# CHAPTER 1

# INTRODUCTION

## Introduction

Rice sheath blight disease, caused by the fungal pathogen *Rhizoctonia solani* Kuhn (Sexual stage: *Thanetophorus cucumeris* [Frank] Donk) (Srichuwong and Suwannarat, 1984), is one of the major production constraints in rice-growing countries of the world and ranks next to blast in causing economical loss (Marshall and Rush, 1980; Ou, 1985). Yield reductions of up to 20% may occur if the disease develops and reaches the uppermost leaves (Teng *et al*., 1990; Pande, 1994). The pathogen survives in the soil from year to year as a hard, weather-resistant structure called a sclerotium. Sclerotia float to the water surface and contact to rice plant. The fungus grows out from the sclerotium and infects leaf sheath. Sclerotia are developed on infected stem surfaces and deposit on the soil. They remain alive in the soil for several years (Reissing *et al*., 1986).

This disease has been controlled using a biocontrol approach (Mew and Rosales, 1986; Vasantha Devi *et al*., 1989; Gnanamanickam and Mew, 1990; Gnanamanickam *et al*., 1992; Kanjanamaneesathian *et al*., 1998; Pengnoo *et al*., 2000; Wiwattanapatapee *et al*., 2004). In most of studies, fresh cells of potential antagonists have been tested for control rice sheath blight disease (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 rice farmers using in the rice field (Pengnoo *et al*., 2000). As a result, bacterial antagonists in various formulation, for example, granule, liquid and powder were prepared and tested (Kanjanamaneesathian *et al*., 1998; Arunyanart *et al*., 2001; Radja Commare *et al*.,

2002; Kusonwiriyawong *et al*., 1999). Although these formulations demonstrate the desired characteristics and provide quite satisfactory protection for rice plants from *R. solani* infection, the numbers of bacterial antagonists in the formulations greatly decline during 6 months storage (Kanjanamaneesathian *et al*., 2000). This undesirable characteristic of the formulation makes it improper for large scale production and commercialization. Recently, floating pellets containing endospores of *Bacillus megaterium* were tested and showed promising result in suppression of sheath blight lesions in greenhouse experiment. The viability of bacteria in the pellets remained high after 6 months storage (Wiwattanapatapee *et al*., 2004). For this reason, further research and development of bacterial formulations are required.

In this study, the formulations were produced in granule and tablet forms and *B. megaterium* endospores were used as a bioactive substance. These products have no inhalation hazard, do not readily drift in the wind and can be quantified easily (Jones and Burges, 1998). Both formulations were produced for either spray application or broadcast application.

## Objectives

1. To develop bacterial formulations in granule and tablet forms for both spray application and broadcast application
2. To evaluate the physical characteristics and viability of the bacteria in the formulations
3. To test the efficacy of the selected bacterial formulations in suppression of rice sheath blight disease in greenhouse conditions

## Review of literatures

### 1. Rice sheath blight disease

Rice sheath blight disease is caused by a fungus, *R. solani* (Sexual stage: *T. cucumeris*) (Srichuwong and Suwannarat, 1984). It is described in Japan and has also been reported in Bangladesh, India, Indonesia, Malaysia, Thailand, Vietnam, Brazil, Suriname, Venezuela, Madagascar and the United States (IRRI, 1993; Pande, 1994; Chaudhary, 2002). It is considered as important production constraints of rice in South and South East Asia and other parts of the world (Ou, 1985). Many factors contribute to the difficulty in controlling this disease. These include an ability of the pathogen to survive for a long time in the soil in the form of sclerotium. Furthermore, infected rice tissue left in rice field unattended is also a good source of inoculum (Cook and Baker, 1983).

Sheath blight is usually worse in thick, lush rice stands because of high relative humidity within the canopy. *R. solani* thrives when the canopy humidity is above 95% and temperatures are between 80- 90$^{o}$F. Initial infection will occur in thin, short stands of rice when humidity within the canopy is low (Reissing *et al.*, 1986). Normally, the disease was serious in temperate regions where dew deposition was heavy for prolonged periods. High tillering varieties, high plant population and heavy use of nitrogen fertilizers, contribute to the spread of sheath blight both vertically and horizontally (IRRI, 1993; Chaudhary, 2002).

