J. Trop. Agric. and Fd. Sc. 38(2)(2010): 249–256

Identification of potential bacteria controlling pathogenic fungi *Rhizoctonia solani* in rice

(Pengecaman bakteria yang berpotensi mengawal fungi perosak *Rhizoctonia solani* tanaman padi)

G.H. Tan*, M.S. Nordin* and A.B. Napsiah*

Keywords: identification, bacteria, pathogenic fungi, *Rhizoctonia solani*, rice

Abstract

This study was to produce highly efficient antagonists to control soil borne plant pathogen *Rhizoctonia solani* in rice. Soil samples from different location in natural ecosystem were collected and characterization for antagonistic efficiency between isolated bacteria and *R. solani* were performed. Antagonistic test in dual culture showed that isolates S3B11 and SW2B49 had the highest antagonistic activities with the inhibition zone of 36.0 mm and 32.2 mm on potato dextrose agar (PDA) medium respectively. These two isolates produced chitinase on M9 agar supplemented with colloidal chitin as the sole carbon source. The results indicated that these two strains hydrolyzed colloidal chitin after 3–4 days. The production of chitinase could be one of the factors which suppressed growth of *R. solani* on the agar medium. From this study, it could be concluded that the two antagonists identified as *Pseudomonas fragi* and *Bacillus pumilus* have the potential to control *R. solani* in rice.

Introduction

Rhizoctonia solani Kuhn. is the causal agent of rice sheath blight, which has become a major constraint to rice production during the last two decades (Kobayashi et al. 1997). The emergence of *R. solani* as an economically important rice pathogen has been attributed to the intensification of rice-cropping systems with the development of new short-statured, high-tillering, high-yielding varieties, high plant densities, and an increase in nitrogen fertilization (Gangopadhay and Chakrabarti 1982; Ou 1985).

This pathogen can survive in soil for many years by producing small (1–3 mm diameter), irregular-shaped, brown to black structures in soil and on plant tissues. Certain rice pathogens of *R. solani* have developed the ability to produce sclerotia with a thick outer layer that allows them to float and survive in water. *Rhizoctonia solani* also survives as mycelium by colonizing soil organic matter as a saprophyte, particularly as a result of plant pathogenic activity (Ghaffar 1988). The sclerotia present in soil and/or on plant tissue germinate to produce vegetative threads (hyphae) of the fungus that can attack a wide range of food and fibre crops. The pathogen is transported in infected soil or through movement of diseased plants or bean pods (Wallwork 1996).

Many researches have been employed to control the sheath blight disease in rice which include searching for high

*Strategic Resources Research Centre, MARDI Headquarters, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
Authors' full names: Tan Geok Hun, Mohd Shukor Nordin and Napsiah A. Rahim
E-mail: tangh@mardi.gov.my
©Malaysian Agricultural Research and Development Institute 2010

quality seeds and avoid seeds that may be contaminated with the pathogen (Farr et al. 1989), preparing for planting areas to increase drainage and prevent water accumulation in the field and addition of compost and organic fertilizers to decrease disease levels (Hawksworth et al. 1995). Fungicides such as methyl thiophanate, PCNB (pentachloronitrobenzene) and chlorothalonil have also been applied to reduce the disease infection. However, so far none of them is found to be effective against this disease in the long term.

Biological control has been developed as an alternative to reduce usage of chemical pesticides to control various plant diseases. By using this strategy, microorganisms themselves or the antibiotics and degrading enzymes that they produce can be used directly against various plant pathogens. Many strains of *B. subtilis* have shown to be potential biocontrol agent against fungal pathogens. It was reported that the principle mechanism of this antifungal involves the production of antibiotics (Fravel 1988). In addition, *B. subtilis* strains produce volatiles that antagonise a range of soil-borne plant pathogen including *R. solani* and *Pythium ultimum*. Thus, the aim of this study was to observe the effectiveness of selected microbes to control *R. solani* in rice.

