EFFICACY OF BENEFICIAL MICROORGANISM FOR PLANT GROWTH STIMULATION AND PLANT IMMUNITY TO DISEASES IN RICE

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หัวข้อวิทยานิพนธ์	ประสิทธิภาพของจุลินทรีย์ที่มีประโยชน์ต่อการกระตุ้นการเจริญเติบโตของพืชและการสร้างภูมิคุ้มกันโรคในข้าว
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บทคัดย่อ

ผลการวิจัยพบว่า *Curvularia lunata* ทำให้เกิดโรคใบจุดในข้าวพันธุ์ กข41 และ พิษณุโลก2 และ *Drechslera oryzae* ทำให้เกิดโรคใบจุดสีน้ำตาลในข้าวพันธุ์ กข47 และ กข57 เชื้อ *C. lunata* และ *D. oryzae* ได้รับการพิสูจน์แล้วว่าสามารถก่อโรคได้ พบแบคทีเรียต่อต้านคือ *Rhodospirillum centenum* สายพันธุ์ SM41, SM61, SM72 และ SM92. เชื้อ *R. centenum* สายพันธุ์ SM41, SM61, SM72 และ SM92 ลักษณะโคโลนีมีสีแดง, โคโลนีนูนเป็นเงา, แกรมลบ, การเคลื่อนที่ของเซลล์เคลื่อนที่ และการทดสอบกิจกรรมเจลาตินเป็นลบ *R. centenum* สายพันธุ์ SM41 และ SM61 แสดงกิจกรรมที่ดีของการหมักกลูโคส แลคโตส และซูโครส และแยก SM72 และ SM92 พบว่าเป็นกิจกรรมที่ไม่ผ่านการหมัก *R. centenum* สายพันธุ์ SM41, SM61, SM72 และ SM92 ยังได้รับการพิสูจน์โดยโมเลกุล phylogenic ผ่านการวิเคราะห์ลำดับยีน 16S rRNA ซึ่งเกี่ยวข้องอย่างใกล้ชิดกับ *Rhodospirillum centenum* SW ใน Genbank No CP00613.2

แบคทีเรียต่อต้านอื่นๆ สายพันธุ์ *Serratia marcescens* LB01 แสดงลักษณะโคโลนีสีแดง, เรียบ, แกรมลบ, รูปแท่ง, สามารถเคลื่อนที่, เจลาตินเป็นบวก *Dietzia cercidiphylli* สายพันธุ์ LB02 แสดงสีส้ม, ผิวเรียบ, แกรมบวก, รูปแท่ง, ไม่เคลื่อนที่ และเจลาตินเป็นลบ ผลการวิจัยเพิ่มเติม *R. centenum* SM41, SM61, SM72, SM92, *S. marcescens* (LB01) และ *D. cercidiphylli* (LB02) ได้รับการพิสูจน์ว่าผลิตอะไมเลส โปรตีเอส และไลเปส ซึ่งพิสูจน์ได้ว่าเป็นกลไกการควบคุม *R. centenum* SM41 ที่ปฏิบัติต่อข้าวพันธุ์ กข41 ส่งเสริมการงอกของเมล็ดข้าวพันธุ์ พิษณุโลก2 และตามด้วย *R. centenum* สายพันธุ์ SM92, SM61 และ SM72 *R. centenum* สายพันธุ์ SM92 ที่ใช้กับข้าวพันธุ์ กข41 ส่งเสริมให้ต้นข้าวงอกและ

เจริญเติมโตดีและสายพันธุ์ *R. centenum* สายพันธุ์ SM41, SM61, SM72 และ SM92 กระตุ้นการงอกของเมล็ดข้าวพันธุ์ กข41 และ พันธุ์พิษณุโลก2 ปฏิกิริยาต่อต้านเชื้อก่อโรคของ *R. centenum* สายพันธุ์ SM41 และ SM61 พบว่าช่วยลดโรคใบจุดและกระตุ้นการเจริญเติบโตของต้นข้าว *S. marcescens* (LB01) และ *D. cercidiphylli* (LB02) พบว่าต่อต้านเชื้อรา *C. lunatra* สาเหตุโรคใบจุดในข้าวพันธุ์ กข41 และส่งเสริมการเจริญเติบโตของข้าวพันธุ์ กข41

พบเชื้อราต่อต้าน *Neosartorya hiratsukae* EU06 ที่สามารถยับยั้งเชื้อ *D. oryzae* ที่ทำให้เกิดโรคใบจุดสีน้ำตาลในข้าวพันธุ์ กข47. *Chaetinium brasiliense* CB01 ได้รับการพิสูจน์แล้วว่าสามารถยับยั้ง *D. oryzae* ที่ทำให้เกิดโรคใบจุดสีน้ำตาลในข้าวพันธุ์ กข47. สารสกัดหยาบของเชื้อ *Ch. brasiliense* CB01 ยับยั้งเชื้อ *D. oryzae* ที่ทำให้เกิดโรคใบจุดสีน้ำตาลของข้าว กข47 สารสกัดหยาบเอทิลอะซีเตตของ *N. hiratsukae* EU06 พิสูจน์แล้วว่าสามารถยับยั้ง *D. oryzae* ที่ก่อให้เกิดโรคใบจุดสีน้ำตาลในข้าวพันธุ์ กข 47 ที่ ED_{50} เท่ากับ 12.18 ppm และตามด้วยสารสกัดหยาบเฮกเซนและเมทานอล ซึ่งค่า ED_{50} เท่ากับ 29.16 และ 43.90 ppm. ตามลำดับ สารสกัดหยาบเอทิลอะซิเตตของ *Ch. brasiliense* CB01 ยับยั้งเชื้อ *D. oryzae* ที่ทำให้เกิดโรคใบจุดสีน้ำตาลของข้าว กข47 ซึ่งมี ED_{50} เท่ากับ 9.64 ppm และสารสกัดหยาบเฮ-กเซนและเมทานอล ซึ่งมีค่า ED_{50} เท่ากับ 14.35 และ 23.35 ppm ตามลำดับ

งานวิจัยเกี่ยวกับนาโนเทคโนโลยีสำหรับการสร้างภูมิคุ้มกันโรคพืชพบว่า Nano-NHM จาก *N. hitsukae* EU06 ยับยั้งเชื้อ *D. oryzae* ที่ทำให้เกิดโรคใบจุดสีน้ำตาลในข้าวพันธุ์ กข47. Nano-NHM, nano-NHE และ nano NHH ยับยั้งการสร้างสปอร์ด้วยค่า ED_{50} ที่ 3.93, 3.97 และ 4.46 ppm ตามลำดับ นอกจากนี้ Nano-CBH, Nano-CBE และ Nano-CBM จาก *Ch. brasiliense* CB01 ยับยั้งการสร้างสปอร์ของ *D. oryzae* ที่ทำให้เกิดโรคใบจุดสีน้ำตาลในข้าวพันธุ์ กข47 ที่มี ED_{50} เป็น 0.83, 1.70 และ 2.59 ppm ตามลำดับ Nano-CBH, Nano-CBE , Nano-CBM ที่ฉีดพ่นในข้าวพันธุ์ กข47 ที่ปลูกเชื้อด้วยเชื้อ *D. oryzae* ทำให้เกิดใบจุดสีน้ำตาล พบว่ามีการสร้างสารต่อต้านการเกิดโรค คือ Oryzalexin C โดยการตรวจโดยวิธีทางโครมาโทกราฟีแบบแผ่นเคลือบ ที่ค่า R_f 0.61