**Figure 1** Rice sheath blight disease (IRRI, 1993).

Sheath blight symptoms appear on rice seedling, especially but usually at internode elongation stage. A water-soaked lesion symptom on leaf sheaths appear at or near the water line. The lesion becomes grayish white center surrounded by a dark purplish or reddish brown margin within two or three days (Figure 1). The fungus grows upward inside the sheath and on the surface when the humidity inside the rice plant canopy increases, causing new lesions. The fungus can also spread to nearby plants when infected tissue contacts the adjacent rice plants, causing horizontal spread (Srichuwong and Suwannarat, 1984; IRRI, 1993). When rice plants have reached maturity and become senescent, lesions become dry and turn grayish to tan with brownish borders. Initially sclerotia is white but turn dark brown at maturity and they are produced superficially on or near the lesions (Figure 2). Sclerotia are loosely attached and easily dislodge from the plant. They survive for a long time in the soil and will float to the surface of flooded rice fields in the subsequent rice crop. The mycelia infect rice plants at the waterline and

continue the disease cycle. They can also attack several weed hosts and cause infection (Srichuwong and Suwannarat, 1984; IRRI, 1993).

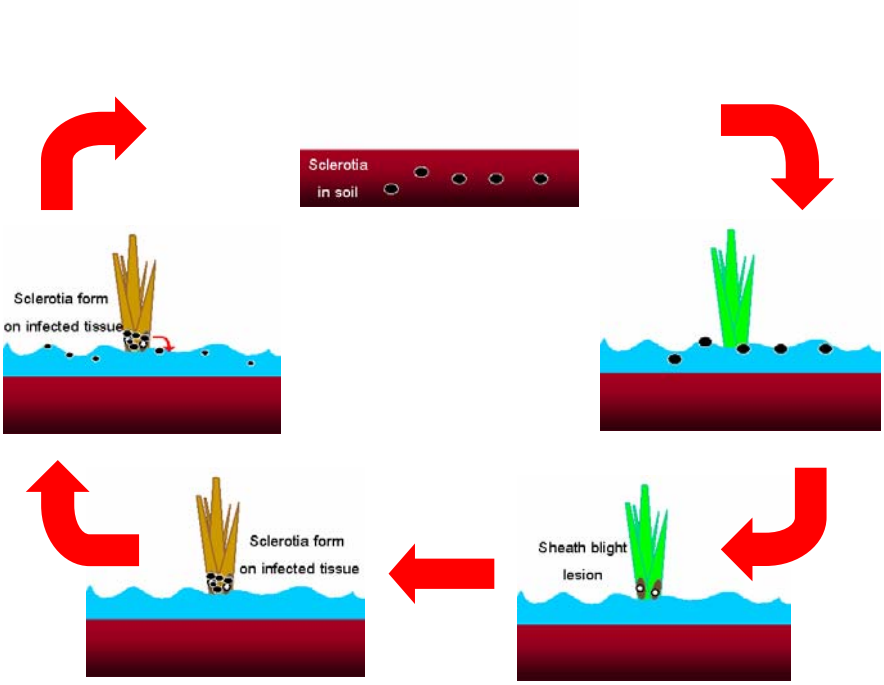


**Figure 2** The cycle of rice sheath blight disease

Damage can range from partial infection of the lower leaves with little effect on grain development to premature plant death. 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; Srichuwong and Suwannarat, 1984).

## 2. Biological control

Bacterial biological control agents that have been demonstrated to suppress plant pathogenic fungi are mainly the genera *Pseudomonas* (Schippers, 1983), *Streptomyces* (Tanaka *et al*., 1987) and *Bacillus* (Katz and Demain, 1977). Antagonism and the mode of action of the biological control agent are categorized as antibiosis, competition for nutrients, competition for space, hyperparasitism and induced resistance (Baker, 1987) but these are only superficial statements and in many cases the precise mechanism of action is not fully understood. Antibiosis is generally the mode of antagonism observed with *Bacillus* spp (Baker, 1987).