Materials and methods

Isolation and preparation of bacterial culture

Bacterial culture was isolated from various sources such as sewages, soils and water samples from Pontian, Tok Bok waterfall, Kelantan and Cameron Highlands. The isolation and screening of pure bacterial culture followed the common Microbiological Protocol as described by Sambrook et al. (1989) and cultured on NBTA medium (nutrient agar supplemented with 0.025 g bromothymol blue and 0.04 g 2,3,5-triphenyltetrazolium chloride per litre, AmBresco, USA). A loopful of 24 h bacterial culture was added to a 250 ml conical flask containing 100 ml of tryptic soy broth and shaken for 24 h at ambient temperature (~28 °C). Subsequently, the bacterial suspension was spun at 4,000 x g for 5 min and the supernatant was discarded. Sterile phosphate buffer (50 mM, pH 7.2) was added to the pellets and mixed thoroughly to obtain a concentrated suspension of the bacterial symbiont. Different cell concentrations were then prepared by dilution of sterile phosphate buffer (50 mM), and the cell number in each suspension was established by counting the colony forming unit (cfu/ml).

Fungi preparation

The isolate of *R. solani* was obtained from sheath blight disease in rice from Biotechnology Research Centre, MARDI. The fungi was cultured on PDA (Potato Dextrose Agar, AmBresco, USA) and incubated in the dark at ambient temperature (~28 °C) for 5 days for complete sporulation, and later stored at 4 °C.

Inhibition of fungal growth by bacteria

One-week-old *R. solani* culture was cut into 5 mm diameter discs and transferred to the PDA petri dishes. In each dish, the same agar medium was inoculated with a 5 mm diameter disc of antagonist positioned diametrically opposite to the 5 mm diameter disc of the *R. solani*. The distance between discs was approximately 60 mm. The cultures were incubated at 28 ± 3 °C and measurement was recorded after 4 days of incubation. The radial growth was measured after 1 week of the observation. The efficiency of antagonist in suppressing radial growth was calculated as follows: $[C-T]/C \times 100$ where C is radial growth measurement of the pathogen in the negative control and T is radial growth of the pathogen in the presence of antagonist. The disc soaked in distilled water was set as negative control. Each test has three replications.

Detection of chitinolytic activity

In order to test the chitinolytic activity on plates, cells of bacteria strains were

inoculated on a chitinase detection agar plate which was prepared by mixing 10 g of colloidal chitin and 20 g of agar in M9 medium (25.6 g Na_2HPO_4 $7H_2O$, 6.0 g KH_2PO_4, 1.0 g NaCl, 2.0 g NH_4 Cl, 1 litre). The plates were incubated at 30 °C for 7 days until clearing zones could be detected around the colonies. Each test has three replications.

Identification of potential bacteria using 16S rDNA analysis

A loopful of bacterial cell was picked up and suspended in TE buffer [100 mM Tris (pH 8.0), 30 mM EDTA (pH 8.0), 360 μl]. Lsozyme (50 mg/ml, 20 μl) was added into the tube and incubated at 37 °C for 30 min. SDS [10% (w/v), 40 μl] was added, and the tube was inverted for 5–6 times, then incubated at 55 °C for 10 min. Phenol: chloroform: isoamyalcohol [25:24:1, 400 μl] was added and mixed well. The mixture was spun at 13,000 rpm for 15 min. The upper solution was transferred to a new microtube. The same procedure was repeated if the solution was not clear. Sodium acetate (3 M, 1/10 volume) and 2 volume of cold ethanol was then added. DNA was then pooled by glass rod and dried for 5–10 min until DNA became clear. Finally, the DNA was dissolved in 100–200 μl of sterilized distilled water and stored at –20 °C.

Polymerase Chain Reaction (PCR) of the 16S rDNA for the bacterial isolates was conducted and the PCR reaction was carried out as followed: sterile distilled water (59.5 μl); 10X PCR buffer (Promega, USA, 10.0 μl); 25 mM $MgCl_2$ (Promega, USA, 8.0 μl); 2 mM dNTP mix (Promega, USA, 10.0 μl); universal primers (Forward: 5' GAG TTT GAT CCT GC TCA G 3'; Reverse: 5' GTT ACC TTG TTA CGA CTT 3'] Invitrogen, USA, 10 pmol/μl, 4.0 μl); Taq Polymerase (5U/μl; 0.5 μl) and genomic DNA as template (4 μl). The PCR tubes was then put into therma cycler and preheat at 94 °C for 3 min, followed by 25 cycles of denaturing at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2 min and final elongation at 72 °C for 3 min. The reactions were kept at 4 ºC until loaded onto the gel. The PCR product was purified, sequenced and compared with the 16S rDNA sequence of bacteria from the NCBI Gene Bank nucleotide sequence. The sequence and identity of each isolate was then double confirmed by constructing the Phylogenetic Tree using BioEdit (CA) and MEGA 4.0.2 software (UK).

