length/tiller and relative lesion height (RLH) are evaluated (Table 3.6). Further studies are required to find out the appropriate dose and frequency of application to get the most effective biocontrol treatment against sheath blight disease.

The fact that the PDA medium incorporated with solution of water-soluble formulations which had been stored for 24 months completely suppressed mycelial growth of *R. solani* indicated that *B. megaterium* still remained viable in the formulation and could still produce antibiotic which inhibited the pathogen. This result indicates that this water-soluble formulation has reasonable shelf life and has potential for commercialization.

CHAPTER 4

PREPARATION AND EVALUATION OF *BACILLUS MEGATERIUM* EFFERVESCENT GRANULES

4.1 Introduction

In the preceding chapter, water-soluble granule formulation has been devised and tested for sheath blight control. However, in terms of research and development, novel formulations should be developed to provide the industry with a better technological alternative for possible production and commercialization of the products. Therefore, fast-released formulations which can readily liberate an adequate number of bacteria for suppressing the disease are developed. Effervescent granule is a novel formulation developed to have better features in term of easy application either by spraying or broadcasting to water.

The principle for producing the effervescent formulation and the techniques involved for improving the efficacy of effervescent formulation have been extensively reviewed (Ansel *et a*l., 1995; Allen *et al*., 2004). These effervescent formulations have been used for administering to the patients (Katare *et al*., 1990, 1995; Eichman and Robinson, 1998) and applied in other systems (Burges, 1998). Nonetheless, this type of formulation has never been produced to control plant diseases before.

This formulation has been devised based upon the ingredients of both acids and bases chemical agents, with produced carbon dioxide upon application to the aquatic environment (Allen *et al*., 2004). Tartaric acid and citric acid are two chemical agents with an acidic property, while sodium bicarbonate is the agents with a basidic property used in the formulation process.

Wet massing is the most important step in the effervescent granule preparation using wet granulation process. The source of binding agent is not the water of crystallization from the citric acid but water added to solvents such as alcohol, propylene glycol or glycerin which act as moistening agents. In the granulation step, the granules themselves are formed by forcing the moistened power through a screen in an oscillating granulator. The granules are dried on the trays

either under hot air circulation oven condition or in fluid and immediately transferred to containers which are tightly sealed.

The objectives of this research were (1) to develop fast-disintegrating bacterial granules which can be applied either by spraying on plant or by broadcasting to suppress sheath blight disease of rice (2) to evaluate the physical and biological characteristics of the formulation and (3) to test the effectiveness of a novel formulation in sheath blight disease suppression in the laboratory and greenhouse conditions.

4.2 Materials and methods

4.2.1 Materials

Citric acid monohydrate, tartaric acid and sodium bicarbonate were from Srichand United Dispensary (Thailand), lactose monohydrate (Pharmatose ® 200M) was from DMV International Distributor (Thailand) and polyvinyl pyrrolidone K-30 (PVP, K-30) was supplied by Vidhyasom (Thailand). Iprodione (Rovral ® Fungicide) was purchased from Bayer CropSciences (Australia). Plate count agar (PCA) (Difco ®) was from Becton, Dickinson and Company Sparks, MD (USA).

4.2.2 Preparation of bacterial endospores suspension

B. megaterium was used *as* a biocontrol agent based upon its ability to inhibit mycelial growth and sclerotial germination of *R. solani* (Kanjanamaneesathian *et al*., 1998). The bacterium was cultured in the fermentor (dimension 30 l) containing 20 l of potato dextrose broth (PDB) at 30-35 ^{0}C, 200 rpm and 1 vvm for 4 days. The bacterial endospores were incubated in water bath at 80 ^{0}C for 20 min to get rid of the vegetative cells.

4.2.3 Preparation of effervescent granule formulations

Effervescent granules containing *B. megaterium* was prepared by wet granulation using different proportions of citric acid (5-10%), tartaric acid (10-20%), sodium bicarbonate (20-30%), PVP (k-30) (0-5%) and lactose monohydrate contained bacterial endospores (35-65%), as listed in Table 4.1. All ingredients were mixed with 15 ml alcohol in a planetary mixer (KitchenAid, USA) until they became a damp mass. Then this mass was passed through a granulator (sieve No.14) and dried in a hot air oven at 60 ^{0}C for 2 h. After that the dried granules were screened through another sieve No.16 to a suitable size. Effervescent granules without bacterial endospores were prepared as blank.

Table 4.1 Composition of ingredients in different effervescent granule formulations

Ingredients	Formulations/100 g					
	EG1	EG2	EG3	EG4	EG5	EG6
Citric acid (g)	10	10	5	10	10	5
Tartaric acid (g)	20	10	10	20	10	10
Sodium bicarbonate (g)	30	30	20	30	30	20
Lactose monohydrate (g)*	35	45	60	40	50	65
PVP (k-30) (g)	5	5	5	-	-	-

* Lactose contained bacterial endospores (9.25 Log. number/g)

4.2.4 Evaluation of physical and biological properties of effervescent granules

4.2.4.1 Disintegration time

One g of effervescent granules from each formulation was placed into 99 ml of distilled water at room temperature (26-30 ^{0}C). Disintegration time of the granules was recorded and three replicates were performed for each formulation.

4.2.4.2 pH

One g of effervescent granules from each formulation was dissolved in 99 ml of distilled water at room temperature (26-30 ^{0}C). The pH value of 1% w/v solution was measured by pH meter (Mettle-Toledo Co., Ltd., Switzerland). Three replicates were performed for each formulation.

4.2.4.3 Friability

Ten g of effervescent granules in size between 16 and 40 meshes were placed in a Roche friabilator. The drum was rotated at 25 rpm for 16 min, each rotation causing the bacterial granules to fall a distance of 15 cm inside the drum. Bacterial granules were then weighed on a 40 mesh screen and then calculated as friability index [Equation (4.1)]. Three

replicates were performed for each test. Formulations with high friability index were chosen for further studies.

Friability index = [(granule wt above 40 mesh)/total wt] x 100 (4.1)

4.2.4.4 Density

Bulk and tapped density of the formulation were measured using a Jolting Apparatus (Vankel, USA). Bulk density was performed by pouring 100 g granules into a 100 ml graduated cylinder and measuring the volume to the nearest ml. Tapped density was performed by dropping graduated cylinder until the granules attained a constant tapped volume and the density value was calculated in g/ml. Three replicates were performed for each test.

4.2.4.5 Moisture content

Ten g of effervescent granules were weighted and dried in hot air oven at 60 ^{0}C until they attained a constant weight. The percent moisture content of effervescent granules was measured. Three replicates were performed.

4.2.4.6 Enumeration of viable bacteria in effervescent granules

Viable bacteria in the formulation were counted using the drop plate method (Zuberer, 1994). The viability tests were carries out after the formulation were produced and during storage at room temperature (20-30 ^{0}C) in plastic containers for 24 months. One g of each bacterial formulation was dissolved in 99 ml of sterile distilled water. The viable bacteria were cultured in PCA at room temperature for 18-24 h after which colony forming unit were counted. The value (Log. number/g) of viable bacteria was the average of six replications (six drops) per dilution.

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

The mycelial growth inhibition of bacterial effervescent granules was tested after production and during storage at room temperature (26-30 ^{0}C) in plastic containers for 24 months. One g of the bacterial effervescent granules was dissolved in 99 ml of sterile distilled

water. One ml of the solution was sampled and mixed with melted potato dextrose agar (PDA) at 1:10 (v/v) in Petri dishes (9.0 cm diameter). The agar plug of *R. solani* was cultured on PDA incorporated with solution of the bacterial formulation to test the inhibition of *R. solani* mycelial growth. Each treatment consisted of six 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 (4.2)] (Gamliel *et al*., 1989).

$$\% \text{ mycelial inhibition} = 100 - [(r^2/R^2) \times 100] \tag{4.2}$$

In equation (4.2), r is colony radius of *R. solani* on PDA incorporated with one percent solution of bacterial effervescent granule formulation and R is colony radius of *R. solani* on PDA incorporated with sterile water.

4.2.5 Evaluation of viable bacteria in water and testing the inhibition of *R. solani* mycelial growth

To study the viability of bacteria in the water after broadcasting, one g of bacterial effervescent granules was placed to 99 ml of sterile distilled water at room temperature. One ml of water sample was taken at 1, 4, and 7 days after broadcasting and the number of bacteria was counted using the drop plate method as mentioned in section 4.2.4.6. The number of viable bacteria per gram of effervescent granules was then calculated and the value was the average of six replications.

The inhibition of *R. solani* was tested using the same method as mentioned in section 4.2.4.7. Each treatment consisted of six replications.

4.2.6 Evaluation of viable bacteria on surface of rice tissues and testing the inhibition of *R. solani* mycelial growth

B. megaterium endospores suspension (fresh cells) and bacterial effervescent granule formulation suspension were sprayed on the rice seedlings in the plastic pots at 100 ml/pot using a hand-held sprayer. One g of leaf sheath or leaf blade from rice plant samples was taken at 1, 4, 7 and 14 days after spraying the formulation. Leaf tissues were placed into 99 ml of

sterile distilled water and were incubated in water bath at 80 ^{0}C for 20 min to get rid of the other microorganisms. The number of bacteria on the leaf sheath or leaf blade was assessed using the drop plate method as mentioned in section 4.2.4.6. The value (Log. number/g of leaf sheath or leaf blade) of viable bacteria was the average of six replications (six drops) per dilution.

The inhibition of *R. solani* was tested using the same method as mentioned in section 4.2.4.7. Each treatment consisted of six replications.

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

4.2.7.1 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 was taken.

4.2.7.2 SEM observation of endospores on plant surface

One percent w/v effervescent granule formulation suspensions were sprayed on the rice seedlings in the plastic pots at 100 ml/pot using a hand-held sprayer. After spraying the selected formulations 1 h, one g of leaf sheath or leaf blade from rice plants was cut. They were then fixed in 2.5% 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 blades were taken.

4.2.8 Testing the efficacy of effervescent granules under greenhouse condition

The efficacy of the effervescent granule formulation against sheath blight was investigated under greenhouse conditions at the Central Laboratory and Greenhouse Complex, Faculty of Natural Resources, Prince of Songkla University, Hat Yai campus, Thailand. Ten

grams of sterile rice seeds infested with *R. solani* were placed in the centre of each plant 60 days after sowing. The effervescent granule formulation was seven treatments as listed in Table 4.2. Each treatment consisted of eight replications (three rice seedlings per replication).

Sheath blight assessment in the greenhouse tests was carried out 30 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 lesion which showed sheath blight symptoms. Percent Relative Lesion Height (% RLH) was calculated [Equation (4.3)] (Anh *et al.*, 1986). The entire length of the lesion on each rice tiller which had sheath blight symptoms was also measured.

$$\% \text{ RLH} = Li/Lt \times 100 \quad (4.3)$$

In equation (4.3), Li is the length of the lesion and Lt is the length of the rice tillers.

Data were subjected to one way analysis of variance compared with Duncan's Multiple Range Test (DMRT) at $p \leq 0.01$ or $p \leq 0.05$.

Table 4.2 Different types of effervescent granule treatment used under greenhouse condition

Treatment	Description
Control EG3	Broadcasted with effervescent granules without bacterial endospore 1, 5 and 10 day after pathogen inoculation (10 g/replication)
Pre-EG3	Broadcasted with bacterial effervescent granules 1 day before pathogen inoculation (10 g/replication) (10.50 Log. number/ replication)
Broadcaste-EG3	Broadcasted with bacterial effervescent granules 1, 5 and 10 day after pathogen inoculation (10 g/replication) (10.50 Log. number/ replication)
Spray-EG3	Sprayed with bacterial effervescent granules 1, 5 and 10 day after pathogen inoculation (100 ml/replication) (10.18 Log. number/ replication)
Fresh cell	Sprayed with fresh cells 1 day after pathogen inoculation (100 ml/ replication) (10.11 Log. number/replication)
Chemical fungicide	Sprayed with 0.15% w/v Iprodione liquid 1 day after pathogen inoculation (100 ml/replication)
Control	Rice plants inoculated only with *R. solani*

4.3 Results

4.3.1 Preparation and development of effervescent granule formulations

Six effervescent granules were successfully prepared using different proportions of PVP (k-30) (0-5%), lactose contained bacterial endospores (35-65%), citric acid (5-10%), tartaric acid (10-20%) and sodium bicarbonate (20-30%). All formulations were white in color. Evaluation of physical properties of all formulations was shown in Table 4.3. The pH values of 1% solution of all formulations were slightly acid (5.33-6.67). They were completely disintegrated in water within 8-35 sec at room temperature. The friability index of EG1-EG3 and EG4-EG6 was in the range of 92-94% and 34-57%, respectively, (Table 4.3) and there was significant difference among various formulations.

Table 4.3 pH, disintegration time and friability index of effervescent granule formulations

Formulations	pH	Disintegration time (sec)	Friability index (%)
EG1	5.33±0.06 c	23.57±0.27 a	93.33±0.24 a
EG2	6.67±0.06 a	18.74±0.30 c	92.53±0.13 a
EG3	6.53±0.08 a	19.71±0.29 b	93.53±0.07 a
EG4	5.39±0.06 c	12.74±0.18 d	56.17±0.11 b
EG5	6.53±0.08 a	9.03±0.36 e	55.97±0.03 b
EG6	6.27±0.02 b	8.70±0.44 e	34.47±0.22 c
Distilled water	6.59±0.14 a	-	-
F-test	**	**	**
C.V. (%)	1.30	2.06	1.78

** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p \leq 0.01$

From the data, formulation EG3 was selected for preparation of effervescent granule formulation containing *B. megaterium* because it has short disintegration time and high friability index. Furthermore, EG3 could incorporate the highest amount of *B. megaterium* endospores than that of other formulations, except EG6 (Table 4.1).

4.3.2 Preparation of effervescent granule formulation containing *B. megaterium*

4.3.2.1 Physical properties of effervescent granules

White effervescent granules containing *B. megaterium* were successfully developed by using 5% PVP (k-30), 60% lactose monohydrate contained bacterial endospores, 5% citric acid, 10% tartaric acid and 20% sodium bicarbonate (Figure 4.1). Bulk density and tapped density of granules were 0.47±0.01 and 0.52±0.01 g/ml, respectively. The friability index was 92.70±0.36%. They were completely disintegrated in water within 30 sec at room temperature. The pH value of bacterial suspension obtained (pH 6.57±0.05) was not different from the pH of distilled water (pH 6.64±0.10). The moisture content was 0.11±0.01%.

Figure 4.1 Bacterial effervescent granule EG3 (a) disintegration of granules in water (b)

4.3.2.2 Enumeration of viable bacteria in effervescent granules

The number of viable bacteria remained high after the formulation was produced. The effervescent granules contained viable bacteria number of 9.69±0.65 Log. number/g. The bacterial number remained high after storage at room temperature for 6, 12, 18 and 24 months (9.14±0.71, 9.06±0.63, 8.44±0.87 and 8.36±0.58 Log. number/g, respectively).

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

The inhibition of *R. solani* mycelial growth was presented in Figure 4.2. The bacterial effervescent granules had ability to inhibit mycelial growth of *R. solani* although the tests were carried out after 0, 6, 12, 18 and 24 months of storage at room temperature (% mycelial inhibition was 98.31±0.59%, 98.01±0.37%, 98.55±0.96%, 98.00±0.00% and 98.19±0.59%, respectively).

Figure 4.2 Inhibition of *R. solani* mycelial growth test on PDA, incorporated with effervescent granule formulation containing bacteria (left) compared with control (right)

4.3.3 Enumeration of viable bacteria in water and testing the inhibition of *R. solani* mycelial growth

The viability and effectiveness of bacteria were monitored after applying effervescent granules into water for 1, 4 and 7 days. The results showed that the bacteria released from effervescent granules could survive up to 7 days in water (at 9 Log. number/g) which were in the same range as the bacterial number of fresh cells (Figure 4.3). This study indicated that

effervescent granule formulation had no effect on the survival of bacterial antagonist in water. In addition, *B. megaterium* released from the granules had the same ability to inhibit *R. solani* mycelial growth as the bacterial fresh cells (Table 4.4).