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ABSTRACT

The research findings found *Curvularia lunata* causing leaf spot of rice var. RD41 and PL2 and *Drechslera oryzae* (brown leaf spot of rice var. RD47 and RD57). *C. lunata* and *D. oryzae* were proved by pathogenicity. The antagonistic bacteria were found as *Rhodospirillum centenum* strains SM41, SM61, SM72 and SM92. *R. centenum* strains SM41, SM61, SM72 and SM92 found to be gram-negative, motile cells, gram negative, expressed reddish color and shiny convex colony. It was negative for gelatin activity test. *R. centenum* strains SM41 and SM61 expressed to good fermentation activities of glucose, sucrose and lactose and isolates SM72 and SM92 found to be non-fermented activity *R. centenum* strains SM41, SM61, SM72 and SM92 were also proved by molecular phylogenic through analysis of 16S rRNA gene sequence which closely related to *Rhodospirillum centenum* SW in Genbank No CP00613.2.

The other antagonistic bacteria, *Serratia marcescens* strain LB01 showed gram-negative, rod shape, motility, gelatin positive, red culture, and smooth appearance. *Dietzia cercidiphylli* strain LB02 showed orange in color, smooth, gram-positive, rod, non-motile, and gelatin negative. Further research findings, *R. centenum* SM41, SM61, SM72, SM92. *S. marcescens* (LB01) and *D. cercidiphylli* (LB02) were shown to produce amylase, protease and lipase which possible proved to be control mechanism. *R. centenum* SM41 treated to rice var. RD41 promoted seed germination of rice var. PL2 and followed by *R. centenum* strains SM92, SM61 and SM72. *R. centenum* strain SM92 applied to rice var. RD41 gave a good plant strand of rice and *R. centenum* strains SM41, SM61, SM72 and SM92 stimulated

seed germination on rice var. RD41 and var. PL2. The antagonistic action of *R. centenum* strains SM41 and SM61 decreased leaf spot disease and stimulated plant growth of rice. *Serratia marcescens* (LB01) and *Dietzia cercidiphylli* (LB02) were found to be antagonize *C. lunatra* causing leaf spot of rice var. RD41 and promoted plant growth of rice var RD41.

The antagonistic fungus, *Neosartorya hiratsukae* (EU06) found to be inhibited *D. oryzae* (brown leaf spot of rice var. RD47). *Chaetinium brasiliense* (CB01) proved to be inhibited the tested *D. oryzae.* Crude extracts of *Ch. brasiliense* (CB01) inhibited the tested *D. oryzae.*Crude ethyl acetate metabolite of *N. hiratsukae* EU06 proved to be inhibited *Drechslera oryzae* causing brown leaf spot of rice var. RD47 the ED_{50} of 12.18 ppm, and followed by crude hexane and methanol metabolite which the ED_{50} of 29.16 and 43.90 ppm, respectively. Crude ethyl acetate extract of *Ch. brasiliense* CB01 showed highest inhibition to the tested *D. oryzae* which the ED_{50} of 9.64 ppm, and followed by crude hexane and methanol metabolites which the ED_{50} were 14.35 and 23.35 ppm, respectively.

Research investigation on nanotechnology for plant immunity was found that nano-NHM from *N. hiratsukae* EU06 inhibited *D. oryzae* (brown leaf spot of rice var. RD47). Nano-NHM, nano-NHE and nano-NHH inhibited sporulation with the ED_{50} values of 3.93, 3.97 and 4.46 ppm, respectively. Morover, nano-CBH, nano-CBE, and nano-CBM from *Ch. brasiliense* CB01 inhibited sporulation of *D. oryzae* with ED_{50} of 0.83, 1.70, and 2.59 ppm, respectively. Nano-CBH, nano-CBE, nano-CBM treated to the inoculated rice var. RD47 with *D. oryzae* which proved to produce Oryzalexin C on the thin layer chromalography where the retention factor (R_f) is 0.61.

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CHAPTER 1

INTRODUCTION

1.1 Statement and significant of the problems

Rice is a monocotyledon plants, *Oryza sativa* (Asian rice) or *Oryza glaberrima* (African rice). It is a cereal grain that most widely consumed as staple food for a large part of the world's human population, especially in Asia and the West Indies. It has the grains with the third-highest worldwide production (FAOSTAT, 2017).

Drechslera oryzae (synonyms *Helminthosporium oryzae*, *Bipolaris oryzae, Cochilobolus miyabeans*) causes brown leaf spot of rice which important to cause yield loss. It is a fungal pathogen that infects seedlings and mature rice plants. The symptom is brown blight and mostly destroyed seedling stage of 10-58% (Rice Department, 2018).

The chemical fungicides have been used by farmers and are known to cause residual toxicity, pathogen resistance, polluted to environment, hazardous to living things including human, animals, beneficial microorganism. Scientists have interested to develop biological control to solve the problems and to serve for environmentally safety. The research for biocontrol control agents have come out in recent years eg, *Trichoderma* spp. (Khalili, 2012) *Bacillus subtilis*, *Chaetomium spp., Chaetomium cochliodes* (Soytong, 2014) for plant disease control.

1.2 Objectives

The general objective was to find beneficial microorganism for plant growth stimulation and plant immunity to disease in rice. Specifically, the study was aimed:

1. To isolate, identify of pathogens from leaf spot and brown leaf spot of rice var. RD41, RD47, RD57 and Pitsanulok 2 (PL2)
2. Pathogenicity tests
3. Isolation, identification of Photosynthetic bacteria
4. Morphological and physiological characterization

5. Enzyme production property of photosynthesizing bacteria
6. Evaluation of Photosynthetic bacterium *Rhodospirillum centenum* strain SM41, SM61, SM72 and SM92 to increase the growth of rice var. RD41 and Pitsanulok 2 (PL2)
7. Effects of Photosythesizing bacteria *Serratia marcescens* (LB01) and *Dietzia cercidiphylli* (LB02) for controlling leaf spot (*Curvularia lunata*) of rice var. RD41 and stimulating plant growth
8. Antagonistic *Chaetomium brasiliense* strain CB01 and *Neosartorya hiratsukae* strain EU06 against brown leaf spot (*Drechsoera oryzae*) of rice var. RD47
9. Bi-culture antagonistic test
10. Active metabolites from *Chaetomium brasiliense* strain CB01 and *Neosartorya hiratsukae* strain EU06 testing for inhibition of leaf spot of rice var. RD47 caused by *Drechslera oryzae*
11. Testing nano-particles from *Chaetomium brasiliense* srtrain CB01 and *Neosartorya hiratsukae* strain EU06 to control brown leaf spot of rice var. RD47 caused by *Drechslera oryzae*
12. Testing nano-particles derived from *Chaetomium brasiliense* strain CB01 (nano-CBH, Nano-CBE and Nano-CBM) to induce plant immunity for brown leaf spot of rice var. RD47 caused by *Drechslera oryzae*