Most *Bacillus* spp. produce antibiotics, many of them have antifungal activity (Katz and Demain, 1977). *Bacillus* spp. also produces endospores which are dormant structures resistant to desiccation, heat, UV irradiation and organic solvents (Roberts and Hitchins, 1969). The production of antibiotics and endospores by *Bacillus* spp. therefore suggests that they may be attractive biological control agents for use against phytopathogenic fungi. These properties are suitable for formulation and commercialization (Rhodes, 1990).

The antibiotics from *Bacillus* spp. are always peptide in nature and generally of low molecular weight. In several cases, D rather than L-amino acids are present and the structure can be cyclic or have a cyclic component (Katz and Demain, 1977). Over a hundred antibiotics from the genus *Bacillus* have been identified (Berdy, 1974) and there is strong potential and scope for use of *Bacillus* spp. in biocontrol systems.

The use of any *Bacillus* products as biocontrol should raise questions over safety. The safety of *B. subtilis* and *B. amyloliquefaciens* has been reviewed (Boer and Diderichsen, 1991). This review specifically examined published incidences of *Bacillus* infections. These infections were not due to direct ingestion of

*Bacillus*, but from other sources. The findings showed that infections most frequently appeared in people with a history of endocarditis, who were immunosuppressed or had recently undergone surgery. While the paper also admits that reported cases of food poisoning by *B. subtilis* are very low, it points out that exact and reliable figures are hard to obtain. This is because hospitals do not necessarily differentiate between *B. cereus* and other species of *Bacillus* as agents of food poisoning. An important aspect of establishing safety of a product is proper taxonomic characterization of the bacteria in the product. One approach is for industry to develop and use objective and science- based guidelines for commercial products. Alternatively, it is likely that governments will recognize the need to impose more stringent regulations regarding these particular microorganisms. It is suggested that they be considered the minimal safety information for companies to bring sporeforming bacteria to market as biocontrol products.

In this study, *B. megaterium* have been used for formulation. It was non pathogenic bacterium (Rourke, 2004 and United Nations, 2005). This bacterium has been shown to suppress some plant pathogenic fungi such as *Sclerotium cepivorum*, the causal agent of onion disease (Wong and Hughes, 1986), *R. solani*, *Fusarium roseum* and *Alternaria alternata* which are the causes of rice diseases (Islam and Nandi, 1985; Kanjanamaneesathian *et al*., 2000; Pengnoo *et al*., 2000; Wiwattanapatapee *et al*., 2004).

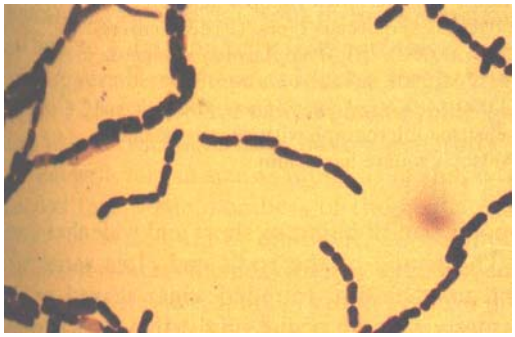

**Figure 3** *B. megaterium*, a rod-shaped bacterium in chains. Gram stain (x600) (Prescott *et al*., 1993).

*B. megaterium* is the gram-positive rod-shaped and usually found in the soil. Its cell wall contains polysaccharide and this makes them to stick together in chains (Figure 3). Morphologically, *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 and produces one endospore per one cell. This bacterium produces important enzymes such as endoproteinase and phospholipase (Brock and Madigam, 1991). Antibiotics are normally produced during stationary phase and some antibiotics are produced during exponential phase (Prescott *et al*., 1993). When *B. megaterium* is cultured in agar, the colony is yellowish white to brown. It produces dark pigment when cultured in broth.

*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 spore 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 spore. The last stage which is not shown, the enzyme destroys

sporangium to release endospore in the complete conditions (Prescott *et al.*, 1993) (Figure 4).