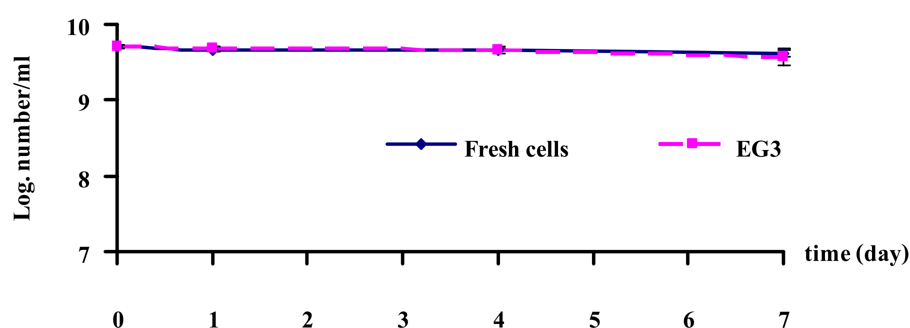

Figure 4.3 Number of viable bacteria in water after broadcast bacterial effervescent granules for 1, 4 and 7 days

Table 4.4 The effectiveness of bacterial formulations in inhibiting mycelial growth of *R. solani* after broadcast for bacterial effervescent granules for 1, 4 and 7 days

Formulation	% mycelia inhibition			
	0	1	4	7
EG3	94.63±0.52 a	98.99±0.26 a	99.42±0.22 a	99.19±0.32 a
Fresh cells	95.59±0.29 a	99.51±0.17 a	99.78±0.45 a	99.50±0.20 a
Sterile distilled water	00.00 b	00.00 b	00.00 b	00.00 b
F-test	**	**	**	**
C.V. (%)	2.43	0.29	0.18	0.31

** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p \leq 0.01$

4.3.4 Evaluation of viable bacteria on surface of rice tissues and testing the inhibition of *R. solani* mycelial growth

After spraying the effervescent granule formulation for 1, 4, 7 and 14 days, the number of bacteria on leaf sheath and leaf blade were counted using the drop plate method. The number of viable bacteria on the leaf sheath remained high (approximately 6 Log. number/g of leaf sheath) at 14 days after spraying with formulation. The bacterial counts on leaf sheath using spray formulation were in the same range as the bacterial counts using fresh cells (Table 4.5). In case of leaf blade, the number of viable bacteria on leaf tissue at 14 days after spraying with formulation was 1 log unit higher than the number of bacteria using fresh cells. In addition, *B. megaterium* from both rice tissue solutions had the highest ability to inhibit *R. solani* mycelial growth compared with the bacterial fresh cells and sterile distilled water at 1, 4, 7 and 14 days after spraying the effervescent granules (Table 4.6).

Table 4.5 Viability of fresh cells and cells of *B. megaterium* in effervescent granules on leaf sheath and leaf blade of rice plant

Formulation (A)	Log. number/g plant								
	(B) 1		4		7		14		Means (A)
	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	
Fresh cells	6.47±0.07	6.60±0.03	6.31±0.08	6.25±0.09	6.34±0.07	5.23±0.03	5.54±0.04	5.68±0.11	6.05±0.48
EG3	6.32±0.07	6.80±0.01	6.08±0.06	6.28±0.03	6.25±0.07	6.20±0.04	5.76±0.02	5.32±0.04	6.13±0.42
Means (B)	6.55±0.19 a		6.23±0.11 b		6.01±0.47 b		5.57±0.18 c		
Means (C)									
Leaf sheath	6.13±0.31								
Leaf blade	6.04±0.55								
F-test	**								
C.V. (%)	4.57								

** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at p≤0.01

Note: A=Formulation (Fresh cell and EG3)

B=Days after spraying formulation (1, 4, 7 and 14 days)

C=Plant tissues were detected (leaf sheath and leaf blade)

Table 4.6 Inhibition of mycelial growth of *R. solani* of fresh cells and effervescent granules after sprayed on leaf sheath and leaf blade of rice plant

Formulation (A)	% mycelial inhibition								
	(B) 1		4		7		14		Means (A)
	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	
Fresh cells	97.58±0.00	98.22±0.00	96.25±1.03	95.97±0.89	95.97±0.89	94.37±0.60	93.99±1.09	93.65±0.66	95.75±1.71 b
EG3	97.79±0.37	98.40±0.31	97.09±0.43	96.56±0.48	96.56±0.48	96.28±0.48	94.72±0.60	93.99±1.09	96.42±1.48 a
Means (B)	98.00±0.40 a		96.47±0.77 b		95.79±1.04 b		94.09±0.86 c		
Means (C)									
Leaf sheath	96.24±1.40								
Leaf blade	95.93±1.82								
F-test	**								
C.V. (%)	0.74								

** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p \leq 0.01$

Note: A=Formulation (Fresh cell and EG3)

B=Days after spraying formulation (1, 4, 7 and 14 days)

C=Plant tissues were detected (leaf sheath and leaf blade)

4.3.5 SEM observation of bacterial endospores on effervescent granule formulation and plant surface

The SEM micrographs of the endospores of *B. megaterium* on the surface of effervescent granule are shown in Figure 4.4a. Numerous normal bacterial cells were found on the surface of the effervescent formulations. These bacteria on the formulation surface were only a few numbers as the majority of the bacteria were encapsulated in the formulation. However, all bacterial cells will be easily released when the effervescent formulation are applied into the water. This is a positive characteristic of this type of formulation.

These endospores of *B. megaterium* were also observed on the surface of leaf sheath after spraying with bacterial formulation (Figure 4.4b). The bacterial endospores were also detected on the surface of leaf blade which possessed hairy texture as shown in Figure 4.4c. The fact that bacterial cells are detected on the rice plant surfaces sprayed with effervescent formulation may indicate that reduction of sheath blight disease incidence occurs because of the antagonistic activity of the bacteria (see also Table 4.7).

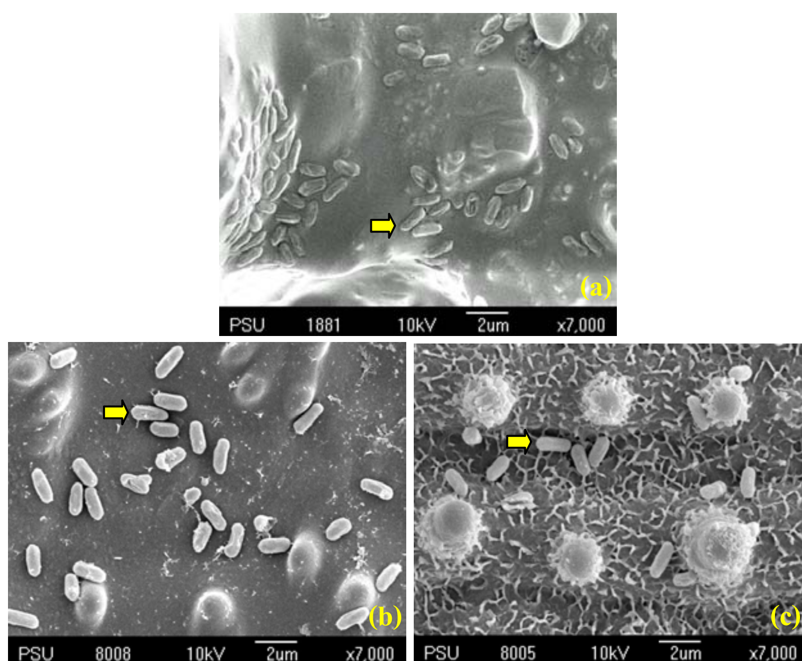

Figure 4.4 Micrographs of bacterial endospores on the surface of effervescent granule (a), leaf sheath (b) and leaf blade (c)

4.3.6 Testing the efficacy of effervescent granule formulations containing *B. megaterium* under greenhouse condition

The application of effervescent granules contains *B. megaterium* by any of these methods; broadcasting 1 day before pathogen inoculation (Pre-EG3), broadcasting 1, 5 and 10 day after pathogen inoculation (Broadcast-EG3) and spraying 1, 5 and 10 day after pathogen inoculation (Spray-EG3), significantly reduced the development of sheath blight lesion (Table 4.7). The number of lesion/tiller and lesion length decreased remarkably when compared to those of control. The severity of disease was greatest in control treatment (inoculated only with the *R. solani*), but it was least in chemical treatment (applied with Iprodione). Application rice plants with Pre-EG3, Broadcast-EG3, Spray-EG3, or fresh cells of the bacterium provided some protection against *R. solani*. The Broadcast-EG3 application had shown the most promising result in deterring sheath blight spread and suppressing sheath blight severity. In this experiment, the efficacy of bacterial effervescent granules was less than that of the chemical fungicide (Iprodione). However, the results indicated that effervescent granule formulation had potential for deterring and suppressing the development of sheath blight disease.

Table 4.7 Efficacy of effervescent granule formulation in suppressing the development of sheath blight disease in the pot test

Treatment	Number of lesion/tiller	Lesion length (cm)/tiller	Severity (% RLH)
Control EG3	11.70±1.46 b	25.51±2.03 a	51.00±3.41 a
Pre-EG3	5.39±2.56 d	19.24±2.56 b	38.79±2.21 bc
Broadcast-EG3	4.43±2.55 d	15.69±2.43 c	29.42±5.67 d
Spray-EG3	4.47±2.08 d	17.27±2.01 bc	38.40±2.27 bc
Fresh cell	7.46±2.89 c	20.68±2.35 b	33.28±3.42 dc
Iprodione	0.55±2.35 e	1.98±2.33 d	4.42±2.08 e
Control	13.63±2.32 a	28.11±1.05 a	51.81±3.02 a
F-test	*	*	*
C.V. (%)	22.68	25.50	27.29

* Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p \leq 0.05$

4.4 Discussions

In this chapter, *B. megaterium* was successfully devised in an effervescent granules by wet-granulation using the mixture of bacterial endospores and lactose monohydrate (at 1:5, v/w): PVP K-30: citric acid monohydrate: tartaric acid: sodium bicarbonate at ratio of 60:5:5:10:20. They were completely disintegrated in water and fast-released bacterial antagonist for suppressing the disease. As a result, this formulation is considered suitable for either broadcasting or spraying

The greenhouse test also indicated that effervescent granule was a suitable ingredient in producing a formulation containing *B. megaterium*. This is because the ingredients used in the formulation do not affect the viability of bacteria in the formulation and the ability of bacterial antagonist to inhibit mycilial growth of *R. solani* (section 4.2.2.2 and 4.2.2.3).

When broadcasted to the rice plant canopy, the bacterium in the effervescent formulations would be released quickly before the formulation deposits at the bottom of rice field due to gravity force. This would enhance the chance in which the bacterium could remain on the water surface and colonize rice plant at the water-rice plant interface to provide protection against pathogen infection and colonization. In addition, it is likely that the effervescent formulations developed for sheath blight disease control in this study, may be also suitable for use to control other plant diseases, particularly plant disease occurring in the closed system. One potentiality is the application of this type of formulation to deal with root diseases of plants grown hydroponically. Effervescent formulations may be applied to nutrient tank used to grow plant hydroponically. The circulated plant nutrients incorporated with antagonistic bacteria in the formulation would suppress root diseases and enhance plant growth.

The fact that the numbers of viable bacteria detected on both leaf sheath and leaf blade after spraying with either effervescent granule formulation or fresh cells are not different may indicate that the ingredients used in producing effervescent granules do not affect water surface tension of the droplets containing bacterial endospores. If water surface

tension of the droplets is increased, it is likely that the droplets may not remain on the surface of rice plant tissues (Bateman, 1999). This would reduce the numbers of bacteria on the surface of rice plant tissues. The deposition of bacteria on plant tissues is an important event which is required before the bacteria can multiply and maintain their population on the target tissues. Any factors affecting the deposition of bacteria onto target plant tissues would affect the initial population of the bacteria and will subsequently affect the number of the bacteria at some point in time (Chapter 3).

The result (in Table 4.5) also indicated that the bacterial population remained more or less the same up to 14 days after spraying with either effervescent or fresh cells of the bacteria. The fact that the bacteria population does not declined rapidly after spraying to plant tissues may indicate that this bacterium is a suitable candidate for formulation study and use to control diseases in the field condition. Survival capability of the bacterium on plant tissues is important because it would increase the potentiality of using this particular strain of bacterium for formulation study and use it successfully to control disease in the field.

For biological control agents to be marketable, the product should still contain high number of effective bacteria after formulation. An adequate shelf life of the biological control product is required to ensure that the product remains effective when it is applied by farmers in field conditions. Preliminary testing of bacterial formulations has been investigated and provided protection for sheath blight of rice in both greenhouse and field tests (Kanjanamaneesathian *et al*., 1998; Kusonwiriyawong *et al*., 1999; Pengnoo *et al*., 2000), but they had a comparatively short life, and the number of bacterial antagonists in the formulations greatly declined during 6 months storage (Pengnoo *et al*., 2000). Although, there is no convention regarding the length of shelf-life of products containing biological agents, it is perhaps sensible to devise formulations which have a shelf-life of preferable not less than 24 moths (Burges and Jones, 1998).

However, the fact that the numbers of bacterium in the formulations remained high after 24 months storage may indicate that the bacterial inoculum, used in the formulation, had only of viable endospores. In addition, the endospores of the bacteria was

detected on the surface of the formulation and plant tissues with SEM (Figure 4.4), these endospores may viable and as a result may be functional to inhibit *R. solani* when applied in the field conditions.

In conclusion, novel effervescent fast-disintegrating bacterial granules with storage stability, commercially feasible and easily applied either by broadcasting or spraying were developed. Viability and effectiveness of *B. megaterium* in inhibiting the mycelial growth of *R. solani* retained high after storage at room temperature more than 24 months. The greenhouse tests indicated that effervescent granules had potential to control rice sheath blight disease. Further studies are required to find out the appropriate dose and frequency of application to get acceptable level of control efficacy against sheath blight disease, when compared with fungicide application.

CHAPTER 5

PREPARATION AND EVALUATION OF *BACILLUS MEGATERIUM* EFFERVESCENT TABLETS

5.1 Introduction

In the previous chapter, novel bacterial effervescent granules which can be easily applied either by broadcasting or spraying were developed. Viability and effectiveness of *B. megaterium* in inhibiting the mycelial growth of *R. solani* retained after 24 months storage at room temperature. The greenhouse tests indicated that fast disintegrating bacterial granules had potential to control rice sheath blight disease (Chapter 4).

Effervescent tablet is an interesting form to be used in rice field. The benefit is that this formulation has specific and pre-determined dose, making it a lot more convenience for handling and application. Effervescent tablets are formulated by compressing granular effervescent in which a lubricant is needed to bring the material to the tablet dies. These lubricants should be water-soluble agents, such as sodium benzoate, polyethylene glycol and apidic acid. However, magnesium stearate is the most typical lubricant which has been used in the tablet formulation. The ideal amount and type of lubricant is one that easy to bring the tablet from the dies, prevent fill material from sticking to the surface of punches and protect worn-out of punches and dies. In this study, polyethylene glycol 6000 (PEG 6000) and magnesium stearate were chosen as lubricants for support the compression of effervescent tablet.

The aims of this study were (1) to develop bacterial effervescent tablet which can be applied by spraying on plant or by direct broadcasting to water, (2) to evaluate the physical and biological characteristics of the formulation and (3) to test the efficacy of bacterial formulation in suppressing sheath blight disease development in greenhouse conditions.