1.3 Scope and location of research work

The research findings are covered the isolation, identification of pathogens from leaf spot and brown leaf spot of rice var. RD41, RD47, RD57 and Pitsanulok 2 (PL2) and their pathogenicity tests. Isolation, and identification of Photosynthetic bacteria were investigated. Enzyme production property of photosynthesizing bacteria were done. Photosynthetic bacterium *Rhodospirillum centenum* strains SM41, SM61, SM72 and SM92 to increase the growth of rice var. RD41 and Pitsanulok 2 (PL2) was evaluated. Effects of Photosythesizing bacteria *Serratia marcescens* (LB01) and *Dietzia cercidiphylli* (LB02) for controlling leaf spot (*Curvularia lunata*) of rice var. RD41 and stimulating plant growth were conducted. Active metabolites from *Chaetomium brasiliense* strain CB01 and *Neosartorya hiratsukae* strain EU06 testing for inhibition of leaf spot of rice var. RD47 caused by *Drechslera oryzae* were done. Testing nano-particles from *Chaetomium brasiliense* srtrain CB01 and *Neosartorya hiratsukae* strain EU06 to control brown leaf spot of rice var. RD47 caused by *Drechslera oryzae* were

tested. Testing nano-particles derived from *Chaetomium brasiliense* strain CB01 (Nano-CBH, Nano-CBE and Nano-CBM) to induce plant immunity for brown leaf spot of rice var. RD47 caused by *Drechslera oryzae* were evaluated.

CHAPTER 2

REVIEW LITERATURE

2.1 Rice

Rice (*Oryza sativa* L.) is a cereal grain which has consumed as staple food for a large part of the world. It can grow to height between 1 to 1.8 m, but also depends on soil fertility, slender leaves of 50–100 cm (20–39 in) in length, and 2–2.5 cm (0.79–0.98 in) broad. The flowers are produced in a branched arching to inflorescence between 30 to 50 cm. Rice are destroyed by plant pathogens and pests to reduce the yield (Jahn *et al*., 2007). Other abiotic factors can reduce rice growth eg. overuse of chemical pesticides, improper irrigation, and overuse nitrogen fertilizer (Jahn *et al*., 2005). Outbreak from insects and plant pathogens eg rice gall midge, army worm outbreaks during high rainfall, thrips during drought. *Curvularia lunata* (leaf spot) is caused and showed many symptoms e.g. grain discoloration, leaf spot, black kernel and seedling blight (Douangboupha *et al*., 2006).

2.2 History, taxonomy and biology of Pathogen

Taxonomy of *Drechslera oryzae*

Classification of fungi (lto, 1930)

Kingdom Fungi

Division Ascomycota

Class Dothideomycetes

Order Pleosporales

Family Pleosporaceae

Form Genus *Drechslera*

The net blotch disease caused by *Drechslera teres*, an imperfect stage *Pyrenophora teres*. *Drechslera oryzae* is classified in the Deuteromycotina (imperfect fungi), class Deuteromycetes, order Moniliales, and family Dematiaceae.

Imperfect stage appears the conidiophores arising singly or in groups, branched or simple, multi-septate, flexuous, sometimes upper part geniculate, brown to black. Conidiogenous nodes dark

brown, smooth or slightly verruculose. Conidia are usually curved, rarely straight, navicular, fusiform, obclavate or cylindrical, hyaline when immature, becoming slightly brown. (Manamgoda, 2014)

Drechslera oryzae are reported to cause brown leaf spot of rice which is important plant pathogen to causes widespread disease leading to yield losses. Brown spot can infect both seedlings and mature plants which causes blight on seedlings and seedling mortality of 10-58% (Rice Department, 2018).

2.3 Photosynthetic bacteria

Photosynthetic bacteria are found to apply in various biotechnological applications. It is used for water purification, bioremediation of chemical, bio-fertilizers, wastewater purifiers, animal feed and aquaculture, biological hydrogen and coenzyme Q production. Koh and Song (2007) found that purple non-sulfur bacteria are produced indole-3-acetic acid (IAA) and 5-aminolevulinic acid (ALA), which act as mechanisms of plant growth stimulants. Lee *et al.* (2008) found that that purple non-sulfur bacterium, *Rhodopseudomonas* sp. can enhanced the germination and growth of tomato seed under axenic conditions which applied as an environment-friendly as biofertilizer to get high quality tomato and other crops.

Photosynthetic bacteria are applied and get high yield of rice which also found to suppress root rot, and it is reported to increase (Kobayashi and Kobayashi, 2000). It is considered to be biofertilizers and ability of photosynthetic bacteria to fix N_2 (Raymond *et al.,* 2004).

2.4 Antagonistic fungi

***Chaetomium* spp.**

Taxonomy of *Chaetomium brasiliense*

Classification of fungi (Kunze, 1817)

Kingdom Fungi

Division Ascomycota

Class Sordariomycetes

Order Sordariales

Family Chaetomiaceae

Genus *Chaetomium*

Chaetomium belongs to Ascomycota, Chaetomiaceae which established by Kunze in 1817 (Arx *et al*., 1986). It is one of the largest genera of saprobic ascomycetes over 300 species worldwide (Arx *et al.*, 1986; Soytong and Quimio, 1989). *Chaetomium* species are reported to degrade cellulose and other organic materials, and as biological agents against plant pathogens. *Chaetomium* species are isolated and screened for controlling several plant pathogens potential biological control agents in Thailand since 1989 (Soytong *et al*., 2001).

Chaetomium brasiliense CB01 and *Chaetomium cupreum* CC03 were antagonized *F. oxysporum* f.sp. *lycopersici* (tomato wilt) (Sibounnavong *et al.*, 2012). Bioactive tests showed that *Ch. brasiliense* CB01 and *C. cupreum* CC03 inhibited *F. oxysporum* f. sp. *lycopersici*. Crude metabolites *Ch. brasiliense* CB01 and *Ch. cupreum* CC03 reported to inhibit *F. oxysporum* f. sp. l*ycopersici*.

***Neosartorya* spp.**

Taxonomy of *Neosartorya hiratsukae*

Classification of fungi (Udagawa *et al*., 1991)

Kingdom Fungi

Division Ascomycota

Class Eurotiomycetes

Order Eurotiales

Family Trichocomaceae

Genus *Neosartorya*

Neosartorya species are imperfect stage of *Aspergillus* species. It reported to produce bioactive compounds which expressed antibacterial and cytotoxicity. It is reported to be antagonize plant pathogen*, Colletotrichum* spp., causing anthracnose in various plants. *Neosartorya fischeri*, *N. glabra*, *N. hiratsukae*, *N. takakii*, and *N. tatenoi* are reported to control anthracnose of chilli caused by *Colletotrichum capsici* (Eamvijarn *et al.,* 2009).