### 3. Production of biological control agents

There are two types of fermentations; solid state and liquid fermentations. Liquid fermentation is the most frequent production technique because it is operationally simple and inexpensive to operate. *B. subtilis* NSRS 89-24 had been reported to produce a lot of antibiotic when cultured in Potato Dextrose Broth, Czapek, Dox Broth and Nutrient Broth (Keawprom, 1996). Musa (1999) reported that *B. subtilis* NSRS 89-24 can be cultured and inhibit mycelial growth of *R. solani* very well when the bacterium is cultured in Mckeen medium.

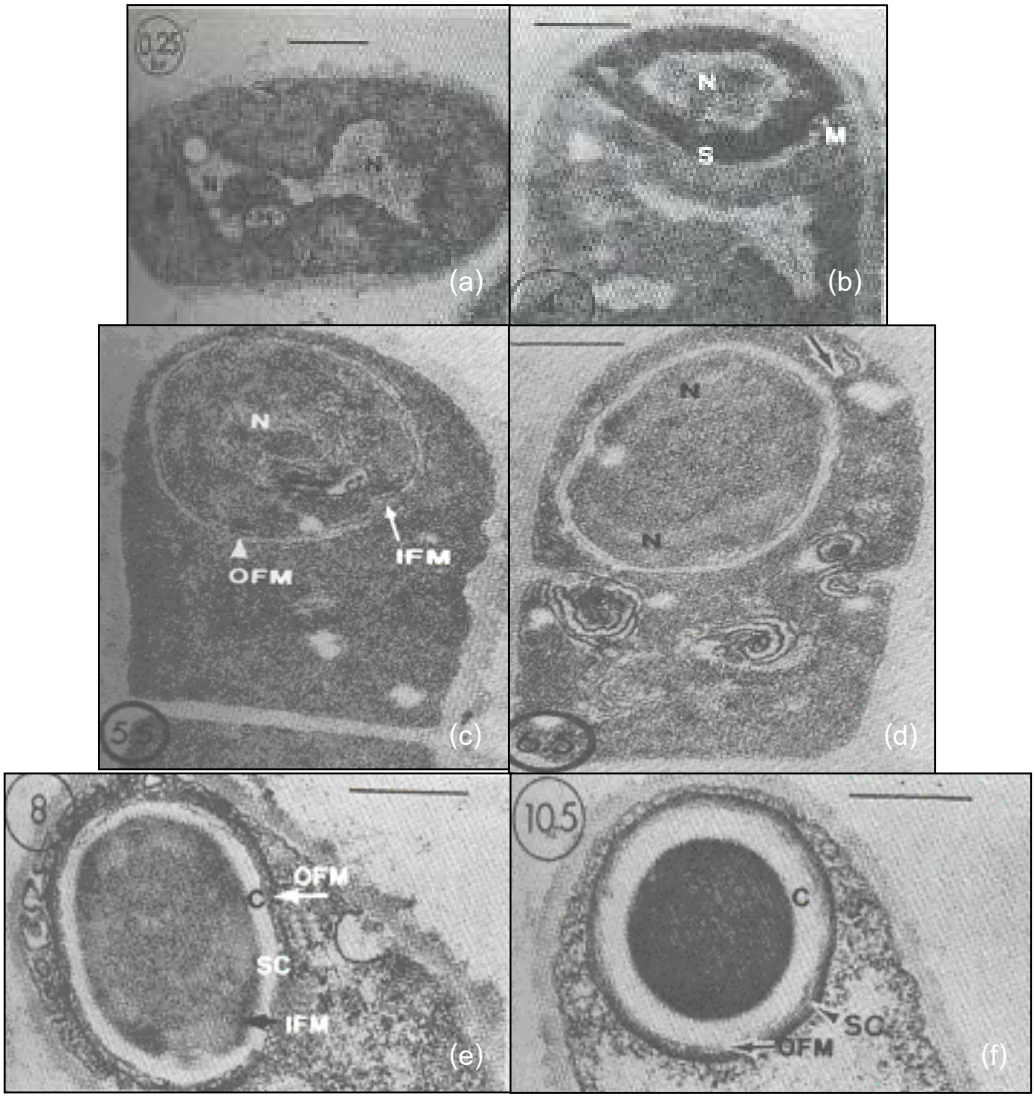


**Figure 4** Stages of sporulation in *B. megaterium* (Prescott *et al*., 1993). The circled numbers refer to the h from the end of the logarithmic phase of growth. (a) 0.25 h: a typical vegetative cell. (b) 4 h: stage II cell, separation. (c) 5.5 h: stage III cell, engulfment. (d) 6.5 h: stage IV cell, cortex formation, (e) 8 h: stage V cell, coat formation. (f) 10.5 h: stage VI cell, mature spore in sporangium. *C*, cortex; *IFM* and *OFM*, inner and outer forespore membranes; *M*, mesosome; *N*, nucleoid; *S*, septum; *SC*, spore coats. The bars indicate 0.5 micron.

## 4. Formulations of microorganism to control plant disease

In general, the obvious advantages of formulation include greater efficacy, increased shelf-life, ease of handling, increased safety, lower production costs and compatibility with agricultural practices (Paau, 1998).

However, for biological control agents to be commercialized, the major difficulty to reach the market and to be competitive with the chemical fungicides is the consistency and the reliability for disease control. Furthermore, shorter shelf-life is also problematic. Both problems can be solved by scientific development of the formulation.

Often a biofungicide formulation comprises many ingredients, such as carriers, diluents, bulking additives, membrane stabilizers, growth and contaminant suppressants, buffering systems, binders, dispersants, lubricants, activators, food sources and coating compounds added for various purposes (Paau, 1998).

The favourable type of formulation is dependent on the objective of application. A liquid formulation would be preferred for application to soilless cultures by appling the inoculant through the drip irrigation system. A granular material would be more appropriate for combining with potting mix, while a wettable powder would be more appropriate for root dips or sprays (Lewis, 1991; Paau, 1998).

### 4.1 Liquid formulations / Fluid suspensions

From early on, microbiologists knew that many soil bacteria such as *Xanthomonas* and *Pseudomonas* species could be harvested from active cultures, rinsed clear of nutrient residues and stored as a concentrated suspension in sterile distilled water at ambient temperature. Bacteria in these concentrated suspensions