5.2 Material and methods

5.2.1 Materials

Citric acid monohydrate, tartaric acid and sodium bicarbonate were from Srichand United Dispensary (Thailand), lactose monohydrate (Pharmatose ® 200M) was from DMV International Distributor (Thailand) and polyvinyl pyrrolidone K-30 (PVP, K-30) was supplied by Vidhyasom (Thailand). Magnesium stearate was from S. Tong Chemical Co., Ltd. (Thailand) and Micronized polyethylene glycol (PEG 6000) was supplied by Vidhyasom (Thailand). Iprodione (Rovral ® Fungicide) was purchased from Bayer CropSciences (Australia). Plate count agar (PCA) (Difco ®) was from Becton, Dickinson and Company Sparks, MD (USA).

5.2.2 Preparation of bacterial endospores suspension

B. megaterium was used *as* a biocontrol agent in this study (Kanjanamaneesathian *et al*., 1998). The bacterium was cultured in the fermentor (dimension 30 l) containing 20 l of potato dextrose broth (PDB) at 30-35 ^{0}C, 200 rpm and 1 vvm for 4 days. The bacterial endospores were incubated in water bath at 80 ^{0}C for 20 min to get rid of the vegetative cells.

5.2.3 Preparation of effervescent tablet formulations (patent pending, No. 0701002092)

Effervescent tablets containing *B. megaterium* were prepared, using the mixture of lactose with bacterial endospores (60%), PVP (k-30) (5%), citric acid (5%), tartaric acid (10%) and sodium bicarbonate (20%). All ingredients were mixed in a planetary mixer until they became a damp mass. This mass was then passed through a granulator (sieve No.14) and dried in a hot air oven at 60 ^{0}C for 2 h. These granules were then passed through a sieve No.16 and mixed with different amount of magnesium stearate (0.5-1.0% w/w) or PEG 6000 (4.0-5.0% w/w) for 2-3 min as listed in Table 5.1. Tablets were compressed using a single punch tablet machine tooled with 1 inch round flat faced punch. Effervescent tablet without bacterial endospores were prepared as blank for the control treatment.

Table 5.1 Composition of lubricant in different effervescent tablet formulations

Lubricant (%)	ET1	ET2	ET3	ET4
Magnesium stearate	0.5	1.0	-	-
Polyethylene glycol (PEG 6000)	-	-	4.0	5.0

5.2.4 Evaluation of physical properties of effervescent tablets

5.2.4.1 Average weight, diameter, thickness and friability

Tablets (n=20) were randomly sampled from each formulation. Each tablet was weighed and the average of weight was calculated for each formulation. The diameter and thickness of each tablet were measured by Erweka tablet hardness tester and the average of thickness was calculated for each formulation. Tablet friability was conducted on 20 tablets using Roche friabilator. 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 (5.1)]. Three replicates were performed for each formulation.

$$\% \text{ friability} = (1 - W/W_0) \times 100 \qquad (5.1)$$

In equation (5.1), W_0 is the initial weight and W is the terminal weight.

5.2.4.2 Disintegration time of effervescent tablets

One effervescent tablet from each formulation was placed into 200 ml of distilled water, at room temperature (26-30 ^{0}C). Disintegration time of the tablet was recorded and six replicates were performed for each formulation.

5.2.4.3 pH

The pH values of dissolved formulation in section 5.2.4.2 were measured by pH meter. Six replicates were performed for each formulation.

5.2.4.4 Moisture content

Tablets (n=10) were weighted and dried in hot air oven at 60 ^{0}C until they attained a constant weight. The percent moisture content of effervescent tablet was measured. Three replicates were performed.

5.2.5 Enumeration of viable bacteria in effervescent tablets

The enumeration was carried out to determine the number of viable bacteria in one tablet of the formulation. The viability tests were carries out after the formulation were produced and during storage at room temperature (20-30 ^{0}C) in plastic containers for 24 months. Viable bacteria in the formulations were counted using the drop plate method (Zuberer, 1994). One tablet of each bacterial formulation was dissolved in 99 ml of sterile distilled water. The viable bacteria were cultured in plate count agar (PCA) at room temperature for 18-24 h after which colony forming unit were counted. The value (Log. number/g) of viable bacteria was the average of six replications (six drops) per dilution.

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

The mycelial growth inhibition of effervescent tablet was tested after production and during storage at room temperature (26-30 ^{0}C) in plastic containers for 24 months. One tablet of the bacterial formulations was dissolved in sterile distilled water 99 ml in a glass container. One ml of the solution was sampled and mixed with melted potato dextrose agar (PDA) at 1:10 (v/v) in Petri dishes (9.0 cm diameter). The agar plug of *R. solani* was cultured on PDA incorporated with solution of the bacterial effervescent tablet formulation to test the inhibition of *R. solani* mycelial growth. Each treatment consisted of six 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 (5.2)] (Gamliel *et al.*, 1989).

$$\% \text{ mycelial inhibition} = 100 - [(r^2/R^2) \times 100] \qquad (5.2)$$

In equation (5.2), r is colony radius of *R. solani* on PDA incorporated with one percent solution of bacterial effervescent tablet and R is colony radius of *R. solani* on PDA incorporated with sterile water.

5.2.7 Evaluation of viable bacteria in water and testing the inhibition of *R. solani* mycelial growth

To study the viability of bacteria in the water after broadcasting, one tablet of bacterial effervescent tablet formulation was placed to 99 ml of sterile distilled water at room temperature. One ml of water sample was taken at 1, 4 and 7 days after broadcasting and the number of bacteria was counted using the drop plate method. The number of viable bacteria per tablet of effervescent tablet was then calculated and the value was the average of six replications.

The inhibition of *R. solani* was tested using the same method as mentioned in section 5.2.6. Each treatment consisted of six replications.

5.2.8 Evaluation of viable bacteria on surface of rice tissues and testing the inhibition of *R. solani* mycelial growth

B. megaterium endospores suspension (fresh cells) and 3% w/v effervescent tablet formulation suspension were sprayed on the rice seedlings in the plastic pots at 100 ml/pot using a hand-held sprayer. One g of leaf sheath or leaf blade from rice plant samples was taken at 1, 4, 7 and 14 days after spraying the formulation. Leaf tissues were placed into 99 ml of sterile distilled water and were incubated in water bath at 80 ^{0}C for 20 min to get rid of the other microorganisms. The number of bacteria on the leaf sheath or leaf blade was assessed using the drop plate method as mentioned in section 5.2.5. The value (Log. number/g of leaf sheath or leaf blade) of viable bacteria was the average of six replications (six drops) per dilution.

The inhibition of *R. solani* was tested using the same method as mentioned in section 5.2.6. Each treatment consisted of six replications.

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

5.2.9.1 SEM observation of the selected formulations

A sample of effervescent tablet formulation was 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 was taken.

5.2.9.2 SEM observation of endospores on plant surface

Three percent w/v bacterial effervescent tablet formulation suspensions were sprayed on the rice seedlings in the plastic pots at 100 ml/pot using a hand-held sprayer. After spraying the selected formulations 1 h, one g of leaf sheath or leaf blade from rice plants was cut. They were then fixed in 2.5% 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 blades were taken.

5.2.10 Testing the efficacy of effervescent tablet formulation containing *B. megaterium* under greenhouse conditions

The efficacy of the effervescent tablet formulation against sheath blight was investigated under greenhouse conditions at the Central Laboratory and Greenhouse Complex, Faculty of Natural Resources, Prince of Songkla University, Hat Yai campus, Thailand. Ten grams of sterile rice seeds infested with *R. solani* were placed in the centre of each plant 60 days after sowing. The effervescent tablet formulation was eight treatments (Table 5.2). Each treatment consisted of eight replications (three rice seedlings per replication).

Sheath blight assessment in the greenhouse tests was carried out 30 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 tiller which showed sheath blight symptoms. Percent Relative Lesion Height (% RLH) was calculated [Equation (5.3)]

(Anh *et al*., 1986). The entire length of the lesion on each rice tiller which had sheath blight symptoms was also measured.

$$\% RLH = Li/Lt \times 100 \quad (5.3)$$

In equation (5.3), Li is the length of the lesion and Lt is the length of the rice tillers.

Data were subjected to one way analysis of variance compared with Duncan's Multiple Range Test (DMRT) at $p \leq 0.01$ or $p \leq 0.05$.

Table 5.2 Different types of effervescent tablet treatment used under greenhouse condition

Treatment	Description
ET3-Bb	Broadcasted with bacterial effervescent tablet 1 day before pathogen inoculation (1 tablet/replication) (9.57 Log. number/ replication)
ET3-Sb	Sprayed with 0.3% bacterial effervescent tablet 1 day before pathogen inoculation (100 ml/replication) (9.57 Log. number/ replication)
ET3-Sa	Sprayed with 0.3% bacterial effervescent tablet 1, 7 and 15 day after pathogen inoculation (100 ml/replication) (9.57 Log. number/replication)
ET3-BS	Broadcasted with bacterial effervescent tablet 1 day before pathogen inoculation (1 tablet/replication), sprayed with 0.3% effervescent tablet 1, 7 and 15 day after pathogen inoculation (100 ml/replication) (9.57 Log. number/replication)
ET3-SS	Sprayed with 0.3% effervescent tablet 1 day before pathogen inoculation (100 ml/replication), sprayed with 0.3% effervescent tablet 1, 7 and 15 day after pathogen inoculation (100 ml/replication) (9.57 Log. number/replication)
Fresh cells	Sprayed with fresh cells 1 day after pathogen inoculation (100 ml/ replication) (9.13 Log. number/replication)
Chemical fungicide	Sprayed with 0.15% w/v Iprodione liquid 1 day after pathogen inoculation (100 ml/replication)
Control	Rice plants inoculated only with *R. solani*

5.3 Results

5.3.1 Preparation and development of effervescent tablet formulation

Four formulas of effervescent tablets for broadcast and spray application were prepared (Table 5.3).

Table 5.3 Evaluated characteristics of each effervescent tablet formulation

Formulations*	Lubricant	Characteristics
ET1	0.5% Magnesium stearate	Binding, incomplete disintegration, a small amount of foam
ET2	1.0% Magnesium stearate	Not sticking with punch and die, incomplete disintegration, lots of foam
ET3	4% PEG 6000	Smooth surface, not sticking with punch and die, complete disintegration
ET4	5% PEG 6000	Sticking, picking and filming, complete disintegration

*All formulations were consisted PVP (k-30) (5%), lactose monohydrate (60%), citric acid (5%), tartaric acid (10%) and sodium bicarbonate (20%)

After the preliminary test, protocol used for devising the formulation ET3 was chosen for preparation of the effervescent tablet formulation containing *B. megaterium* as a result of its desired characteristics such as having smooth tablet surface and non-adhesive to the punch with low friable percentage.

5.3.2. Physical properties of effervescent tablets containing *B. megaterium*

Effervescent tablets containing *B. megaterium* were successfully developed by using proportion of 5% PVP (k-30), 60% lactose monohydrate contained bacterial endospores, 5% citric acid, 10% tartaric acid, 20% sodium bicarbonate and 4% PEG 6000 (Figure 5.1). Tablets had smooth surface and white in color. The results of average weight, diameter and

thickness were 3.31±0.11 g, 26.26±1.03 mm and 4.99±0.21 mm, respectively. They were completely disintegrated in water within 3 min at room temperature (Figure 5.2). The friability was 0.18±0.09%. The pH value of bacterial suspension obtained was pH 6.03±0.08. The moisture content was 0.22±0.01%.

Figure 5.1 Bacterial effervescent tablets

Figure 5.2 Disintegration of bacterial effervescent tablet formulation

5.3.3 Enumeration of viable bacteria in effervescent tablets

The number of viable bacteria remained high after the formulation was produced. The effervescent tablets contained viable bacteria number of 9.57±0.50 Log. number/tablet. The bacterial number remained high after storage at room temperature for 6, 12, 18 and 24 months (9.37±0.50, 9.71±0.75, 9.17±0.58, and 9.44±1.00 Log. number/tablet, respectively).

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

The inhibition of *R. solani* mycelial growth was presented in Figure 5.3. The bacterial effervescent tablets had ability to inhibit mycelial growth of *R. solani* although the tests were carried out after 0, 6, 12, 18 and 24 months of storage at room temperature (% mycelial inhibition was 99.95±0.00%, 99.85±0.09%, 99.56±0.00%, 99.64±0.14% and 99.90±0.09%, respectively).

Figure 5.3 Inhibition of *R. solani* mycelial growth test on PDA, incorporated with effervescent tablet formulation containing bacteria (right) compared with control (left)

5.3.5 Enumeration of viable bacteria in water and testing the inhibition of *R. solani* mycelial growth

The viability and effectiveness of bacteria were monitored after applying effervescent tablet into water for 1, 4 and 7 days. The results showed that the bacteria released from tablets could survive up to 7 days in water (at 9 Log. number/g) which were in the same range as the bacterial number of fresh cells (Figure 5.4). This study indicated that effervescent

tablet formulation had no effect on the survival of bacterial antagonist in water. In addition, *B. megaterium* released from the tablets had the same ability to inhibit *R. solani* mycelial growth as the bacterial fresh cells (Table 5.4).

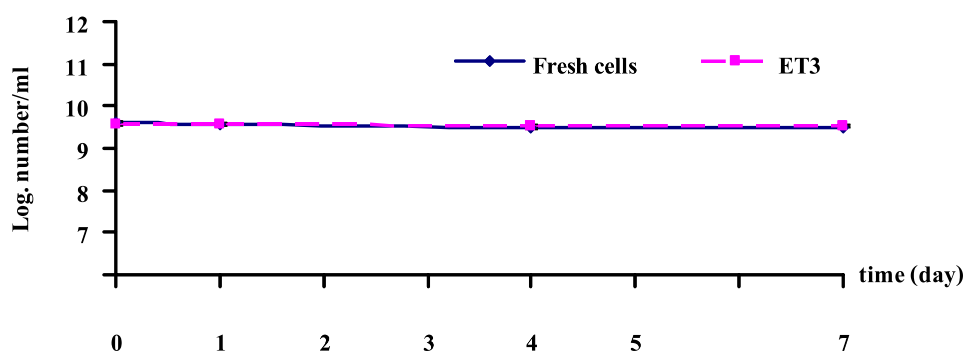

Figure 5.4 Number of viable bacteria in water after broadcast bacterial effervescent tablets for 1, 4 and 7 days

Table 5.4 The effectiveness of bacterial effervescent tablet in inhibiting mycelial growth of *R. solani* after broadcast for bacterial effervescent tablet formulation for 1, 4 and 7 days

Formulation	% mycelia inhibition			
	0	1	4	7
ET3	99.95±0.00 a	99.90±0.09 a	99.77±0.20 a	99.85±0.09 a
Fresh cells	99.90±0.09 a	99.85±0.09 a	99.72±0.14 a	99.72±0.14 a
Sterile distilled water	00.00 b	00.00 b	00.00 b	00.00 b
F-test	**	**	**	**
C.V. (%)	0.08	0.11	0.21	0.14

** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p \leq 0.01$

5.3. Evaluation of viable bacteria on surface of rice tissues and testing the inhibition of *R. solani* mycelial growth

The number of bacteria on leaf sheath and leaf blade was counted using the drop plate method after spraying the effervescent tablet formulation for 1, 4, 7 and 14 days. The bacterial population was declined on both rice tissues which had been sprayed with the fresh cells or solution of bacterial effervescent tablets (Table 5.5). However, the number of viable bacteria on surface of both rice tissues remained high (approximately 5 Log. number/g plant) at 14 days after spraying. The bacterial counts on rice tissues using spray formulation were in the same range as the bacterial counts using fresh cells (Table 5.5). The number of bacteria on a leaf sheath was also higher than that on the leaf blade when rice plants were sprayed with both types of inoculums (Table 5.5).

In addition, the solution of bacteria from leaf sheath or leaf blade had the highest ability to inhibit *R. solani* mycelial growth using dual culture method compared with the bacterial fresh cells and sterile distilled water at 1, 4, 7 and 14 days after spraying the effervescent tablet solution (Table 5.6).