2.5 Phytoalexins in rice

Phytoalexins are inducible secondary metabolites as antimicrobial activity toward phytopathogens (Ahuja *et al*., 2012; Großkinsky *et al*., 2012). The finding of momilactones A and B are identified from rice, and diterpenoid including momilactones, phytocassanes and oryzalexins are identified from pathogen-infected rice (Cartwright *et al.,* 1981; Akatsuka *et al.,* 1985; Sekido *et al,* 1986; Kato *et al.,* 1993; Kato *et al.,* 1994; Koga *et al.,* 1995 and Valletta *et al.,* 2023) (Figure 2.1-2.4). Sakuranetin is 7-methylated flavanone as phenolic phytoalexin in rice (Kodama *et al*., 1992, Park *et al.,* 2013). *N-p*-Coumaroylserotonin (CouSer), *N*-feruloyltryptamine (FerTrp) and N-feruyolserotonin (FerSer) were identified from rice leaves infected by brown spot pathogen (*Bipolaris oryzae*) (Ishihara *et al*., 2008, 2011). N-cinnamoyltyramine (CinTyr), N-benzoyltryptamine (BenTrp) and N-cinnamoyltryptamine (CinTrp) are identified from UV irradiated rice leaves (Park *et al.,* 2013, 2014) to produce phenolic compounds in subclass phenylamides (Martin-Tunguy *et al.,* 1978, Edreva *et al*., 2007). Amine moieties found in rice phenylamide phytoalexins include arylmonoamines tyramine, tryptamine and serotonin.

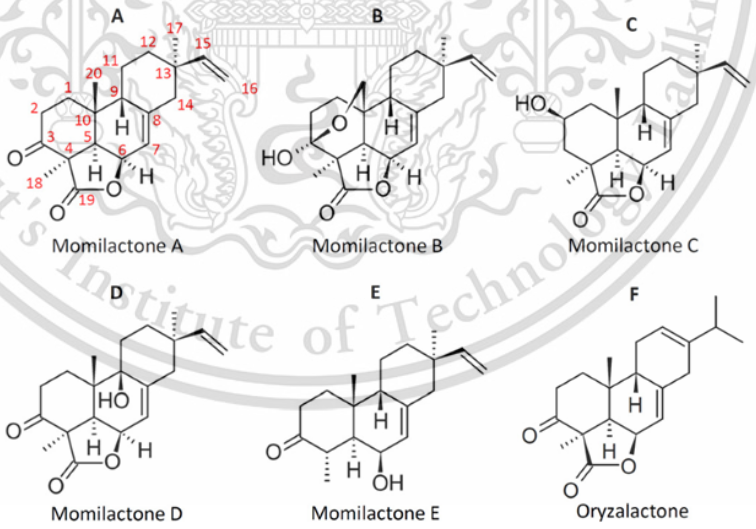


Figure 2.1. Momilactones so far isolated from rice (A–E) and oryzalactone (F).

The dual role of phytoalexins and allelochemicals are demonstrated for momilactone A and B. Momilactones share a pimarane skeleton, while oryzalactone exhibits an abietane skeleton.

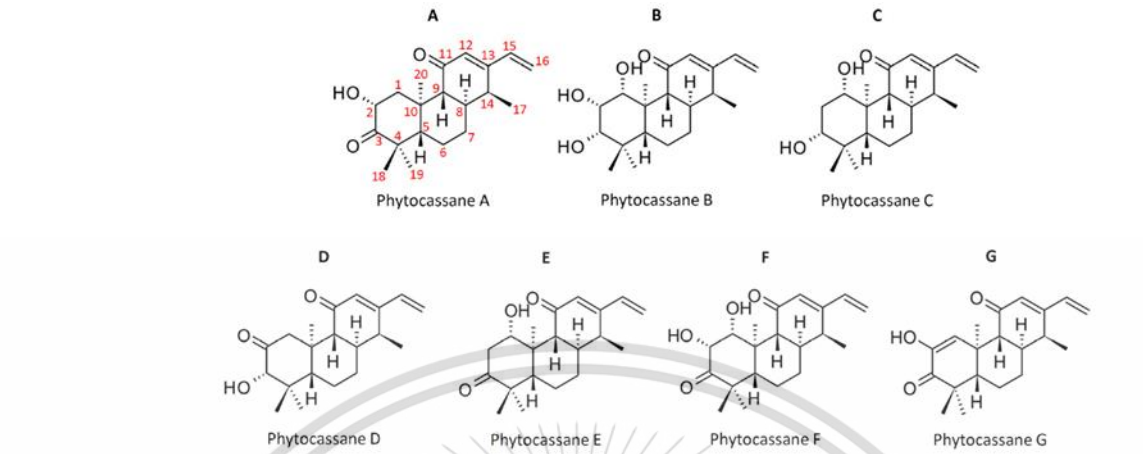


Figure 2.2 Phytocassanes so far isolated from rice.

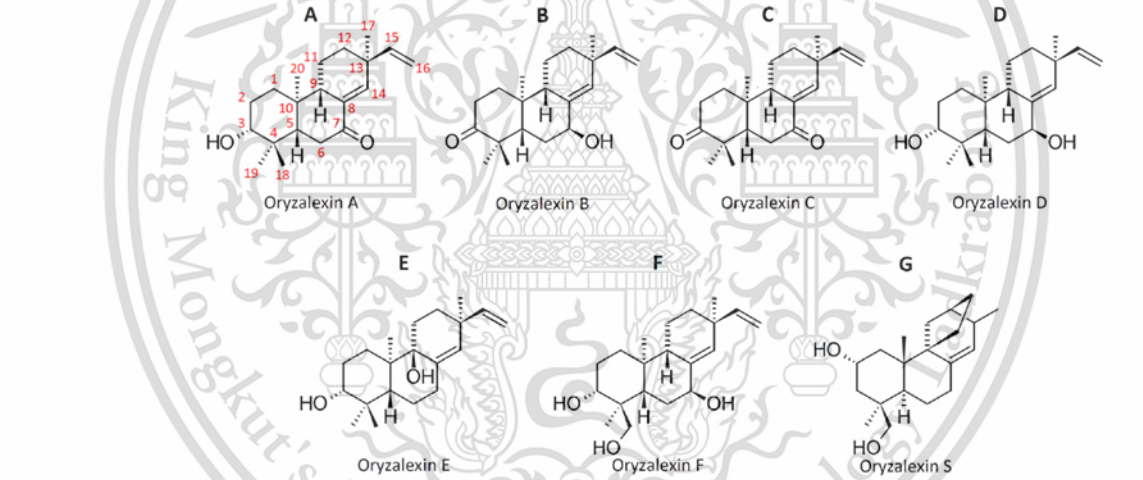


Figure 2.3 Oryzalexins so far isolated from rice.

Despite the name, oryzalexins (A–F) are distinguished from oryzalexin S both by the chemical structure (ent-sandaracopimaradiene- and stemarane-type, respectively) and by the metabolic intermediate which derive (ent-sandaracopimara-8(14),15 diene and syn-stemar-13-ene, respectively; vide infra).