Results and discussion

The interaction between target fungi (*R. solani*) and bacteria culture was recorded after 4 days of incubation once the two cultures were placed on the same petri dish. A total of 25 bacteria isolates (out of 43 isolated from different sources) were examined as potential inhibitor against *R. solani* with different antagonistic efficiency (*Figure 1*). Based on their performance, isolate S3B11 and SW2B49 showed the highest inhibition performance against *R. solani* as shown in *Plate 1*. These two isolates were further tested for chitinolytic production on M9 media supplemented with 0.1% colloidal chitin. The result showed the appearance of clear zone around the colony as shown in *Plate 2* indicating the chitinase activity of the culture.

Several bacteria were reported to have the potential to produce thermo stable chitinase (Sakai et al. 1998). In this study, *Pseudomonas fragi* and *Bacillus pumilus* required 3 to 4 days to produce thermo stable chitinase when incubated at 60 °C. A similar finding was reported by Khiyami and Masmali (2008) where production of chitinase by *P. aeruginosa* K-187 and *Bacillus* strain MH-1 took about 4 days at 58 °C. Chitinase is able to degrade chitin in the fungi cell wall and inhibited growth of the fungi. Chitinase producing microorganisms have been reported as biocontrol agent for different kinds of plant fungal diseases (Chernin et al. 1995, Freeman et al. 2004; Liu et al. 2008).

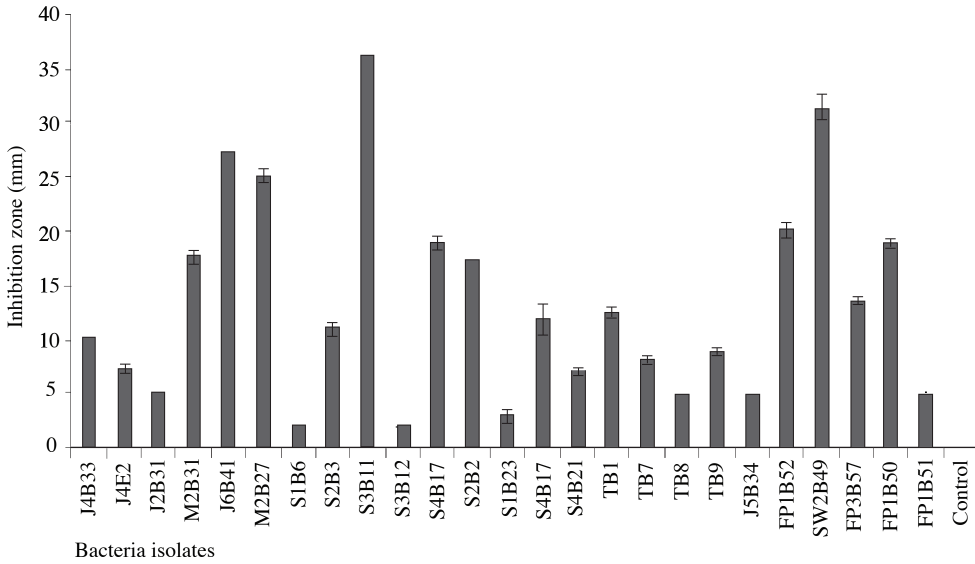


Figure 1. Inhibition zone (mm) formed by bacteria isolates against ***Rhizoctonia solani*** *on plate after 1 week of incubation. Assays were performed in triplicates and the error bars represent the standard error from the arithmetic mean*