Table 5.5 Viability of fresh cells and cells of *B. megaterium* in effervescent tablet formulation on leaf sheath and leaf blade of rice plant

Formulation (A)	Log. number/g plant								
	(B) 1		4		7		14		Means (A)
	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	
Fresh cells	6.05±0.08	6.17±0.03	6.15±0.15	5.62±0.07	5.92±0.03	5.84±0.04	5.54±0.05	5.40±0.05	5.83±0.28
ET3	6.44±0.02	6.45±0.03	6.18±0.06	5.16±0.17	5.78±0.01	5.64±0.04	5.60±0.02	5.48±0.03	5.84±0.45
Means (B)	6.27±0.18 a		5.78±0.45 b		5.79±0.11 b		5.50±0.09 c		
Means (C)									
Leaf sheath	5.96±0.30 a								
Leaf blade	5.72±0.40 b								
F-test **									
C.V. (%) 3.96									

** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p \leq 0.01$

Note: A=Formulation (Fresh cell and ET3)

B=Days after spraying formulation (1, 4, 7 and 14 days)

C=Plant tissues were detected (leaf sheath and leaf blade)

Table 5. Inhibition of mycelial growth of *R. solani* of fresh cells and effervescent tablet after sprayed on leaf sheath and leaf blade of rice plant

Formulation (A)	% mycelial inhibition								
	(B) 1		4		7		14		Means (A)
	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	
Fresh cells	92.28±1.05	83.13±0.21	89.81±1.60	86.75±0.69	87.34±1.38	89.23±1.35	84.51±0.60	83.33±0.94	87.05±3.26
ET3	88.89±0.00	90.95±0.91	88.38±0.88	87.33±1.58	87.84±1.82	86.01±0.83	89.30±1.20	90.29±1.38	88.62±1.83
Means (B)	88.81±3.70		88.07±1.62		87.60±1.69		86.86±3.25		
Means (C)									
Leaf sheath	88.54±2.34								
Leaf blade	87.13±2.96								
F-test	ns								
C.V. (%)	2.97								

ns=means in the same column are not statistical significantly different

Note: A=Formulation (Fresh cell and ET3)

B=Days after spraying formulation (1, 4, 7 and 14 days)

C=Plant tissues were detected (leaf sheath and leaf blade)

5.3.7 SEM observation of bacterial endospores on effervescent tablet formulation and plant surface

Endospores SEM micrographs of *B. megaterium* on the surface and cross-section of effervescent tablet were shown in Figure 5.5a and 5.5b, respectively. The shape of endospores was rod or cylinder. Numerous bacterial cells were found on the surface of the effervescent formulations. Nonetheless, a large number of the bacteria were encapsulated in the formulation. The encapsulated bacterial cells will be, however, liberated from the encapsulation upon being applied to the water.

The endospores of *B. megaterium* were also observed on the surface of leaf sheath after spraying the rice plants with bacterial formulation (Figure 5.5c). The bacterial endospores were also detected on the surface of leaf blade which possessed hairy texture as shown in Figure 5.5d.

The presence of these bacteria on leaf sheath and leaf blade may attribute to the reduced sheath blight disease incidence (see also Table 5.6).

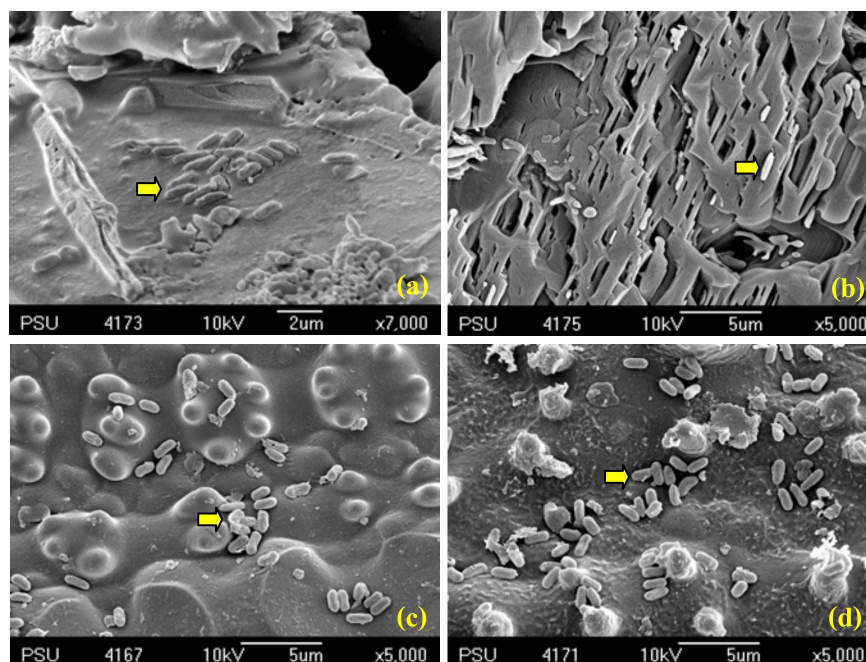

Figure 5.5 Micrographs of bacterial endospores on the surface (a) and cross-section (b) of effervescent tablet, leaf sheath (c) and leaf blade (d)

5.3.8 Testing the efficacy of effervescent tablet formulation containing *B. megaterium* under greenhouse conditions

Biological control of rice sheath blight disease was confirmable in the assessment of the lesion length/tiller with sheath blight symptoms where treatment with broadcasting 1 day before pathogen inoculation follow by spraying 1, 7 and 15 day after pathogen inoculation (ET3-BS) had the lowest lesion length/tiller (14.01±1.24 cm). There was also no statistical difference among ET3-SS treatment (14.05±1.30 cm) that spraying 1 day before pathogen inoculation follow by spraying 1, 7 and 15 day after pathogen inoculation (Table 5.7). When % RLH was compared, the ET3-SS (27.07±1.28%) and ET3-BS (29.65±2.72%) treatments were the most effective formulation in deterring sheath blight spread and suppressing sheath blight severity (Table 5.7).

Bacterial effervescent tablet significantly reduced the incidence of disease compared with the infected control. This formulation was also effective against *R. solani*, although less active than chemical fungicide (Iprodione). In this experiment, the results indicated that broadcasting or spraying with bacterial effervescent tablet 1 day before pathogen inoculation provided some prevention against *R. solani* to infect the rice plant. Bacterial effervescent tablet had potential for controlling the development of rice sheath blight disease when spraying 1, 7 and 15 day after pathogen inoculation.

Table 5.7 Efficacy of effervescent tablet formulation in suppressing the development of sheath blight disease in the pot test

Treatment	Lesion length (cm)/tiller	Severity (% RLH)
ET3-Bb	17.25±1.78 b	38.28±1.71 b
ET3-Sb	18.03±2.15 b	36.85±2.69 b
ET3-Sa	15.27±0.98 c	29.86±2.94 c
ET3-BS	14.01±1.24 c	29.65±2.72 c
ET3-SS	14.05±1.30 c	27.07±1.28 c
Fresh cell	17.77±1.57 b	37.22±2.44 b
Chemical fungicide	7.06±1.32 d	15.46±2.99 d
Control	35.40±2.45 a	67.56±2.52 a
F-test	*	*
C.V. (%)	9.58	7.03

* Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p \leq 0.05$

5.4 Discussions

In the formulation study, *B. megaterium* effervescent tablet was developed using a pharmaceutical technology to suppress rice sheath blight disease. This formulation is considered suitable for broadcast and spray application. Although effervescent granules has been developed for both application in Chapter 4, effervescent tablet is a novel interesting form to be used in rice field because of its convenient use and accurate dose adjustment (Ansel *et al*., 1995). As a result, special consideration is desired in term of selecting chemical agents for use as a lubricant material in the formulation. These compounds should enhance the flow of the material into the tablet dies, minimize wear of the punches and dies, prevent fill material from sticking to the punches and dies and produce tablets with sheen (Allen *et al*., 2004).

This study showed that formulation ET3, containing 4.0% PEG 6000 as a lubricant agent, have superior physical characteristics to formulation ET1, ET2 and ET4 which contained 0.5, 1.0% magnesium stearate and 5% PEG 6000, respectively (Table 5.3). Formulation ET1 was binding with punch and die because 0.5% magnesium stearate lubricant agent might not be enough for compression. Although formulation ET2 with 1.0% magnesium stearate was not sticking with punch and die, using magnesium stearate as the lubricant might cause incomplete disintegration and lots of foam. Formulation ET3 and ET4 showed complete disintegration because PEG 6000 was water-soluble lubricant. However, formulation ET4 was sticking, picking and filming with punch and die, while formulation ET3 was not (Table 5.3). The beneficial characteristics included having a non-adhesive and smooth tablet surface with low moisture content. The tablet was not prone to friable and quickly disintegrated in water. The tablet had high bacterial viability with good inhibitory activity against the mycelial growth of *R. solani*.

In the greenhouse test, the application of bacterial effervescent tablets by ET3-Bb, ET3-Sb, ET3-Sa, ET3-BS and ET3-SS methods had lesion length decreased remarkably when compared to those of control. The severity of disease was greatest in control treatment (inoculated only with the *R. solani*), but it was least in chemical treatment

(applied with Iprodione). The ET3-SS and ET3-BS treatments are more effective formulation to suppress sheath blight disease than the other treatments (Table 5.7). When spray or broadcast with bacterial effervescent tablet 1 day before pathogen inoculation that the bacteria offered preventively as opposed to curatively pathogen infection and colonization. Followed by spraying 1, 7 and 15 day after pathogen inoculation, the bacteria in the effervescent tablets might be deposited on various rice plant tissues and the bacterial antagonist may have inhibited mycelial growth and subsequently reduced infection and disease incidence both horizontally and vertically. The fact that the number of viable bacteria and their efficacy on rice tissues were remained high to 14 days after spraying with bacterial effervescent tablet (Table 5.5, Table 5.6). The results also indicated that this bacterium is a suitable applicant for formulation study and use to suppress disease in the field condition. In addition, the bacterial effervescent tablet may be also suitable for use to combat root disease of plants grown hydroponically. Due to the bacterial effervescent tablet is fast released an antagonistic bacterium to provide protection against pathogen when broadcasted to the water.

In terms of shelf-life study, the number of bacteria in the effervescent tablet formulation, which was assessed after the formulation process, was an approximately 9 Log. number/tablet. The viability of bacteria in this formulation still remained high (approximately 9 Log. number/tablet) after 24 months storage in plastic bag at room temperature. This is perhaps because only bacterial endospores have been used in the formulation process (Wiwattanapatapee *et al*., 2004).

More study in rice field, which have sheath blight disease problem are needed to determine the effective dose and frequency of application of this effervescent tablet formulation in suppressing rice sheath blight disease. This data is critical as it useful for the farmers who will decide to replace chemical fungicide with biological control product for controlling sheath blight disease of rice.

CHAPTER 6

EFFICACY OF *BACILLUS MEGATERIUM* FORMULATIONS UNDER FIELD CONDITION

6.1 Introduction

The formulations of *B. megaterium*, such as water-soluble granule and effervescent forms, have been tested to suppress rice sheath blight disease under laboratory and greenhouse conditions (Chapter 3, 4 and 5). However, conditions in laboratory and greenhouse are not the same as in nature, field trials are essential to truly see the potential of theses bacterial antagonist formulations. Rice plants in nature are subject to several different stresses, and to really study the efficiency of the bacterial formulation, plants have to be monitored during a lifetime of fungal infection, pest attacks, drought, and all other facets of plant life. Seed yield, which may be considered to be the foremost indicator of plant fitness, is an important factor to study. Lumsden *et al*. (1995) stated that biological control systems consist of interactions among antagonists-pathogens-other soil micro flora-hosts, compounding the problems of variability in both greenhouse and field conditions, although variability of efficacy is especially acute in field application. Poor correlation between *in vitro* and *in vivo* results may also be related to inactivation of antibiotics which can occur in the natural environment (Knudsen *et al*., 1997).

In greenhouse test, bacterial floating pellets showed promising results in suppression of the development of sheath blight (Wiwattanapatapee *et al*., 2004). In contrast, this formulation may become less effective in the field test. 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 condition (Kanjanamaneesathian *et al*., 2007).

B. subtilis was used successfully to control *Pythium* and *Rhizoctonia* diseases and increased the yield of ornamental plants grown in sterilized soil (Broadbent *et al*., 1977). However, tests in field soils by this strain of *B. subtilis* did not always increase the yield. Because

some microorganisms which produce antibiotics in sterilized soil condition fail to produce so under unsterilized condition (Shoda, 2000).

Ngugi *et al*. (2005) shown that application of Serenade, a commercial biofungicide formulation containing *B. subtilis*, had the efficacy to suppress the mummy berry fungus *Monilinia vaccinii-corymbosi* and did not negatively affect on fruit characteristics when evaluated in laboratory or greenhouse. However, field data indicated a negative impact on both fruit set and seed numbers, implying that caution should be exercised in applying this product in circumstances or conditions otherwise unfavorable for adequate pollination.

In field studies, the commercial product of *B. subtilis* (Kodiak) did not control black scurf or stem canker of potato (Larkin, 2001; Brewer and Larkin, 2005). Spore counts were conducted on the formulations to confirm viability. All counts were consistent with the spore counts provided on label by the manufacturer. The commercial formulation may not have been successful because of the specific soil type used in these experiments, the other specific environmental conditions of the product, or perhaps it is not the best formulation for use with potato.

The majority of strategies for biocontrol of rice sheath blight disease, caused by *R. solani*, rely on a single method application may be less effective because *R. solani* survives in soil as sclerotia and in rice plant tissues as mycelia (Ou, 19 5). Only, seed treatment or broadcasting or spraying application of bacterial antagonist formulation is not likely to perform consistently against all pathogens of the crop or under diverse rhizosphere and soil environmental conditions. A combination of method application is more likely to have a greater variety of traits responsible for disease suppression and also is likely to have these traits expressed over a wide range of environmental conditions.

Water-soluble granule formulation was highly soluble, with optimal viscosity, high-bacterial viability and high-activity against mycelium growth of *R. solani*. The formulation would be suitable for spray application (Chapter 3). This formulation, when used as seed treatment, also had no effect on the germination of rice seeds and did not cause abnormality of seedling shape (unpublished data). The effervescent formulations had good physical and biological properties. They were completely disintegrated in water which would be suitable for broadcast application (Chapter 4 and 5). Nevertheless, field trials to validate the efficacy of these

formulations are required because the field environments may be varied and different from the greenhouse setting.

The objective of this chapter was to test the efficacy of water-soluble granules (by spraying and seed treatment) and effervescent granules (by broadcasting) in sheath blight disease suppression in field condition.

6.2 Material and methods

6.2.1 Experimental design and treatments

The efficacy of the formulations against sheath blight was investigated under field conditions at Chian-Yai District, Nakhornsithammarat Province, Thailand in 2006. There were eight treatments in the field trial. Each treatment consisted of six blocks (replications), with 64 rice hills/subplot. The experiment was arranged in a Randomized Complete Block Design (RCBD). The details of the treatments for the field tests are shown in Table 6.1.

Table 6.1 Different types of treatment used under field condition

Treatment*	Description
1. SG2 (normal)	Sprayed with 3% water-soluble granules 60, 65 and 70 days after sowing without pathogen inoculation (0.6 l/subplot)
2. EG3 (normal)	Broadcasted with effervescent granules 60, 65 and 70 day after sowing without pathogen inoculation (1 g/subplot)
3. Chemical fungicide (normal)	Sprayed with 0.15% w/v Iprodione liquid 60 days after sowing without pathogen inoculation (0.5 l/subplot)
4. Control (normal)	Rice plants without *R. solani*
5. SG2	Sprayed with 3% water-soluble granules 1, 5 and 10 day after pathogen inoculation (0.6 l/subplot)
6. EG3	Broadcasted with effervescent granules 1, 5 and 10 day after pathogen inoculation (1 g/subplot)
7. Chemical fungicide	Sprayed with 0.15% Iprodione liquid 1 day after pathogen inoculation (0.5 l/subplot)
. Control	Rice plants inoculated only with *R. solani*

* In treatment 1, 2, 3 and 4, rice plants were not inoculated with *R. solani*, while treatment 5, 6, 7 and rice plants were inoculated with *R. solani*

6.2.2 Rice field preparation

The rice plot 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 (replications). Each block was further partitioned into subplots (2x2 m) by 30 cm width earth embankment to prevent water movement among the subplots (Figure 6.1). The rice filed was flooded again and the water level was maintained by opening or closing a small gate on each subplot embankment.