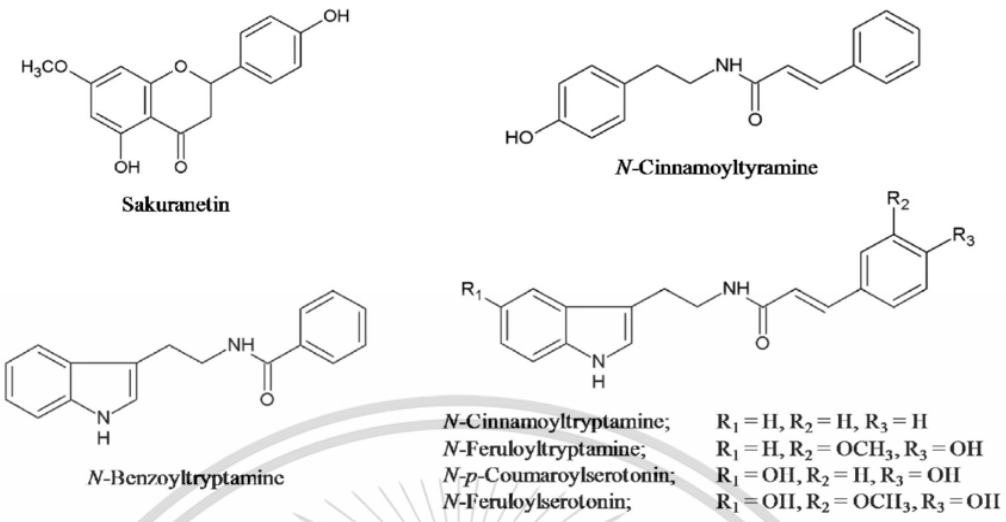


Figure 2.4 Antimicrobial phenolic compounds, methylflavonoid sakuranetin and phenylamides

CHAPTER 3

RESEARCH METHODOLOPGY

3.1 Isolation, Identification of pathogen from leaf spot and brown leaf spot of rice var. RD41, RD47, Pitsanulok 2 (PL2) and RD57

The samples of rice diseased were collected in the fields at Chachengsao province, Thailand. They were isolated by tissue transplanting (Soytong ,1992). Symptoms are surface disinfectant and cut in between diseased part and healthy part to small pieces, soaked sterilized water, 1% sodium hypochlorite for 3 min. The pieces were moved to water agar and incubated to observe hyphal tips then transferred to PDA until get pure culture. Culture was morphologically identified under compound microscope. It was proved pathogenicity in rice varieties of RD41 RD47, RD57 and Pitsanulok 2 (PL2) following Koch'Postuate method.

Pathogen isolates were morphological identified under compound microscope and fungal morphology were recorded as mycelia structure, shape and size of conidia.

3.1.1 Pathogenicity tests

The experiment was done by Completely Randomized Design (CRD) with four replications. Each pathogen was tested for pathogenicity by detached leaf followed Koch's Postulate. The pathogenicity test was proved for *Drechslera oryzae* and *Curvularia lunata*. The mycelia of *Drechslera oryzae* or *Curvularia* sp. were separately moved to sterilized water and spore suspension which adjusted to 5×10^6 conidia/ml using haemacytometer. Rice seedlings var. RD41, RD47, RD57 and Pitsanulok 2 (PL2) were grown in pots for 15 days, and inoculated each isolate to the wounded leaves (3 leaves/seedling). The inoculated leaves were observed the infected leaves. The inoculated leaves with sterilize water served as controls. Disease Index (DI) is rated according to International Rice Research Institute (IRRI, 2002) as follows:

1 = No incidence

2 = symptom on leaves, less than 1%

3 = symptom on leaves, 1-3%

4 = symptom on leaves, 4-5%

5 = symptom on leaves, 11-15%

6 = symptom on leaves, 16-25%

7 = symptom on leaves, 26-50%

8 = symptom on leaves, 51-75%

9 = symptom on leaves, 76-100%

3.2 Isolation, identification of Photosynthetic bacteria

The samples of wastewater were collected from the fields in Samutprakan, Thailand. Each samples was isolated by dilution plate. The dilution was done by cross streak in soft-agar tubes G-5 that consisted of peptone (5.0 g), L-glutamic acid (4.0 g), yeast extract (5.0 g), KH_2PO_4 (0.12 g), malic acid (3.5 g), and K_2HPO_4, (0.18 g) according to the work of Kohlmiler and Gest (1951). The tested tubes were placed for incubation for anaerobic at illumination of 1,000-1,500 lux at 28-30 ^{o}C , 7-12 days. A loopful of culture from pinkish, reddish or brownish colony was sub-cultured by single streak in G-5 agar, incubated for 7 days. Single colony was sub-cultured to G-5 agar plate to get pure culture. The tubes were incubated under anaerobic at 30-35 ^{o}C for 5-10 days under illumination of 100 W incandescent lamp statically. A loopful of culture with pinkish, reddish or brownish culture both was streaked onto G-5 agar, placed in anaerobic jars. Each colony was pick up and streaked onto new G-5 agar plates until get pure culture.

Serratia marcescens (LB01) and *Dietzia cercidiphylli* (LB02) are offered from Assoc. Prof. Dr. Kasem Soytong. They were observed the growing colony on nutrient agar (NA) and morphological characters under binocular compound microscope and scanning electron microscopy (SEM).

3.2.1 Morphological and physiological characterization

The physiological and biochemical tests of photosynthesizing bacteria were done as described by Nastee (2552). All isolates were cultured on Nutrient Agar (NA). Gram-reaction, mortality, shape and color of colony, gelatin hydrolysis, oxidase activities, nutrients reduction and carbon utilization

tests were investigated as described in Bergey's Manual of Systematic Bacteriology (Imhoff and Trüper, 1989). Morphology was observed using a scanning electron microscopy (SEM).

Bacterial isolates were morphological identified according to Bergey's Manual of Systematic Bacteriology (Imhoff and Trüper, 1989). It was tested for gram-reaction, and observed for mortality, colony shape and color using light microscopy and scanning electroscopy.

Molecular phylogeny was used to distinquish *pufM* amplification. Genomic DNA was extracted using Alkaline Lysis activity was done according to manufacturer's protocol (Sambrook, 1989). Primers is determined using *pufM* gene region amplification as 557F (5'-CGCACCTGGACTGGAC-3') and 750R (5'-CCCATGGTCCAGCGCCAGAA-3'). The PCR products were done using electrophoresis with 1% agarose gel in 1xTBE buffer at 100 V to purity which the size is approximately 193 bp. Nucleotide sequence analysis was done by purification. The PCR products were sequenced and distinguished with National Center for Biotechnology Information (NCBI) Genbank by using the BLAST. The phylogenetic tree is constructed using MEGA11 software.

3.2.2 Enzyme production property of photosynthesizing bacteria

The activiity of bacteria was tested to produce amylase, protease and lipase. Completely Randomized Design (CRD) was used for the experiment with four replications. Non-transferred plates served as negative controls.

Amylase- Starch agar medium is consisted of sodium chloride (5 g.), peptone (5 g.), HM peptone B (1.5 g.), yeast extract (1.5 g.), starch, soluble (2 g.) and agar (15 g.). Culture was streaked to starch agar, incubated at 37°C for 24 h. The colony was shown, then flooded the transferred plate in solution of Lugol's iodine. Clear zone surrounding the colony was observed. The starch is hydrolyzed by amylase, then it appeared clear zone around colony. A blue or purple zone informs that starch is not hydrolyzed (Harrigan and McCance, 1976).

Protease- Skim milk agar medium is consisted of skim milk powder (28 g.), casein enzymic hydrolysate (5 g.), Yeast extract (2.5 g.), Dextrose (1 g.) and agar (15 g.) which prepared and streaked on the plates, incubated at 37°C for 24 h., and clear zone surrounding the colony was observed. When colonies were appeared and inspected the plates for clear zones to observe caseinase, it indicates as positive reaction (Frazier and Rupp, 1928).