remain viable in a physiologically dormant state for a long time and could be recovered years later for experimentation. This long term viability had been exploited to satisfy the long shelf-life requirement of a commercial product. All liquid formulations were prepared as a suspension of the living organisms for application to soil either by in-furrow or overhead spray and drip applications (Paau, 1998).

The earliest commercial *Rhizobium* product was just a jar with an agar substrate at the bottom and rhizobia growing on the agar surface (Burton, 1967). This unformulated product, although often effective, was difficult to regulate the number of bacteria during application. It was also very difficult to store, transport, and required time-consuming handling before use. The growth stage of the organisms in the inoculant was also not controlled and the organisms may have been in various growth phases during application.

To avoid these obstacles, frozen concentrates of bacterial cultures were sometimes used. This formulation also eliminated the need to transport and store agar which might dry and shorten the shelf-life of the inoculant. Storage and transport temperature were either below or just above freezing to lower metabolism of the organisms, achieving a physiologically uniform population with extended shelf-life. The low temperature also discouraged contaminant growth. These concentrates were diluted with water before use to spray into the soil. However, the need for low storage and transport temperature was costly and incompatible with most agricultural field practices (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.

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 regrowth until reactivation with moisture, and thus the products were less likely to be overgrown by contaminant during storage and transport (Paau, 1998).

### 4.2 Solid formulations

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 (I) appling directly to soil with no further manipulations; (II) suspending in water or other carriers for spray applications; or (III) dusting onto seeds to deliver the organisms. The two general types of powders are easily distinguished by their moisture content (Paau, 1998).

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.

Like powder, 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).

Entrapment of biocontrol organisms in calcium alginate granules, termed prill, 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 dropwise 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 pellet.

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 carboxymethylcellulose.