6.2.3 Rice seedling and rice plant preparation

Seeds of rice (cv. RD-23) were treated with 1% w/w of water-soluble granule formulation and incubated in the moistened sack for 1 day. Rice seedlings were raised in the seedling bed of a farmer's field. After 15 days, these rice seedlings were transplanted into the rice field with 20 cm spacing between and within rows in each 2x2 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 subplot 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 (Figure 6.1).

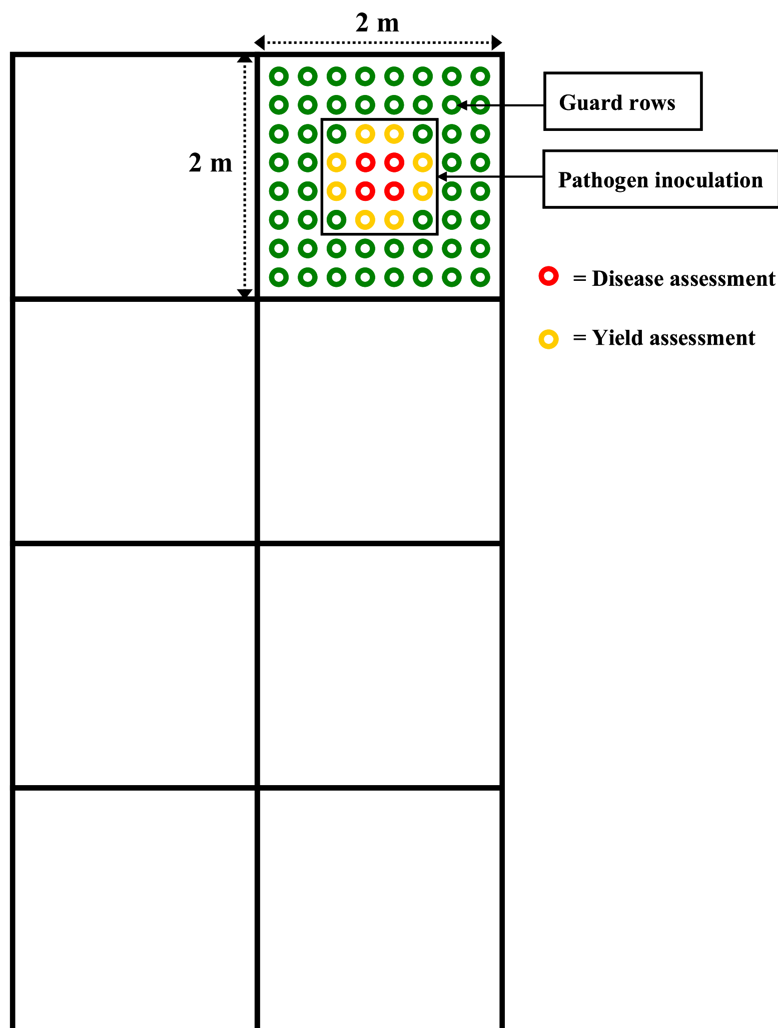

Figure 6.1 Eight subplots in each block (replication) of rice field trials

6.2.4 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 (Figure 6.1). Ten g 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 (Figure 6.2). The water level in the subplot was maintained at the same level throughout the experiment.

Figure 6.2 Pathogen inoculation

6.2.5 Disease and yield assessment

For sheath blight disease assessment, only the 4 innermost rice hills were uprooted, and sheath blight symptoms were assessed 14 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. Percent Relative Lesion Height (% RLH) was calculated [Equation (6.1)] (Anh *et al.*, 19 6). The entire length of the lesion on each rice tiller which had sheath blight symptoms was also measured.

$$\% \text{ RLH} = \text{Li}/\text{Lt} \times 100 \tag{6.1}$$

In equation (6.1), Li is the length of the lesion and Lt is the length of the rice tillers.

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 (Figure 6.3a). Fresh and dry of 1,000 total kernels (undeveloped kernels and whole kernels) and 1,000 whole kernels were weighed (Figure 6.3b, 6.3c). Four replicates were used for each treatment.

Figure 6.3 Panicles (a), total kernels (b) and whole kernels (c)

6.2.6 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. Data was compared with Duncan’s Multiple Range Test (DMRT) at $p \leq 0.01$ or $p \leq 0.05$.

6.3 Results

In the field tests, the application of novel formulations containing *B. megaterium* by either spray or broadcast 1, 5 and 10 days after pathogen inoculation, significantly reduced the development of sheath blight lesion when % tillers with sheath blight symptom was assessed (Table 6.2). The water-soluble granule formulation (at 0.6 l/subplot) and effervescent granule formulation (at 1 g/subplot) were more effective than the chemical fungicide (Iprodione) in sheath blight suppression. The severity of disease was greatest in control treatment (inoculated only with the *R. solani*) and chemical fungicide treatment (applied with Iprodione). In this experiment, the results indicated that selected bacterial formulations had potential for deterring and suppressing the development of sheath blight disease.

At the end of the experiment (110 days after transplanting), rice plants applied with either water-soluble granule (at 0.6 l/subplot) and effervescent granule (at 1 g/subplot) formulations, both containing *B. megaterium*, had quite high fresh panicle weights, fresh and dry weights of 1,000 whole kernels. These were significantly different from those with chemical fungicide (Iprodione) or control (inoculated only with *R. solani*) (Table 6.3).

Table 6.2 Efficacy of novel formulations in suppressing the development of sheath blight disease in the field test

Treatment**	% of tiller with sheath blight symptom	Severity (% RLH)
1. SG2 (normal)***	0.00±0.00 d	0.00±0.00 d
2. EG3 (normal)	0.00±0.00 d	0.00±0.00 d
3. Chemical fungicide (normal)	0.00±0.00 d	0.00±0.00 d
4. Control (normal)	2.04±0. 7 d	0.47±0.25 d
5. SG2	1 .67±2.64 c	5.36±1.62 c
6. EG3	25.69±3.30 c	7.76±2.44 c
7. Chemical fungicide	49.32±2.4 b	19.24±2.42 b
. Control (only *R. solani*)	100.00±0.00 a	42.17±1.56 a
F-test	*	*
C.V. (%)	20.99	29.05

* Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at p≤0.05

** Rice seeds of all treatments (except treatment 4) were treated with bacterial formulation before broadcasting in the field

*** In treatment 1, 2, 3 and 4, rice plants were not inoculated with *R. solani*, while treatment 5, 6, 7 and rice plants were inoculated with *R. solani*

Table 6.3 Efficacy of novel formulations on fresh weight of rice panicles and fresh and dry weight of 1,000 total kernels and 1,000 whole kernels

Treatment**	Panicle weight (g)[V]	1,000 total kernels		1,000 whole kernels	
		Fresh weight (g)[W]	Dry weight (g)[X]	Fresh weight (g)[Y]	Dry weight (g)[Z]
1. SG2 (normal)***	6.79 bcd	17.06 bc	15.27 b	24.27 cd	21. 0 cd
2. EG3 (normal)	6.75 bcd	17.12 b	15.32 b	25. 0 ab	23.14 ab
3. Chemical fungicide (normal)	6.04 cde	15. 0 d	14.14 c	23.13 e	20.75 de
4. Control (normal)	5.02 e	15.24 d	13.6 c	25.14 bc	22.5 bc
5. SG2	.67 a	19.5 a	17.54 a	26. 3 a	24.0 a
6. EG3	7.95 ab	15.69 d	14.0 c	23.77 de	21.34 de
7. Chemical fungicide	7.27 abc	14.94 d	13.43 c	25.2 bc	22.75 bc
. Control (only *R. solani*)	5.67 de	15.99 cd	14.39 bc	22. 9 e	20.61 e
F-test	*	*	*	*	*
C.V. (%)	14.25	4.63	4.59	3.14	3.57

* Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p \leq 0.05$

** Rice seeds of all treatments (except treatment 4) were treated with bacterial formulation before broadcasting in the field

*** In treatment 1, 2, 3 and 4, rice plants were not inoculated with *R. solani*, while treatment 5, 6, 7 and rice plants were inoculated with *R. solani*

[V] Panicle weight is the average of rice hills which were inoculated with novel formulations and *R. solani* inculum

[W, X] Fresh and dry weight are the average of 1,000 total kernels (undeveloped kernels and whole kernels) which were inoculated with novel formulations and *R. solani* inculum

[Y, Z] Fresh and dry weight are the average of 1,000 whole kernels which were inoculated with novel formulations and *R. solani* inculum

6.4 Discussions

In this experiment, the water-soluble granules (0.6 l/subplot) or effervescent granules (1 g/subplot) performed better than the chemical fungicide (Iprodione) in sheath blight suppression when % of tiller with sheath blight symptom and % severity was evaluated (Table 6.2). While, the efficacy of these bacterial formulations was less than that of the chemical fungicide under greenhouse conditions (Chapter 3, 4 and 5). Enhanced control of sheath blight of rice may also be provided by combining seed treatment with either spray or broadcast application of bacterial formulation. However, this performance is achieved through the application of a bacterial antagonist formulation three times comparing with the application of fungicide only once after the pathogen inoculation. As the cost of these formulations is relatively cheap, frequent application of these formulations is reasonable and acceptable. Moreover, these formulations are easy to produce, handle and apply, have no inhalation hazard, and do not readily drift in the wind. Farmers will be more like to accept biological control measures because they are familiar with their handling properties and can use conventional equipment to apply them.

In the field, treating rice seeds with the water-soluble granule formulation prior to sowing significantly reduced disease incidence of sheath blight compared with the pathogen infected control. Seed treatments set the stage for early colonization of germinating seedlings by the antagonistic bacterium and it has been shown to be an important determinant for rhizosphere and rhizoplane colonization (Lewis, 1991; Mcintyre and Press, 1991; Lumsden *et al.*, 1995).

Interestingly, all treatments which treated seed with formulation decreased the disease incidence and increased the yield of rice. This could be due to the high density of propagules of the antagonist provided by seed treatment and these propagules might have proliferated faster at the seed application site, colonized the rhizosphere of the developing seedling and, thereby, protected the plant from pathogen attack for a long time. While, spray or broadcast application would enhance the chance in which the bacterium could remain on the water surface and colonize rice plant at the water-rice plant interface to provide protection against *R. solani* pathogen infection and colonization.

Yield of rice plants sprayed with the water-soluble formulation is also greater than yield of rice plants sprayed with fungicide (Table 6.3). It is possible that *B. megaterium* may contribute to the increase in the yield of rice. Stimulation of different crops by *Bacillus* rhizobacterial inoculation has also been demonstrated by other studies, both in laboratory and field trials. For example, it was reported that wheat yield increased up to 43% with *Bacillus* inoculants (Kloepper *et al*. 19 9) and a 10-20% yield increase in the same crop was reported in field trials using a combination of *B. megaterium* and *Azotobacter chroococcum* (Brown, 1974).

In addition, the bacterial formulations also had potential for deterring and suppressing the development of rice blast disease caused by *Pyricularia grisea* (data not shown). This result supported the study of Leelasuphakul *et al*. (2006) which showed that *B. subtilis* NSRS 9-24 produced an antibiotic effective in inhibiting the mycelial growth of *R. solani* and *P. grisea* in laboratory.

Results of the field experiment showed that the using of *B. megaterium* formulation significantly was more effective to decrease both sheath blight and blast diseases of rice than Iprodione fungicide. The modern fungicides, although effective, had specific activity against the pathogens. For instance, tricyclazole, a systemic fungicide is effective for blast disease but is ineffective against sheath blight. Similar narrow spectrum activity was reported with some bacterial metabolites such as blasticidin S and kasugamycin, which are effective only against blast of rice and validamycin A is effective only against sheath blight of rice (Yamaguchi, 1996). In this context, applying formulation of bacterium which possesses broad activity against plant pathogens is a sound approach for disease control.

In conclusion, combination of seed treatment with either spray or broadcast application of bacterial antagonist formulations is effective to deter sheath blight caused by *R. solani* and blast caused by *P. grisea*. Further research is warranted to determine the efficacy of *B. megaterium* formulations against different plant diseases under field condition. The field test should also be supported with the ecological study in which the fate of the applied *B. megaterium* is determined both spatially and temporally using appropriate molecular biology techniques such as green fluorescent protein (GFP). The mechanism governing the interaction between rice plant and *B. megaterium* should also be examined.

CHAPTER 7

MECHANISM STUDIES OF BIOCONTROL OF *BACILLUS MEGATERIUM*

7.1 Introduction

The use of biological agents to protect plant against pathogens represents an ecologically friendly alternative to pesticides repeatedly used to control plant diseases. The success of a biological control agent in suppressing plant disease depends on their ability to establish metabolically active populations that could mediate host protection and/or compete directly or indirectly with the pathogens for nutrient resources.

Several studies have reported the use of endophytic and plant growth promoting bacteria, including *Bacillus* spp. and *Pseudomonas* spp. against soil borne pathogens (Benhamou and Nicole, 1999; Jeun *et al*., 2004). *Bacillus* spp. is involved in the control of plant diseases through a variety of mechanisms of action, such as competition, systemic resistance induction and antibiotic production. The mechanism of antibiosis has been shown to be one of the most important (Tomashow and Weller, 1996).

The use of *Bacillus* sp. as biocontrol agents has been extensively studied (Souto *et al*., 2004). The heat and desiccation-resistant structures of spore producing Gram-positive bacteria can be readily formulated into stable products (Handelsman and Stabb, 1996; Kavitha *et al*., 2005). In particular, different *Bacillus* species excrete peptides and lipopeptides to the culture medium, such as fungicine, iturin, bacillomicine and others, having antifungal properties (Yu *et al*., 2002; Chitarra *et al*., 2003, Cho *et al*., 2003). Whereas rhizoctocin A produced by *B. subtilis* ATCC 6633 is a phosphono-oligopeptide (Kluger *et al*., 1990) and fungicin M-4 produced by *B. licheniformis* M-4 is composed of 34 amino acid residues of seven types (Lebbadi *et al*., 1994).

Some of *Bacillus* species could produce antibiotics against phytopathogenic fungi, most notably the nonribosomally synthesized cyclic lipopeptides surfactin, iturin and fengycin. These amphiphilic cyclic biosurfactants are already suggested to play an important role in biological activities. They have many advantages over other pesticides: low toxicity, high

biodegradability, environmentally friendly characteristics (Kim *et al*., 2004; Maget-Dana and Peypoux, 1994; Stein, 2005; Yoshida *et al*., 2001; Yu *et al*., 2002; Wang *et al*., 2008). In addition, *Bacillus* species are known to produce starch hydrolyzing enzymes.

In this research, we showed that *B. megaterium* was effective *in vitro* and *in vivo* against rice sheath blight disease. Strain of *B. megaterium* used in this study was selected based on the study by Kanjanamaneesathian *et al*. (1998). *B. megaterium* was identified using biochemical and physiological tests (Mac Faddin, 1976).

The objective of this study was to determine the possible mechanism of *B. megaterium* to suppress *R. solani*, a causal agent of rice sheath blight disease.

7.2 Material and Methods

7.2.1 Antagonism

7.2.1.1 Dual cultures

A dual culture method was used to test the potential *B. megaterium* that were effective in inhibiting mycelial growth of *R. solani*. A plug of agar of *R. solani* was placed at the center of potato dextrose agar (PDA) plate and 10 μl endospore suspension of *B. megaterium* (9 Log. number/ml) was inoculated half way between the rim of the Petri-dish and plug of a *R. solani* with triangular characteristics at room temperature (26-30 ^{0}C) for 7 days. Observation was done daily to detect antagonistic activity of *B. megaterium* antagonist toward *R. solani*. The efficacy of *B. megaterium* was evaluated using clear zone (inhibition zone) and the number of sclerotia of *R. solani* produced as criteria. Culture of *R. solani* on PDA incorporated with sterile water and 0.15% Iprodione was used as control and positive control, respectively. SEM observation of the inhibited mycelia of *R. solani* was observed.