Lipase- Lipase activity test was tested by growing the isolates on Polysorbate 80 agar which consisted of animal tissue (10 g.), Polysorbate 80 (10 g), Agar (15 g.). Cultures were observed after incubation at 37°C for 24 h. A positive reaction was appeared as precipitated fatty acid crystals around the colony (Favero, 1967).

3.2.3 Evaluation of Photosynthetic bacterium *Rhodospirillum centenum* to increase the growth of rice var. RD41 and Pitsanulok 2 (PL2)

Rhodospirillum centenum strains SM41, SM61, SM72, and SM92 were evaluated the germinated seeds of rice var. RD41 and Pitsanulok 2 (PL2), Seeds were surface sterilized with 95% ethanol for 5 minutes, 2% sodium hypochlorite for 2 minutes, rinsed in sterilized water, air dried for 1 h., inoculated with spore suspension of pathogen at 1 $X10^6$ spore/ml for 20 minutes. The isolate was grown in nutrient (NA). Bacterial cells were collected to sterile water to reach $1x10^6$ cells/ml by optical density (OD) (Koch, 1970), (OD_{600}=1). Each rice seed variety was inoculated with pathogen inocula by soaking overnight. The soaked seeds in sterilized water served controls. The experiment was performed using 2 factors factorial in CRD in four replicates. Factor A represented rice var. (RD41 and Pitsanulok 2 (PL2). Factor B represented SM41, SM61, SM72 and SM92. Each treatment was planted 10 rice seeds put onto petri dishes with sterilized filter paper (Whatman # 102). Seeds were checked germination in 3 days and the root and shoot length were measured after 7 days at 25°C.

Data were analyzed the variance and treatment means were compared by Duncan's Multiple Range Test at P =0.05 and 0.01 using statistical package for social science (IBM, SPSS) program.

The emergence of seedling was calculated with the following formula:

$$\text{Emergence (\%)} = \frac{\text{Number of emerged seedlings}}{\text{Number of seeds grown}} \times 100$$

Vigour index = (mean root length + mean shoot length) × percentage germination

(Abdul-Baki and Anderson 1973).

3.2.4 Effects of Photosythesizing bacteria *Serratia marcescens* (LB01) and *Dietzia cercidiphylli* (LB02) for controlling leaf spot (*Curvularia lunata*) of rice var. RD41 and stimulating plant growth

Dual culture was investigated using *Serratia marcescens* (LB01) and *Dietzia cercidiphylli* (LB02) against *Curvularia lunata* (leaf spot of rice var. RD41). The pathogen was cultured on PDA at 25°C for five days, mycelial disc was cut and placed on the one side of a Pertri dish containing NRA consisted of peptone 5 g., beef extract 3 g., rice flour 10 g., dextrose 2.5 g. and agar 18 g. followed the method of Fokkema (1978). A full loop of bacteria was streaked at distance of 5 cm. away from the mycelia plug of pathogen on the same dish. Inhibition zones were evaluated after incubation at 25°C for 7 days.

Serratia marcescens (LB01) and *Dietzia cercidiphylli* (LB02) were evaluated for plant growth promoting agents for the growth of rice var RD41 in the pot experiment. Cell suspension was obtained from a 48 h. culture broth with adjusted to 10^7 cfu/ml and determined by dilution plating at 560 nm optic density (El Khaldi *et al.,* 2015). The experiment was done using Randomized Block Design (RCBD) with four repeated experiments. Treatments were as follows:-

T1 = non treated one (inoculated *Curvularia lunata*)

T2 = photosynthetic bacteria (LB01) at concentration of $1x10^7$ cells/ml.

T3 = photosynthetic bacteria (LB02) at concentration of $1x10^7$ cells/ml.

T4 =Tebuconazole 20cc/20L of water.

All treatments were inoculated each bacterial suspension at every 15 days until harvest. Data were recorded as plant weight g), weight of grain yield (g), plant height (cm),and number of tiller. Data were computed analysis of variance. Treatment means were compared using Duncan's Multiple Range Test (DMRT) at P=0.05 and P=0.01.

3.3 Antagonistic *Chaetomium brasiliense* (CB01) and *Neosartorya hiratsukae* (EU06) against brown leaf spot (*Drechslera oryzae*) of rice var. RD47

3.3.1 Bi-culture antagonistic tests

Chaetomium brasiliense (CB01) and *Neosartorya hiratsukae* (EU06) were tested against *Drechslera oryzae* (brown leaf spot of rice var. RD47) which tested on PDA using bi-culture (Fokkema, 1978). The fungi and pathogen were separately cultured on PDA at room temperature (30–32ºC) for seven days. A 0.5 cm. diameter of sterilized cork borer was made agar plugs from the actively growing edge of pathogen culture and the antagonistic fungus. An agar plug of the pathogen was placed on one site, and antagonistic fungus was placed in opposite site. The PDA plates were transferred a single plug of an antagonistic fungus or the pathogen served as the controls. The bi-culture plates were incubated at room temperature (28-30^{o}C) and observed for 30 days. The experiment was done with four replications.

The abnormal and nornal spores of pathogen were observed under compound microscope. Colony diameter and number of conidia of pathogen were recorded. Growth and conidia inhibition of pathogen were calculated using formula below:

$$\text{Inhibition (\%)} = \frac{A-B}{A} \times 100$$

A = colony diameter or conidia number of pathogen in control

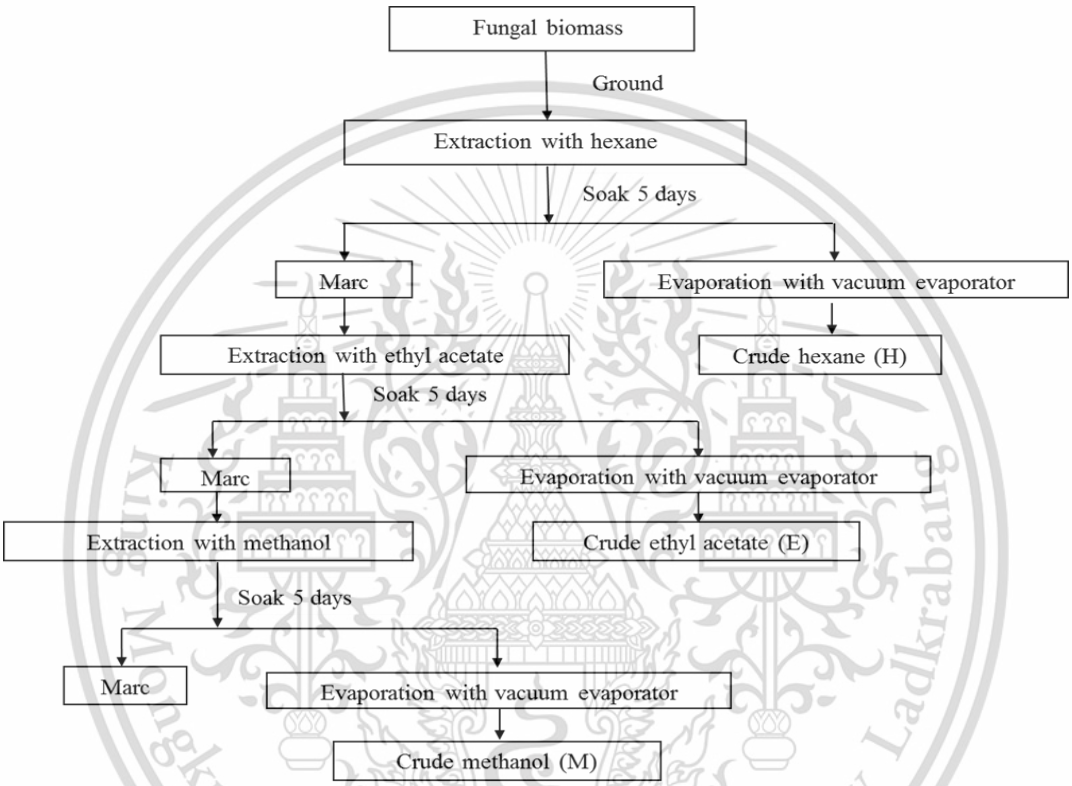
B = colony diameter or conidia number of pathogen in control in dual culture plate