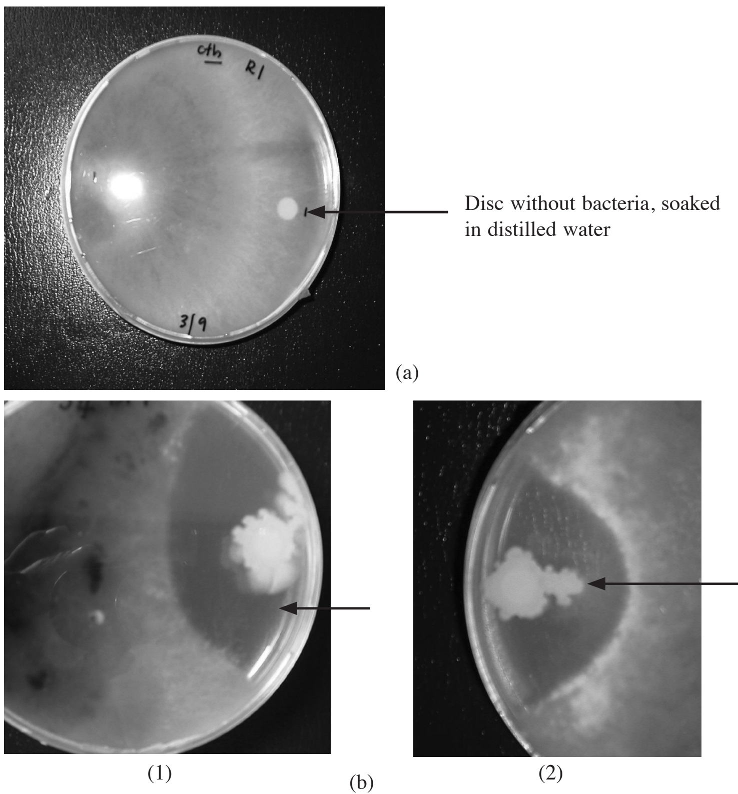
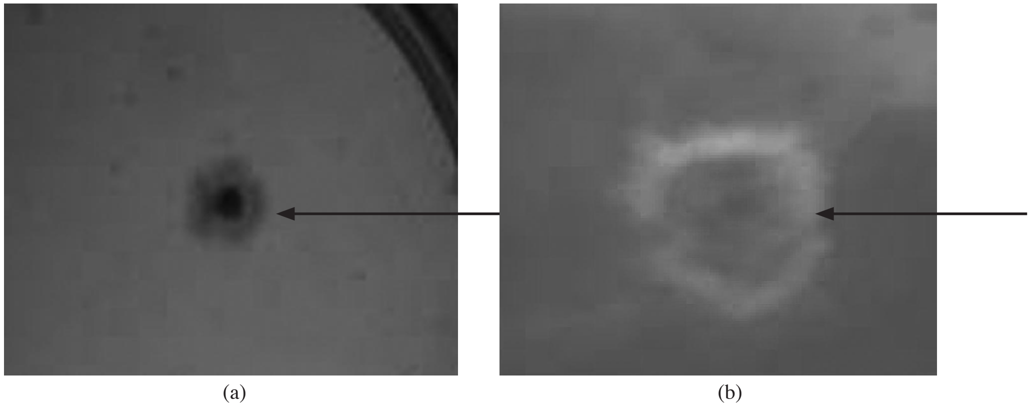


Plate 1. Some examples of the antagonistic effect of bacteria in dual culture. (a) The control disc without bacteria. (b) Disc soaked in (1) S3B11 and (2) SW2B49, a zone of inhibition in a lawn of ***Rhizoctonia solani*** *surrounds a spot-inoculum of antagonists (indicated by arrow)*



(a) (b)