Either 1 ml of endospore suspension of *B. megaterium* (9 Log.number/ml) or 1 ml of 0.15% chemical fungicide (Iprodione) were loaded on the PDA plates that had been inoculated with *R. solani*. After inoculated at room temperature (26-30 ^{0}C) for 4 days the samples for scanning electron microscope (SEM) analysis were isolated from the inhibition zone. Primary fixation was performed by soaking the samples in 2.5% glutaraldehyde solution for 2 h. The washed samples were dehydrated in a series of ethanol concentrations (50, 60, 70, 80, 90, 100, and 100%) and dried in a Critical Point Drier. The resulting samples were mounted on the stub, coated with gold particles, and photographed with SEM.

7.2.1.2 Production of volatile antibiotics

The ability of *B. megaterium* antagonist to produce volatile antibiotics was evaluated using the procedure described by Montealegre *et al*. (2003). One hundred μl of an antagonistic bacterial suspension (9 Log. number/ml) were spread at the center of one half Petri dish contain PDA medium, and a 0.5 cm diameter agar block of four days old pure culture of *R. solani* was placed at the center of another Petri dish containing PDA. Both half plates were

placed face to face preventing any physical contact between the pathogen and the bacterial endospore suspension, and were sealed to isolate the inside atmosphere and to prevent loss of volatiles formed. Plates were incubated at room temperature (26-30 ^{0}C) for 7 days and the growth of the pathogen was measured and compared to control developed in the absence of the bacterial antagonist (distilled water). Each treatment was repeated at least three replications. Results were expressed as means of the percentage of inhibition of growth of *R. solani*, and the number of sclerotia of *R. solani* produced was counted in the presence and absence of the bacterial isolate.

7.2.1.3 Production of diffusible antibiotics

The methodology of Montealegre *et al* (2003), with modifications, was used to determine fungal growth inhibition capacity of *B. megaterium*. Potato dextrose agar (PDA) plates, covered with cellophane membrane, were inoculated in the center with 100 μl of bacterial suspension endospores (9 Log. number/ml). After incubation for 72 h at room temperature (26-30 ^{0}C), the membrane with the grown bacterial isolate was removed, and the plate was inoculated in the middle with a 0.5 cm diameter agar block of a pure culture of *R. solani*. Plate was further incubated at room temperature (26-30 ^{0}C) for 7 days and the growth of the pathogen was measured. Controls were run with mocked inoculated PDA containing plates on the cellophane membrane (replacing the bacterial suspension by sterile distilled water), and further inoculated with *R. solani*. Each experiment was run in triplicates and was repeated at least three times. Results are expressed as means of % inhibition ± S.D. of growth of *R. solani* in the presence and absence of antagonistic bacterial isolate.

7.2.2 Persistence and efficacy of culture filtrates at difference high temperature and time

B. megaterium was cultured in potato dextrose broth (PDB) on a rotary shaker (150 rpm) at room temperature (26-30 ^{0}C) for 0-7 days, after which bacterial cells were centrifuged at 3,200 rpm for 30 min. The supernatant of this culture was filtrated through membrane filter (0.45 μm pore size) and then divided into 2 portions. One portion of this supernatant was heated at 60, 80, 100 and 121 ^{0}C for 20 min, but the other was not heated. Either heated or non-heated supernatant was mixed with melted PDA double strength medium at 1:1 (v/v) in Petri-dish. After the agar was solidified, an agar plug of *R. solani* was placed at the center

of these plates to assess the effect of either sterilized or non-sterilized cultures filtrates of *B. megaterium* to inhibit mycelial growth of *R. solani* at room temperature (26-30 ^{0}C).

Treatments consisted of culture of *R. solani* inoculated on PDA double strength incorporated with either heated or non-heated samples of each bacterial supernatant. Each treatment consisted of 4 replications. Culture of *R. solani* on normal PDA double strength was used as a control. Percent mycelia inhibition of *R. solani* was calculated 36 h after incubation as described by Gamliel *et al.* (1989).

7.2.3 Identification of antibiotic of *B. megaterium*

There are numerous reports of the production of antibiotic antifungal metabolites produced by *Bacillus* sp. *in vitro* that may also have activity *in vivo* (Yu *et al*., 2002; Kim *et al*., 2004; Wang *et al*., 2009). These include protein; enzyme and toxin. *B. megaterium* inhibited *R. solani* that antibiotic production was involved in disease suppression. Identification of antibiotics produced may improve our understanding of the mechanism involved in this and other biocontrol systems. The goal of this study was to purify and identify certain antibiotics produced by *B. megaterium* using chromatographic, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and other chemical analytical techniques.

7.2.3.1 Preparation of crude enzyme

B. megaterium were cultured in PDB for 6 days using the same method as mentioned in section 7.2.2. The supernatant of this culture was filtrated through membrane filter (0.45 µm pore size) and then divided into 2 portions. One portion of this supernatant was heated at 121 ^{0}C for 20 min, but the other was not heated. Enzymes were precipitated from either heated or non-heated supernatant with 80% saturation of ammonium sulphate and centrifuged using Amicon Ultra-15 Centrifugal Filter Devices with molecular weight cutoff (MWCO) of 10,000 kDa (Millipore Corporation, USA) at 3,200 rpm for 20 min. The crude enzymes were washed three times with sterile distilled water. The freeze-drying of either heated or non-heated crude enzymes was carried out in a freeze drier. Dry crude enzymes were collected and stored in a refrigerator at 4 ^{0}C.

7.2.3.2 Enzyme purification

The either heated or non-heated crude enzyme was dissolved in 50 mM Tris-HCl buffer, pH 7.5 and dialyzed in the same buffer. The enzyme solution was loaded onto a (4.0x 25 cm) column with DEAE-Sephacel (Pharmacia) equilibrated with 50 mM Tris-HCl buffer, pH 7.5 at a flow rate of 3 ml/min. The column was washed with 500 ml of the same buffer and the proteins were eluted with 4.0 ml of a linear 0-0.5 M NaCl gradient collecting 10 ml fractions. Elution was monitored at 280 nm with a diode array UV monitor. Active peaks were selected for further study. Fractions containing active peaks were pooled, dialyzed against the same buffer (with MWCO of 12,000 kDa) and tested their efficacy in mycelia inhibition of *R. solani* before concentrated using freeze-drying method.

7.2.3.3 Enzyme characterization

SDS-PAGE was carried out for the determination of purity and molecular weight of the enzyme as described by Laemmli (1970), using a 5% (w/v) stacking gel and a 15% (w/v) separating gel. Samples were prepared by mixing the purified enzyme at 1:5 (v/v) rations with distilled water containing 10 mM Tris-HCl pH 8.0, 2.5% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.002% bromophenol blue. Samples were heathed at 100 ^{0}C for 5 min before electrophoresis. Gels were stained with 0.25% Coomassie Brilliant Blue R-250 in 45% ethanol-10% acetic acid and destained with 5% ethanol-7.5% acetic acid. The molecular weight standards (Pharmacia); 14-97 kDA were used for SDS-PAGE.

7.2.3.4 Antifungal activity of enzyme preparations

The minimum inhibitory concentration (MIC) and 50 percent inhibition concentration (IC_{50}) of enzyme were carried out (Picman *et al*., 1990). The enzyme solutions were serially diluted with 50 mM Tris-HCl buffer, pH 7.5 (0.01-10 mg/ml) and filtered through a sterile Millipore membrane filter (0.45 µm) before mixing (1:1) with sterile melted potato dextrose agar (45 ^{0}C). Approximately 1 ml of the enzyme mixed agar was dropped into sterile 5.0 cm diameter plates using eight replications for each enzyme concentration and 50 mM Tris-HCl buffer, pH 7.5 was used as a control. Small agar plugs (1 mm) of an actively growing fungal mycelium of *R. solani* were placed on the center of the test agars. The fungal cultures were

incubated under moist conditions at 25 ^{0}C for 24 h. The radial growth of each fungal colony was measured. The percentage of hyphal growth inhibition was calculated as described by Gamliel *et al*. (1989). The minimal concentration that significantly inhibited hyphal growth was recorded as MICs (Picman *et al*., 1990). Concentration response curves were obtained in which the percentage of inhibition was plotted against enzyme concentration. The IC_{50} was calculated from the dose response curve.

7.2.3.5 Enzyme activity measurement

Enzyme activity was measured by hydrolysis of starch substrate method (Yoo *et al*., 1987). 0.1 ml of 1% w/v enzyme sample in 50 mM Tris-HCl was added to 0.5 ml 1% soluble starch solution in phosphate buffer, pH 7.0 and mixed for 3 min. The mixture was incubated for 5 min at 37 ^{0}C in an incubator. After the incubation period, a portion of this mixture was combined with 0.5 ml acidic iodine. The acid stopped the enzymatic reaction and the iodine reacted with the starch to produce the blue color. The intensity of the blue color was quantified spectrophotometrically by measuring its absorbance at 620 nm. The greater the change in absorbance between a sample containing the initial amount of starch (without enzyme) and the hydrolyzed mixture containing the enzyme, the greater the amount of starch degraded by the enzyme, therefore the greater the activity of the enzyme being measured.

7.3 Results

7.3.1 Antagonistic mechanisms

7.3.1.1 Direct antagonism through dual cultures

B. megaterium antagonist and Iprodione fungicide were tested on the basis of their ability to inhibit *R. solani* mycelial growth and suppress sclerotial production. *B. megaterium* inhibited mycelial growth of *R. solani* by creating clear zone (more than 6 mm) while, Iprodione created the highest inhibition zone at 7.6 mm against *R. solani* (Table 7.1). However, bacterial antagonist had higher potential to suppress sclerotial production of *R. solani* than Iprodione for approximately 2 times (Table 7.1). Figure 7.1 showed the mycelial growth and sclerotial production of a pure culture of *R. solani* in PDA (Figure 7.1a). When, *R. solani* was challenged with the chemical fungicide (Figure 7.1b) or the bacterial culture (Figure 7.1c) growth inhibition of mycelia accursed accompanied by a decreased sclerotial production.

The positive control treatment inoculated only with *R. solani* showed no clear zone (Figure 7.1a). The end hyphae of *R. solani* were attached with the Iprodione showed rise fluffy the mycelial growth over the plate (Figure 7.1b). Bacteria attached end-on to hyphae and extensively colonized the surfaces of *R. solani* including their mycelium. Fibrillar material was seen connecting bacterial cells to each other and to the surfaces of the hyphae of *R. solani*. Partial or total degradation of hyphae was evident indicated by an extensive collapse of cell walls and breakage of hyphae (Figure 7.1c). On the other hand, a change in mycelial color was observed closed to the colony end of *R. solani*, being this one of a darker brown than the one observed at the center of the colony.

SEM observation of this zone, allowed detecting cell wall leakage that could be observed up to the hyphal septum, resulting in deformation and sliming of their apex up to 1/10 of its original size (Figure 7.2). SEM analysis showed that the presence of active bacteria damaged the mycelium of *R. solani*. Normal mycelium could be observed in the absence of any antibiotics (Figure 7.2a), while Iprodione was observed to inhibit the mycelium of *R. solani* by swelling and broken of the fungal cell wall (Figure 7.2b). Treatment with bacterial endospores led to disruption of *R. solani* mycelium with the shrink and lysis of the fungal cell wall (Figure 7.2c).

Table 7.1 Clear zone and number of sclerotia produced in dual culture between *B. megaterium* bacterial antagonist or fungicide and *R. solani*

Treatments	Width of clear zone (mm)	Number of sclearotia produced
B. megaterium	6.40±0.49 b	18.00±1.41 c
Iprodione	7.60±0.80 a	35.67±3.09 b
Control (sterile distilled water)	0.00±0.00 c	149.67±2.87 a
F-test	**	**
C.V. (%)	12.98	4.64

** Means followed by the same letter are not significantly different by Duncan's Multiple range Test at $p \leq 0.01$

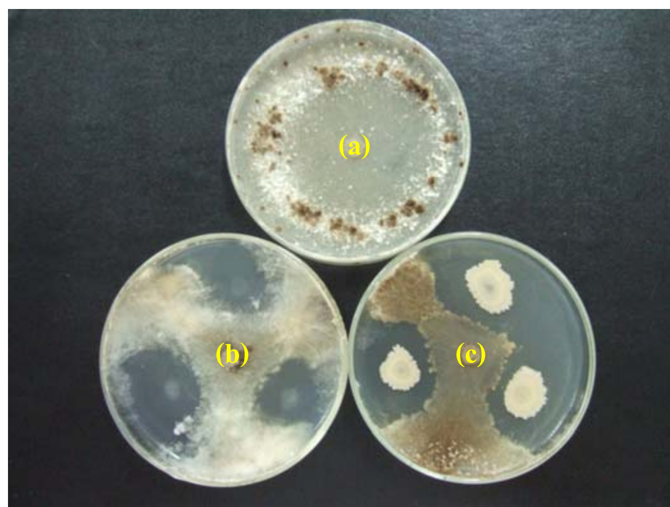

Figure 7.1 Dual culture of *R. solani* on PDA incorporated with (a) sterile distilled water, (b) Iprodione and (c) *B. megaterium*

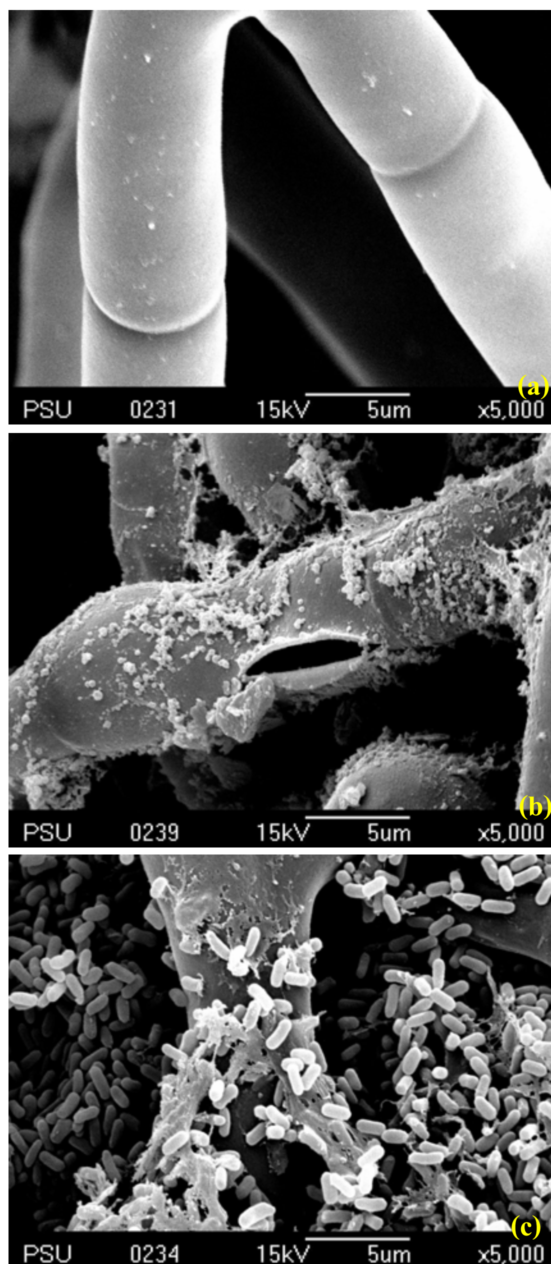

Figure 7.2 Antimicrobial activity of *B. megaterium* and scanning electron microscopy of *R. solani* grown on PDA plate (a) normal mycelium (b) disrupted mycelium by Iprodione or (c) *B. megaterium*

7.3.1.2 Production of volatile antibiotics

From Table 7.2, *B. megaterium* produced volatile antibiotic that showed the best inhibitory effect more than chemical fungicide (Iprodione) and control (sterile distilled water) on the growth and sclerotial production of *R. solani* at 7 days culture age.