Data were computed for analysis of variance (ANOVA) and mean comparison was compared by using DMRT at P=0.01 and P=0.05.

3.3.2 Active metabolites from *Chaetomium brasiliense* CB01 and *Neosartorya hiratsukae* EU06 testing for inhibition of leaf spot of rice var. RD47 caused by *Drechslera oryzae*

Chaetomium brasiliense (CB01) and *Neosartorya hiratsukae* (EU06) were cultured in potato dextrose broth (PDB) at room temperature for 30 days. The biomass was moved from PDB, filtered

and air-dried overnight. Fresh and dry weight were weighted. The dried biomass was ground with electrical blender, placed in flask, and dissolved with equal volume of hexane for 5 days at room temperature. The biomass was filtered through Whatman filter paper No 4. The solvent was evaporated in vacuo to yield crude hexane. The marc was extracted with ethyl acetate and methanol using the same procedure.



The crude metabolites were assayed for pathogen inhibition. Factorial in CRD with and four replications wasperformed. Factor A was solvents, A1= crude hexane, A2= crude ethyl acetate and A3= crude methanol. Factor B was concentrations of crude extracts (ppm) which consisted of B1 = 0, B2 = 10, B3 = 50, B4 = 100, B5 = 500 and B6 = 1000 ppm. Each crude extract was dissolved in 2% of dimethyl sulfoxide (DMSO) incorporated with PDA before autoclaved at 121^0C, 15lbs/inch^2 for 30 minutes. The colony margin of tested pathogen grown in PDA was cut by sterilized cork borer (3 mm diameter), and moved to the middle of PDA Petri-dishes (5.5 cm in diameter) and incubated at room temperature (28-30^0C) until the control grown to full plate. Abnormal and normal spores were observed under compound microscope.

The number of spores and colony diameter were recorded. Data were computed analysis of variance. The treatment means were compared using DMRT at P=0.01 and 0.05. ED_{50} was done by using probit analysis.

3.3.3 Testing nano-particles from *Chaetomium brasiliense* CB01 and *Neosartorya hiratsukae* EU06 to control brown leaf spot of rice var. RD47 caused by *Drechslera oryzae*

Nano-particles constructed from *Chaetomium brasiliense* strain CB01 to get Nano-CBH, Nano-CBE and Nano-CBM and *Neosartorya hiratsukae* strain EU06 to get Nano-NHH, Nano-NHE and Nano-NHM using the method of Dar and Soytong (2014). Two factors factorial in CRD was done with four replications. Factor A represented nanoparticles and factor B represented 0, 1, 3, 5, 7 and 10 ppm. Each nano-particle was dissolved in 2% DMSO, mixed to PDA, autoclaved at 121^0C, 15 lbs/inch2 for 30 minutes. The colony of *D. oryzae* was cut at the peripheral colony with sterilized cock borer (3mm), moved to the middle of PDA incorporated with each concentration. The plates were incubated at 28-30^0C, observed the pathogen in control growing full plate, recorded colony diameter and number of spores. Abnormal spores were observed under compound microscope. ED_{50} was calculated using probit analysis.

3.4 Testing nano-particles derived from *Chaetomium brasiliense* strain CB01 (Nano-CBH, Nano-CBE and Nano-CBM) to induce plant immunity for brown leaf spot of rice var. RD47 caused by *Drechslera oryzae*

The experiment was done using RCBD with four replications. The 20 days seedlings of rice var. RD47 planted in pots were inoculated with spore of *Drechslera oryzae* at $1x10^5$ spores/ml, except for non-inoculated with pathogen and non-treated control. Each treatment was sprayed nanoparticles at 15 ppm. The treatments were as follows:

T 1 = non-inoculated with pathogen and non-treated control

T 2 = inoculated with pathogen and non-treated control

T 3= Inoculated with pathogen and treated with nano-CBH 15 ppm

T 4 = Inoculated with pathogen and treated with nano-CBE 15 ppm

T 5 = Inoculated with pathogen and treated with nano-CBM 15 ppm

T 6 = Inoculated with pathogen and treated with Propiconazole at application rate of 1 ml/L

The leaf samples were collected at 3, 6 and 9 days. Leaf sample was collected 1.5 g in each sample, and cleaned in 70% methanol before cut to small pieces. Each sample was blended and soaked in 10 mL methanol in waterbath at 50 °C for 10 minutes. The sample was filtered through Whatman filter paper No. 4 to yield filtrate. The filtrate was evaporated with Rotary Vacuum Evaporator to get a purified crude extract. The purified crude extract was added 3 mL methanol. Phytoalexin is detected by running on TLC in the combination of solvents as benzene and ethyl acetate at the ratio of 10:1. TLC tank was filled 2 mL. Each purified crude extract was spotted on TLC plates. Phytoalexin standard was compared. TLC plate was examined under UV light at 365 nm. It was soaked in anisaldehyde solvent, dried , heated until spots appeared. Retention factor (R_f) value was calculated as : R_f = distance spoy travels / distance mobile phase travels.

CHAPTER 4

RESULTS

4.1 Isolation, Identification of pathogen from leaf spot and brown leaf spot of rice var. RD41, RD47, RD57 and Pitsanulok 2 (PL2)

Leaf spot of rice var RD41 and Pitsanulok 2 (PL2) were proved to be *Curvularia lunata.* Pure culture on PDA showed septate mycelia-with generally brown color in appearance. Conidiophores are brown, erect, simple or branched. geniculate, conidia in sympodial order. Conidia are averaged 7.54-11.84 X 13.32-21.12 µm., ellipsoidal, often gently curved, brown in color with 3 to 4 septa (Figures 4.1 and 4.2).