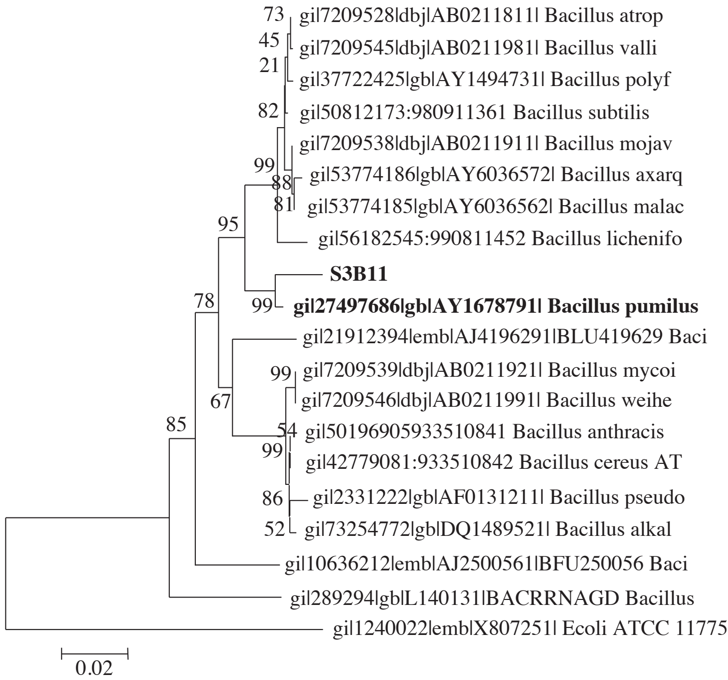
Plate 2. (a). Detection of chitinase production of S3B11 on M9 medium containing 0.1% colloidal chitin, without congo red, (b). The clearing zones of colloidal chitin formed by chitinase, stained with 0.1% Congo red solution (indicated by black arrow)

Based on previous studies, there are more than 167 biological compounds produced by *Bacillus* species that are active against bacteria, fungi, protozoa and viruses (Berdy 1974; Katz and Demain 1977; Karuse et al. 1990; Cordovilla et al. 1993). Most of the antibacterias are peptides and are active against Gram-positive species while compounds such as polymyxin and colistin are active against Gram-negative species. *Bacillus subtilis* BNI strain is effective against *Macrophomina phaseolina* (Smith et al. 1986), *R. solani* and *P. ultimum* (Wright and Thompson 1985; Fiddaman and Rossal 1993). *Bacillus cereus* 28-9 excreted two chitinases which had inhibitory activity against *Botritis elipitica* (Huang et al. 2005).

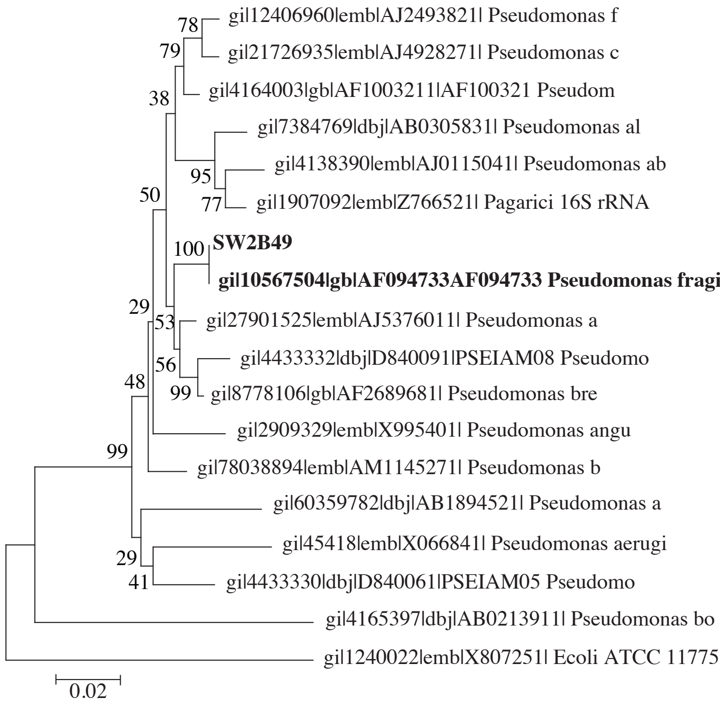
Based on 16S rDNA sequence analysis, the isolate S3B11 was identified as *Bacillus pumilus* and SW2B49 as *Pseudomonas fragi*. These sequences were further confirmed by constructing the phylogenetic tree to correlate with the family tree of both species. The known genus sequence of *Bacillus* and *Pseudomonas* were downloaded from the Gene Bank and computed using BioEdit (CA) and MEGA 4.0.2 software (UK). The final phylogenetic trees (*Figure 2*) showed that the new sequence of S3B11 and SW2B49 are closely related to *Bacillus pumillus* with 99% and *Pseudomoas fragi* with 100% of similarity respectively.