Table 7.2 Efficacy of volatile antibiotics secreted by *B. megaterium* on radial growth and sclerotial production of *R. solani*

Treatments	% inhibition of mycelial growth	Number of sclerotia produced
B. megaterium	99.72±0.12 a	0.00±0.00 c
Iprodione	0.00±0.00 b	5.33±0.47 b
Control (sterile distilled water)	0.00±0.00 b	30.33±0.47 a
F-test	**	**
C.V. (%)	0.24	3.97

** Means followed by the same letter are not significantly different by Duncan's Multiple range Test at $p \leq 0.01$

7.3.1.3 Production of diffusible antibiotics

Results, similar to those obtained when testing the effect of volatile antibiotics, were obtained when the effect of diffusible antibiotics was tested (Table 7.3). The bacterial antagonist evaluated was significantly different in relation to the control. *B. megaterium* and Iprodione showed high inhibitory effect on *R. solani* growth with a percentage of inhibition of more than 90% and suppressed *R. solani* sclerotial production at 7 days culture age (Table 7.3).

Table 7.3 Efficacy of diffusible antibiotics secreted by *B. megaterium* on radial growth and sclerotial production of *R. solani*

Treatments	% inhibition of mycelial growth	Number of sclerotia produced
B. megaterium	99.95±0.00 a	0.00±0.00 b
Iprodione	99.80±0.00 a	0.00±0.00 b
Control (sterile distilled water)	0.00±0.00 b	131.67±1.70 a
F-test	**	**
C.V. (%)	0.00	2.74

** Means followed by the same letter are not significantly different by Duncan's Multiple range Test at $p \leq 0.01$

7.3.2 Persistence and efficacy of culture filtrates at difference high temperature and time

The supernatant of *B. megaterium* was exposed to various temperature for 20 min and residual activity was measured (Figure 7.3, Figure 7.4). Heat treatment of the broth showed the antimicrobial activity was stable at a temperature of 121 ^{0}C for 20 min and antimicrobial activity remained above 90% when broth was treated at 121 ^{0}C for 20 min. Antimicrobial activity against *R. solani* was found to be stable at 121 ^{0}C with 90% of original activity remaining. The six-days culture filtrates of the *B. megaterium* contained a high level of the enzyme activity.

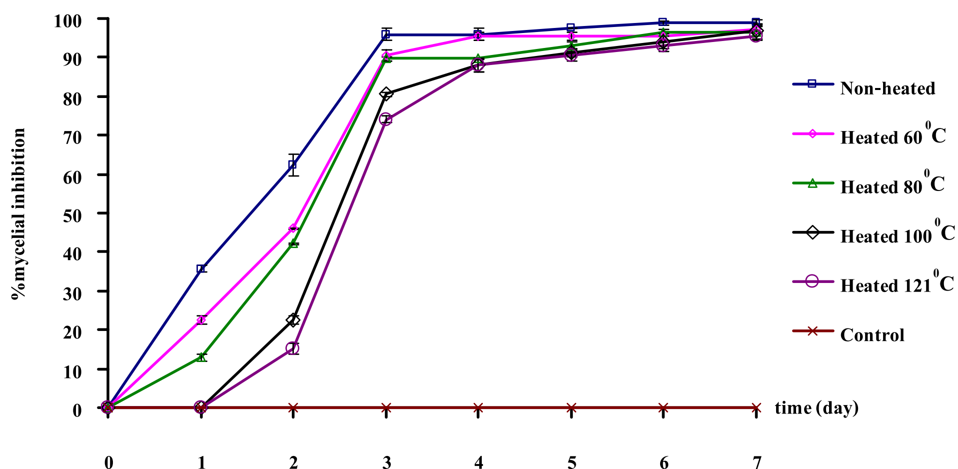

Figure 7.3 Inhibition of *R. solani* by heat treated metabolites of *B. megaterium* at 60, 80, 100 and 121 ^{0}C

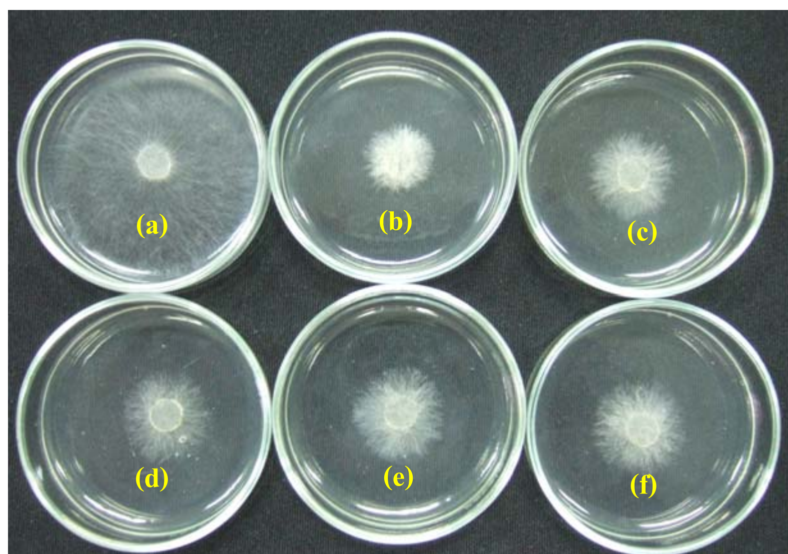

Figure 7.4 Inhibition of *R. solani* by (a) control treated, (b) non-heated treated and heat treated metabolites of *B. megaterium* at (c) 60, (d) 80, (e) 100 and (f) 121 ^{0}C

7.3.3 Purification of enzyme

Four fractions included heated enzyme-unbounded-DEAE, heated enzyme-bounded-DEAE, non-heated enzyme-unbounded-DEAE and non-heated enzyme-bounded-DEAE fractions were collected. Either enzyme fraction was monitored by absorbance at 280 nm as shown in Figure 7.5. Heated enzyme-unbounded-DEAE fraction had the highest the amount of enzyme while, there was no great difference in the amount of enzyme by other fractions. However, all fractions showed high activity against *R. solani* mycelial growth (more than 90%) and suppress sclerotial production (Table 7.4).

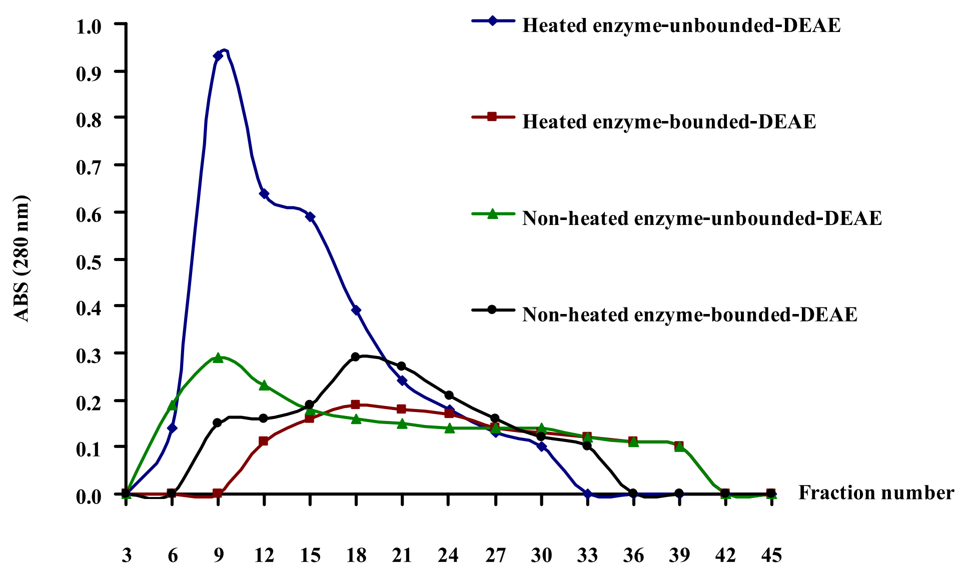

Figure 7.5 Anion-exchange chromatography of heated or non-heated crude enzyme preparation of *B. megaterium* on DEAE-Sephacel

Table 7.4 % inhibition and number of sclerotia produced in dual culture between enzyme fraction and *R. solani*

Treatments	% inhibition of mycelial growth	Number of sclearotia produced
Heated-enzyme-bounded-DEAE	99.41±0.74 a	0.00±0.00 b
Heated-enzyme-unbounded-DEAE	98.45±0.96 a	0.00±0.00 b
Non-heated enzyme-bounded-DEAE	96.00±0.00 b	0.00±0.00 b
Non-heated enzyme-unbounded-DEAE	90.56±1.39 bc	0.00±0.00 b
Control A (50 mM Tris-HCl)	0.00±0.00 d	82.00±2.00 a
Control B (0.5 M NaCl)	0.00±0.00 d	83.00±2.00 a
Control C (sterile distilled water)	0.00±0.00 d	85.00±4.58 a
F-test	**	**
C.V. (%)	1.27	5.70

** Means followed by the same letter are not significantly different by Duncan's Multiple range Test at $p \leq 0.01$

The purified enzyme was analyzed by SDS-PAGE under reducing conditions. As shown in Figure 7.6, the enzyme gave a single band on SDS-PAGE with a molecular weight of approximately 58 kDa, except lane 6: non-heat enzyme-bounded-DEAE showed that the dissociated enzyme produced two bands of proteins with a molecular weight of approximately 58 and 37 kDa (Figure 7.6).

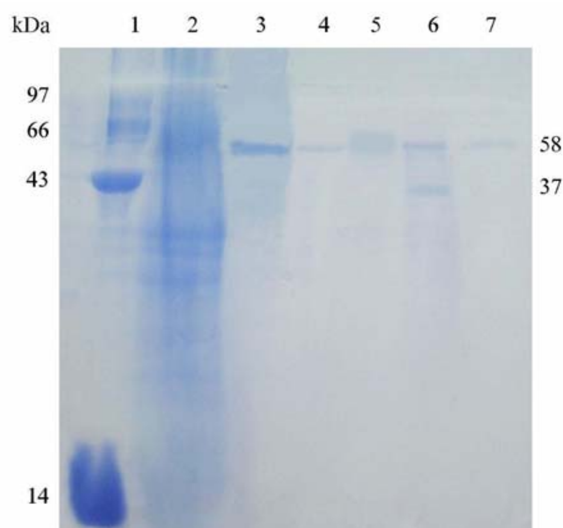

Figure 7.6 SDS-PAGE of enzyme purified from *B. megaterium* filtrate; lane 1: molecular mass markers (14-97 kDa); lane 2: non-heated crude enzyme; lane 3: heated crude enzyme; lane 4: heated enzyme-bounded-DEAE; lane 5: heated enzyme-unbounded-DEAE; lane 6: non-heated enzyme-bounded-DEAE; lane 7: non-heated enzyme-unbounded-DEAE

7.3.4 Antifungal activities of enzyme extract from *B. megaterium*

MICs and IC_{50} of the antibiotic extract against *R. solani* were showed in Table 7.5. MICs of heated enzyme-unbounded-DEAE, heated enzyme-bounded-DEAE and non-heated enzyme-bounded-DEAE were 0.50 μg/ml, which has higher efficiency than other fractions in inhibiting the growth of *R. solani*. While the IC_{50} of heated enzyme-bounded-DEAE showed the lowest value (0.34 mg/ml) followed by heated enzyme-unbounded-DEAE (0.69 mg/ml) and non-heated enzyme-bounded-DEAE (1.50 mg/ml).

Table 7.5 MICs and IC_{50} of antibiotic extract from culture filtrates of *B. megaterium* against growth of *R. solani*

Treatments	MIC (µg/ml)	IC_{50} (mg/ml)	Regression equation	R^2
Heated crude enzyme	25.00	4.42	y=8.6057x+11.964	0.8290
Heated enzyme-unbounded-DEAE	0.50	0.69	y=5.5989x+46.129	0.8171
Heated enzyme-bounded-DEAE	0.50	0.34	y=5.5178x+48.101	0.9363
Non-heated crude enzyme	5.00	3.37	y=6.6599x+27.582	0.9699
Non-heated enzyme-unbounded-DEAE	2.50	2.62	y=7.0064x+31.62	0.9860
Non-heated enzyme-bounded-DEAE	0.50	1.50	y=5.8421x+41.24	0.8489

For hydrolysis of soluble starch, heated enzyme-unbounded-DEAE, non-heated enzyme-bounded-DEAE and non-heated enzyme-unbounded-DEAE were more effective than other treatments as 0.236±0.000, 0.236±0.003 and 0.244±0.004 mg enzyme per g starch per min, respectively (Table 7.6).

Table 7.6 Enzyme activity in starch hydrolysis

Treatments	mg enzyme/1 g starch/min
Heated crude enzyme	0.641±0.012 a
Heated enzyme-unbounded-DEAE	0.236±0.000 d
Heated enzyme-bounded-DEAE	0.255±0.007 c
Non-heated crude enzyme	0.565±0.004 b
Non-heated enzyme-unbounded-DEAE	0.244±0.004 d
Non-heated enzyme-bounded-DEAE	0.236±0.003 d
F-test	*
C.V. (%)	1.72

* Means followed by the same letter are not significantly different by Duncan's Multiple range Test at $p \leq 0.05$

7.4 Discussion

The antibiotics that *Bacillus* spp. produces are generally assumed to be responsible for the control activity of plant pathogens (Helbig *et al*., 1998; Krebs *et al*., 1998). The inhibitory effect of *Bacillus* sp. on phytopathogenic fungus can also be associated with enzyme production that can act against the fungal cell wall (Asaka and Shoda, 1996; Mavingui and Heulin, 1994). The secondary metabolites produced by contain species and strains of the genus *Bacillus* have been found to show antibacterial and or antifungal activity microorganisms (Shoji, 1978; Smirnor *et al*., 1986). It has been reported that *Bacillus* sp. secreted several antifungal metabolites such as bacitracin, bacillin and bacilomycin, which belong to the iturine family (Alippi and Monaco, 1994). Bacteria of the genus *Bacillus* are known as producers of a number of peptides with antibiotic properties effective against bacteria, fungi and yeast (Katz and Demain, 1977).

In this study, different testing methods were used to evaluate the mode of action of *B. megaterium* for the control of *R. solani*. Preliminary *in vitro* assay on PDA culture media was used to measure the antagonistic potential of the strain. The result showed that the bacterium had an inhibitory effect (clear zone), with the discoloration of mycelium at the margin of *R. solani* colony and inhibition of sclerotial production. Abnormal deflation, deformation and degradation of the hyphal tip of *R. solani* were observed when *R. solani* was challenged with *B. megaterium* in the dual culture method. Under attached with the Iprodione, the end vegetative hyphae of *R. solani* had rise fluffy myceial growth over the plate (Figure 7.1). This change allows the affected fungus to survive the fungicide. In time, this surviving organism is likely to become the dominant strain when the same fungicide is used frequently and exclusively with it that becomes more difficult to control (Fuchs and Drandarevski, 2005).

It was also found that *B. megaterium* excreted metabolites (both volatile and diffusible substances) with antifungal activity against mycelial growth of *R. solani*. These compounds efficiently inhibited mycelial growth and sclerotial production of *R. solani*. Fiddaman and Rossall (1993) observed that hyphal vacuolization and deformation *R. solani* and in *Pythium ultimum* occurred as a result of treating these pathogens with a *B. subtilis* strain which secreted a volatile metabolite with fungicide properties. Kuzma *et al*., 1995 reported that *Bacillus* sp.

produced the volatile hydrocarbon isoprene (2-methyl-l, 3-butadiene) under rich media and 45 ^{0}C temperature conditions. *B. subtilis* successfully restricted the growth of six pathogenic fungi in dual cultures, and induced morphological abnormalities such as mycelial and conidial deviations by produced diffusible and volatile antifungal compounds. The inhibitory effect caused by volatiles was greater than that by diffusible compounds (Chaurasia *et al*., 2005).

In this research, *B. megaterium* filtrated suspension was assayed for its thermo-stability. The result showed that culture filtrate was effective in inhibiting the mycelial growth of *R. solani*. Different thermal treatments did not affect the antifungal activity of the culture filtrate. The enzyme in culture filtrate is highly thermo-stable and remained active in a broad range of temperature (at 60-121 ^{0}C). There was no great difference in the percentage of the inhibition zones produced by both heated and non-heated culture filtrate on PDA plates (Figure 7.3). A possible explanation is that some of antibiotics and some toxins are thermostable and remained active after sterilization. A wide rang of temperature (35-100 ^{0}C) has been reported for optimum growth and α-amylase production in *Bacillus* sp. Ts-23 (Lin *et al*., 1998), *Bacillus* sp. ASMIA-2 (Teodoro and Martin, 2000) and *B. subtilis* JS-2004 (Asgher *et al*., 2007). Thermostability for 4 h at 100 ^{0}C has been found for α-amylase from *B. licheniformis* CUMC305 (Krishnan and Chandra, 1983). *Bacillus* sp. ANT-6 α-amylase was stable after overnight (85.5%) and 24 h (55%) incubation at 100 ^{0}C (Burhan *et al*., 2003). Maldonado *et al* (2009) reported that *Bacillus* sp. IBA 33 produced antifungal proteins that remained active after sterilization at 121 ^{0}C for 15 min.

The extracellular thermostable substance produced by *B. megaterium* was purified to homogeneity by ammonium sulphate precipitation and by gel filtration through DEAE-Sephacel. All purified protein fractions showed potent antifungal activity against *R. solani* (Table 7.4). Either fraction of purified protein was homogenous on SDS-PAGE and its molecular weight was estimated to be 58 kDa, except non-heated enzyme-bound-DEAE showed two subunits with different molecular weight of 58 and 37 kDa (Figure 7.6).

Several authors have reported that the thermostable enzyme activity produced by other *Bacillus* species consisting of approximately 58 kDa molecular weight was α-amylase enzyme (Ivanova *et al*., 1993; Mendu *et al*., 2005; Ezeji and Bahl, 2006; Hmidet *et al*., 2008).

Moreover, all fraction of heated and non-heated enzyme had potential in hydrolysis of soluble starch more than heated crude and non-heated crude enzyme (Table 7.6).

The heated enzyme showed potent inhibition of fungal mycelial growth of *R. solani* at 0.5 μg/ml MIC and 0.34 mg/ml IC_{50} that more than non-heated enzyme (Table 7.5). The results indicated that heated enzyme may increase the efficacy of enzyme in suppressing mycelial growth of *R. solani*. There has been a similar report of a heat stable fungicide produced by *B. subtilis* NSRS 89-24 (Leelasuphakul *et al*., 2006). Several studies have demonstrated that extracellular lytic enzymes and antibiotics from *Bacillus* sp. were potent biocontrol agents of fungi acting alone or synergistically through degradation of the fungal cell walls (Leelasuphakul *et al*., 2006).

The mode of action of the antifungal compounds produced by *B. megaterium* against the pathogenic fungi of rice is probably similar to that of other biocontrol agents (Chernin and Chet, 2002). Microscopic observations after treating the mycelium with these agents have shown that the fungus had abnormal hyphal deflation, degraded hyphal tip and arrested sclerotial germination. These observations may indicate that cell wall at each hyphal apex was the target of this substance. The hyphal apex is composed of chitin, β-glucans and other oligosaccharide compounds. The substances produced by this strain of *B. megaterium* may play a crucial role in disrupting the integrity of fungal cell walls.

In conclusion, *B. megaterium* performed high mycelial growth inhibition of *R. solani* and suppressed sclerotial production of *R. solani*, because it produced volatile and diffusible antibiotics and heated stable enzyme which hydrolyzed soluble starch. In the future, the method which is highly helpful to bioprocess engineers to purify effective antibiotic and enzyme more economical is need, it can purify in a short period, industrially applicable, high recover and highly pure the commercial fungicide preparation.

CHAPTER 8

CONCLUSIONS

This thesis described the protocol for producing and formulating the bacterial antagonist (*Bacillus megaterium* isolate No. 16) and subsequently tested the formulations to suppress sheath blight disease of rice in greenhouse and field conditions.

Although this bacterium isolate has been reported to be effective against sheath blight disease, most of the works has been carried out using fresh cells of the bacterium as an inoculum (Kanjanamaneesathian *et al*., 1998; Pengnoo *et al*., 2000). To bring research into practical usage, this thesis has put the emphasis on formulating the bacterium into the products with the features that are required for possible commercialization.

The upstream work was done with the evaluation of the durability of *B. megaterium* endospores against UV irradiation, high temperature and pH. Endospores remained active after treating with UV irradiation (48 h), high temperature at 80 ^{0}C (36 h) and a wide range of pH (2-10) for 15 days. In small-scale fermentation, the screening trial to determine the possibility of using cheap substrates to produce the biomass of *B. megaterium* was studied. It was found that cassava root had potential to use as a substrate for broth medium preparation for bacterial cultivation. The number and size of *B. megaterium* endospores obtained from broth medium prepared from cassava root are similar to those obtained from broth prepared from other materials (potato, sweet potato, rice, brown rice and job's tears), except sticky rice, which provided the highest width of endospores. The types of substrates used for broth medium preparation had no effect on length of the endospores. However, cassava root costs a lot less than other materials (19.9 baht/l). In 30 l fermentation, bacterial antagonist cultured in potato dextrose broth at 30-35 ^{0}C, 200 rpm, 1 vvm for 4 days provided the highest number of endospores.

Three types of formulations, such as water-soluble granule, effervescent granule and effervescent tablet formulations, had been developed (Figure 8.1) and their physical and biological characteristics had been determined (Table 8.1). All formulated products developed in this study were compared in Table 8.2.

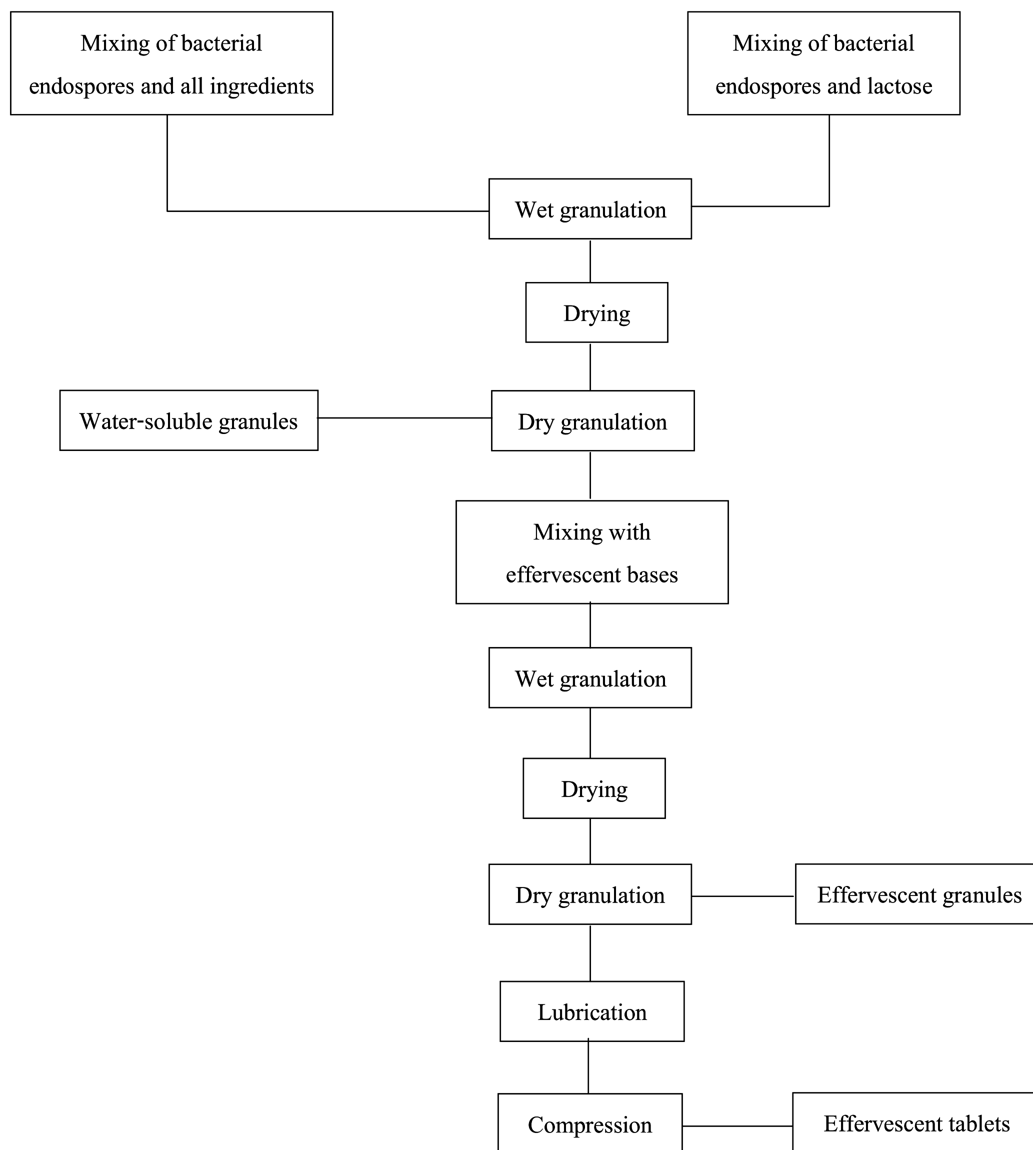

Figure 8.1 Production of water-soluble granules, effervescent granules and effervescent tablets

Table 8.1 Physical and biological characteristics of *B. megaterium* antagonist formulations

Characteristics/Formulations	Water-soluble granules (SG2)	Effervescent granules (EG3)	Effervescent tablets (ET3)
pH of 3% w/v solution	5.93±0.01	6.57±0.05	6.03±0.08
Dissolution time (min)	6.97±0.37	-	-
Disintegration time (min)	-	<1	<3
Viscosity of 3% w/v solution (cp)	5.32±0.07	-	-
Friability index (%)	96.30±0.10	92.70±0.36	99.82±0.09
ulk density (g/ml)	0.46±0.01	0.47±0.01	-
Tapped density (g/ml)	0.51±0.01	0.52±0.01	-
Moisture content (%)	0.25±0.02	0.11±0.01	0.22±0.01
Weight (g/tablet)	-	-	3.31±0.11
Diameter (mm/tablet)	-	-	26.26±1.03
Thickness (mm/tablet)	-	-	4.99±0.21
Shelf-life (24 months)			
-Viable bacteria (Log. number/g)	9.22±0.65	8.36±0.58	9.44±1.00
- Inhibition of *R. solani* mycelial growth (%)	99.64±0.14	98.19±0.59	99.90±0.09

Table 8.2 Typical product characteristics of *B. megaterium* antagonist formulations

Typical characteristics /Formulations	Water-soluble granules (SG2)	Effervescent granules (EG3)	Effervescent tablets (ET3)
Number of ingredients	3	5	6
Number of equipments	3	3	4
Number of processes	4	8	10
Stability	++++	+++	+++
Ease of transportation	++++	++	+++
Application (ease of application)	Spray (+++)	roadcast (+++)	roadcast (++++)
	Seed treatment (++++)	Spray (+++)	Spray (++++)
Applicability to control plant diseases in other regimes	++++ (foliage, soil)	+++ (water, foliage)	+++ (water, foliage)
Approximately cost (baht/ kg product)	280	300	400
Ease of commercial production	++++	+++	++

++ (good) +++ (very good) ++++ (excellent)

These formulations had a good characteristic in both physical and biological properties. There were four basic features for the formulation to be acceptable for plausible usage and commercialization. These were (1) to stabilize the bacterial antagonist during production, distribution and storage, (2) to aid in handling and application of the product so that it is easily delivered to the target in the most appropriate manner and form, (3) to protect the bacterial antagonist from harmful environmental factors at the target site, thereby increasing persistence and (4) to enhance activity of the bacterial antagonist at the target site by increasing activity, reproduction, contact and interaction with the disease organism.

However, not all formulations may have only the positive features. Water-soluble granules (SG2) are dried formulations that are consisted of larger particle than powder and are typically much less dusty. They are highly soluble with optimal viscosity that would be suitable for spray and seed treatment application. This form can be easily produced and the cost of the formulation is relatively cheaper than other formulations. However, water-soluble granules are prepared by wet method using sodium alginate as a binder. This requires thorough mixing process which contributes to the homogeneity of the formulation.

Effervescent granules (EG3) are prepared as an aggregate of the smaller particles of powder which contains bacterial antagonist in a dry mixture composed of lactose, PVP k-30, sodium bicarbonate, citric acid and tartaric acid. These bacterial granules are stable formulation and can be easily applied by broadcasting and spraying. The negative feature of this formulation is that it requires special attention during storage as moisture would destabilize the formulation and eventually affect its efficacy.

Effervescent tablets (ET3) are prepared by compressing granular effervescent that release gas when the water is present. This highly soluble tablet is thus suitable for broadcasting and spraying. In addition, this bacterial formulation has prefabricated dose feature which is very practicable to use to suppress sheath blight disease. However, care must be taken to ensure that the ambient humidity surrounding the area in which this formulation has been produced should be kept low and the tablets must be stored in the air-tight container.

The testing to determine the efficacy of these formulations showed that all formulations have satisfactory efficacy, reducing the sheath blight disease severity and incidence by 28.20, 29.42 and 27.07% using SG2, EG3 and ET3, respectively, under greenhouse condition. Application to rice plant with bacterial formulation before pathogen inoculation provides some protection against *R. solani*. Nevertheless, applying the bacterial formulation thrice after pathogen inoculation is the most promising in deterring sheath blight spread and controlling sheath blight severity. In the field test, application of SG2 seed treatment combined with either SG2 spray or EG3 broadcast 1, 5 and 10 day after pathogen inoculation are more effective than the chemical treatment (Iprodione). This formulation is also effective in controlling rice blast disease caused by *P. grisea* as well (data not shown).

In term of the quality, all formulations of *B. megaterium* had a shelf life of 24 months at room temperature (26-30 ^{0}C) which would be acceptable for commercialization. The stability of the formulation may be because the formulation using only with endospores of the bacterial antagonists, they would have a prolonged shelf-life and be much more resistant to manipulation and application procedure than vegetative cells. In addition, the formulation contained lactose and other chemicals in solid form which can be easy storage, transport and application. Lactose is used as a filler or diluent in formulation. ecause lactose has inert property, it is suitable for being used as filler. In this study, lactose was used as filler in all formulations because it is water-soluble, available, cheap and inert ingredient.

Preliminary investigation to reveal the mechanism in which *B. megaterium* effectively suppress sheath blight shows that the bacterium produced diffusible and volatile antifungal compounds. Antifungal compound in the culture filtrate remains effective to inhibit the mycelial growth of *R. solani* after subjecting it to 121 ^{0}C for 20 min. The heat stable component is preliminary characterized as a protein with a molecular weight of approximately 58 kDa by SDS-PAGE. The MIC and IC_{50} values effective in inhibiting *R. solani* are 0.50 μg/ml and 0.34 mg/ml, respectively. Moreover, heat stable antibiotic have been shown to hydrolyzed soluble starch. This antibiotic may belong to a thermostable α-amylase group.

All in all, this thesis has established a protocol to produce and formulate an antagonistic bacterium, *B. megaterium*, and has showed that these formulations have the required features for commercialization and use by the farmers. The use of bacterial antagonist formulations to combat disease has the potential to become a complement or alternative to more traditional chemical treatment. This is a more environmental friendly option than chemical fungicide. These formulations may be suitable for use to control other plant diseases, such as disease in plants grown hydroponically to produce the pesticide free vegetables.

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