Table 1. The identification of positive antagonists against *Rhizoctonia solani* by 16S rDNA analysis

Bacteria isolates	ID (% similarity with NCBI Gene Bank)
J4B33	*Bacillus thuringiensis* (98%)
J4E2	*Bacillus* sp. (80%)
J2B31	*Bacillus* sp. (80%)
M2B31	*Bacillus* sp. (80%)
J6B41	*Bacillus fusiformis* (95%)
M2B27	*Kluyvera Georgiana* (90%)
S1B6	*Baccilus cereus* (95%)
S2B3	*Lysinibacillus sphaericus* (90%)
S3B12	*Bacillus thuringiensis* (90%)
S4B17	*Bacillus* sp. (80%)
S2B2	*Lysinibacillus sphaericus* (90%)
S1B23	*Bacillus macroides* (98%)
S4B21	*Bacillus* sp. (80%)
TB1	*Bacillus* sp. (80%)
TB7	*Bacillus cereus* (95%)
TB8	*Bacillus* sp. (85%)
TB9	*Bacillus* sp. (85%)
J5B34	*Bacillus cereus* (95%)
FP1B52	*Corynebacterium urealyticum* (85%)
FP3B57	*Aeromonas allosaccharophila* (80%)
FP1B50	*Aeromonas* sp. (80%)
FP1B51	*Bacillus cereus* (90%)



(a) *Bacillus genus*



(b) *Pseudomonas genus*

Figure 2. Phylogenetic tree of (a) ***Bacillus*** *genus and (b)* ***Pseudomonas*** *genus. S3B11 and SW2B49 are closely related to* ***Bacillus pumillus*** *with 99% and* ***Pseudomoas fragi*** *with 100% of similarity, respectively*

The other isolates were also identified using the same method and listed in *Table 1*. However, the phylogenetic data of isolate was not shown.

Conclusion

This study concluded that out of 25 bacteria isolates from various sources, two antagonists namely S3B11 and SW2B49 inhibit the growth of *R. solani* due to the production of chitinase enzyme in the medium. They were identified as *Bacillus pumilus* and *Pseudomonas fragi*. Their potential in suppressing fungal growth should be exploited as a complement or an alternative to chemical control for sheath blight disease in rice.

Acknowledgement

The authors would like to thank MARDI and MOSTI for funding the study under the Bilateral Project between MARDI and Malaysia-Agriculture Genetic Institute (AGI). Many thanks to Dr Marzuki Hashim and Ms Noriha Mat Amin, Biotechnology Research Centre, MARDI for providing the *R. solani* culture.

References

Berdy, J. (1974). Recent developments of antibiotic research and classification of antibiotics according to chemical structure. *Adv Appl Microbiol* 18: 309–406

Chernin, L., Ismailov, Z., Haran, S. and Chet, I. (1995). Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. *Appl. Environ. Microbiol.* 61: 1720–1726

Cordovilla, P., Valdivia, E., Gonzalez-Segura, A., Galvez, A., Martinez-Bueno, M. and Maqueda, M. (1993). Antagonistic action of the bacterium *Bacillus licheniformis* M-4 toward the amoeba *Naegleria fowleri*. *J. Eukaryot Microbiol* 40: 323–328

Farr, D.F., Bills, G.F., Chamuris, G.P. and Rossman, A.Y. (1989). *Fungi on plants and plant products in the United States*. St. Paul, Minnesota: APS Press

Fravel, D.R. (1988). Role of antibiosis in the biocontrol of diseases. *Ann. Rev. Phytopathol.* 26: 75–91

Fiddaman, P.J. and Rossal, S. (1993). The production of antifungal volatiles by *Bacillus subtilis*. *J. Bacteriol.* 74: 119–126

Freeman, S., Minzm, O., Kolesnik, I., Barbul, O., Zveibil, A., Maymon, M., Nitzani, Y., Kirshner, B., Rav-David, D., Bilu, A., Dag, A., Shafir, S. and Elad, Y. (2004). *Trichoderma* biocontrol of *Colletotrichum acutatum* and *Botrytis cinerea* and survival in strawberry. *Eur. J. Plant Pathol.* 110: 361–370

Gangopadhay, S. and Chakrabarti, N.K. (1982). Sheath blight on rice. *Rev. Plant Pathol.* 61: 451–460

Ghaffar, A. (1988). Soil borne disease research centre, Final Research report. Department of Botany University of Karachi 75270 Pakistan. 111 p.

Hawksworth, D.L., Kirk, P.M., Sutton, B.C. and Pegler, D.N. (1995). *Ainsworth and Bisby's Dictionary of the Fungi*. CAB International. Cambridge, Oxon, UK: University Press

Huang, C.J., Wang, T.K., Chung, S.C. and Chen, C.Y. (2005). Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus* 28–9. *J. Biochem. Molecular. Biol.* 38(10): 82–88

Karuse, N., Tenyo, O., Kobaru, S., Kamei, H., Miyaki, T., Konishi, M. and Oki, T. (1990). Pumilucidin, a complex of new antiviral antibiotics. Production, isolation, chemical properties, structure and biological activity. *J. Antibiot* 43: 267–280

Katz, E. and Demain, A.L. (1977). The peptide antibiotics of Bacillus: chemistry, biogenesis and possible functions. *Bacteriol Rev.* 40: 449–474

Khiyami, M. and Masmali, I. (2008). Characteristics of thermostable chitinase enzymes of *Bacillus licheniformis* isolated from red palm weavil gut. *Australian Journal of Basic and Applied Sciences* 2: 943–948

Kobayashi, T., Mew, T.W. and Hashiba, T. (1997). Relationship between incidence of rice sheath blight and primary inoculum in the Philippines: Mycelia in plant debris and sclerotia. *Ann. Phytopathol. Soc. Jpn.* 63: 324–327

Liu, Z.H., Yang, Q., Hu, S., Zhang, J.D. and Ma, J. (2008). Cloning and characterization of a novel chitinase gene (*chi6*) from *Chaetomium globosum* and identification of its biological activity. *Appl. Microbial Biotechnol.* 80: 241–252

Ou, S.H. (1985). *Rice diseases*. Richmond, Surrey, Great Britain: Commonwealth Mycological Institute

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular cloning: a laboratory manual*. Cold Spring Harbour, New York: Cold Spring Harbor Laboratory Press.
Sakai, K., Yakota, A., Kurokawa, H., Wakayama, M. and Moriguchi, M. (1998). Purification and characterization of three thermostable endochitinases of *Bacillus* noble strain MH-1 isolated from chitin containing compost. *Appl. Environ. Microbiol*. 64(9): 3340–3397
Smith, M.D., Flicknger, J.L., Lineberger, D.W. and Schmidth, D.B. (1986). Protoplast transformation in coryneform Bacteria and introduction of an oLAmylase Gene from *Bacillus amyloliquefaciens* into *Brevibacterium lactofermentum*. *Appl. Environ. Microbiol*. 51(3): 3634–3639
Wallwork, H. (1996). *Cereal root and crown diseases*, p. 14–16. Perth, Australia: Kondinin Group
Wright, S.J.L. and Thompson, R.J. (1985). *Bacillus* volatiles antagonize cyanobacteria. *FEMS Microbiol. Lett*. 30: 263–267

Abstrak
Kajian ini bertujuan untuk menghasilkan antagonis yang cekap mengawal fungi perosak dalam tanaman padi, iaitu *Rhizoctonia solani*. Pensampelan tanah dan pengasingan bakteria dari beberapa lokasi dan tindak balas antagonis antara pencilan bakteria dengan *R. solani* telah dijalankan. Ujian antagonis menunjukkan bahawa pencilan S3B11 dan SW2B49 mempamerkan aktiviti antagonis yang paling tinggi dengan menghasilkan zon jernih masing-masing pada 36.0 mm dan 32.2 mm dalam medium agar dektrosa kentang (PDA). Kedua-dua pencilan mampu menghasilkan kitinase dalam agar M9 yang ditambahkan dengan kitin berkoloid sebagai sumber karbon. Keputusan menunjukkan kedua-dua pencilan tersebut mampu menghidrolisis kitin berkoloid selepas 3–4 hari. Penghasilan kitinas merupakan salah satu faktor yang merencatkan pertumbuhan *R. solani* pada medium agar. Daripada kajian ini, kedua-dua antagonis yang dikenal pasti sebagai *Pseudomonas fragi* dan *Bacillus pumilus* berpotensi mengawal *R. solani* dalam tanaman padi.

Accepted for publication on 27 October 2010