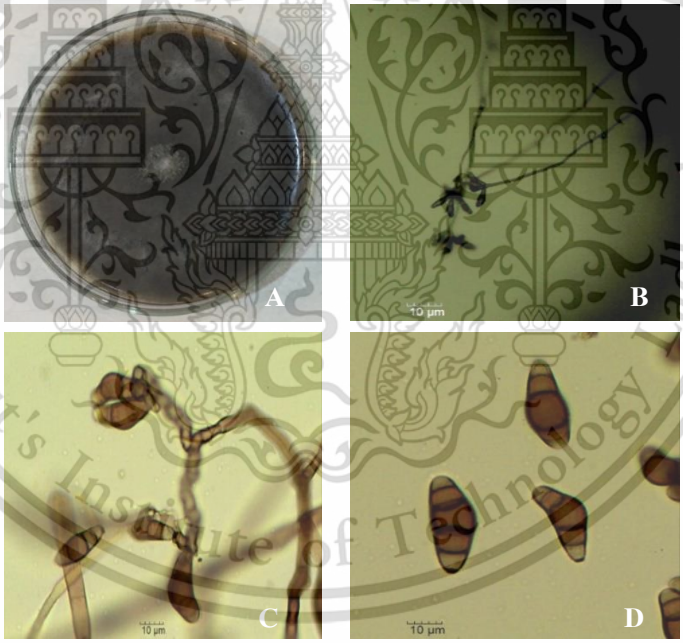


Figure 4.1 Morphology of *Curvularia lunata* of rice var. RD41 A = Colony on PDA after 6 days, B and C =conidiophores and conidia, D = conidia (400X)

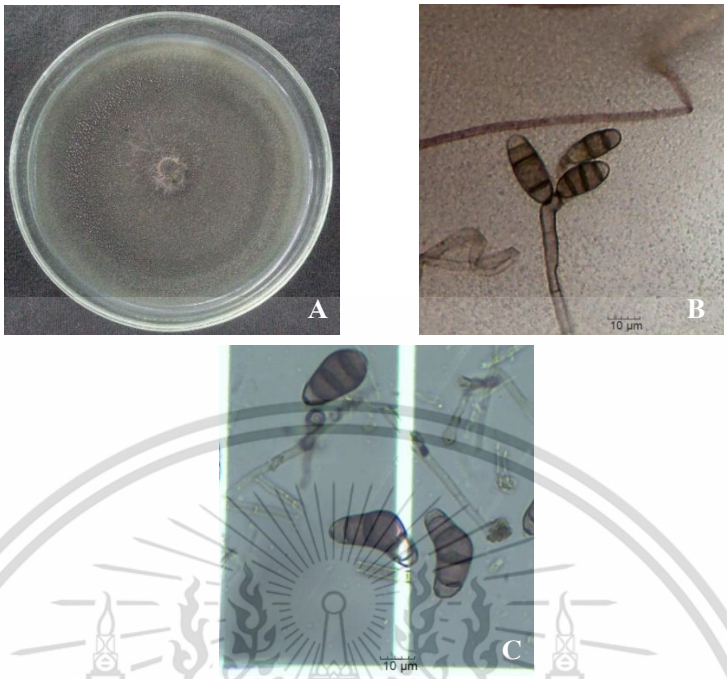


Figure 4.2 Morphology of *Curvularia lunata* of rice var. Pitsanulok 2 (PL2) A = Colony on PDA after 6 days, B conidiophore and conidia, C = conidia (400X)

Brown leaf spot of rice var. RD47 and RD57 was proved to be *Drechslera oryzae.* Colony on PDA is dark brown with fluffy growth. Conidia are averaged 3.39-5.13 X 12.24-32.32, usually slightly curved, fusoid or obclavate shape, occasionally almost cylindrical, pale to golden brown, 5 to 6 septa with hilum (Figures 4.3 and 4.4).

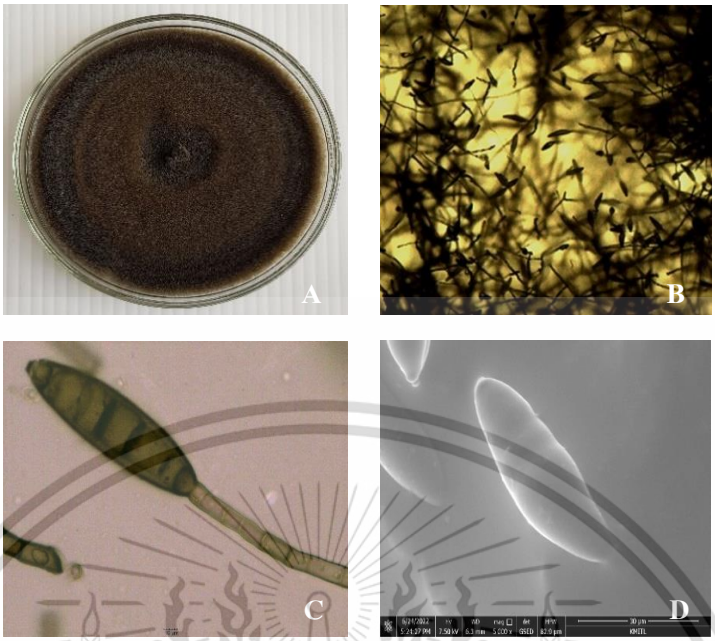


Figure 4.3 Morphological character of *Drechslera oryzae* of rice var. RD47; A = colony on PDA, B = thalli, C = conidiophore and conidium, (100X) and D = SEM photograph of conidium (5000X)

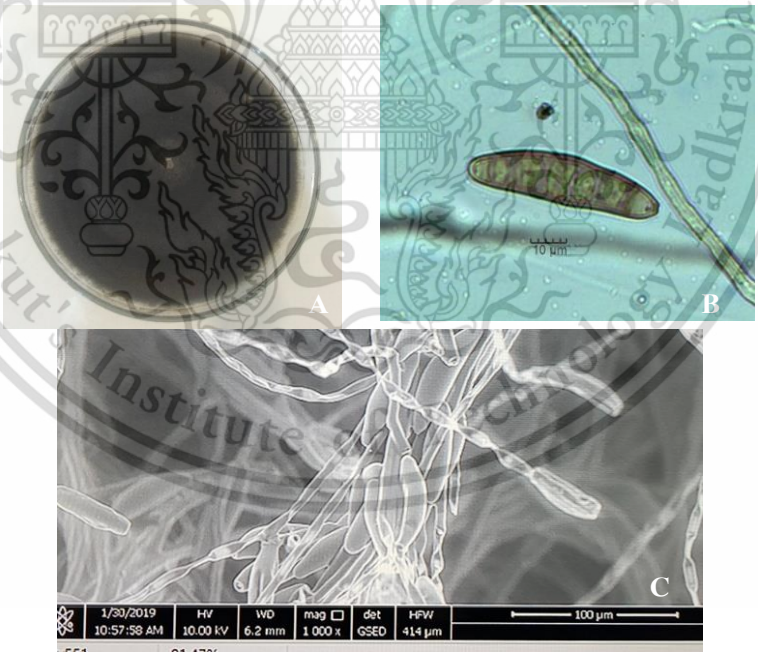


Figure 4.4 Morphological character of *Drechslera oryzae* of rice var. RD57 A = colony on PDA, B = conidium (100X), C = SEM photograph of conidium (1000X).

4.1.1 Pathogenicity tests

Pathogenicity were proved that *Curvularia lunata* isolate was aggressive isolate to cause leaf spot of rice RD41 and Pitsanulok 2 (PL2). The leaf spot showed symptom in 5 days after inoculation (Figure 4.5)

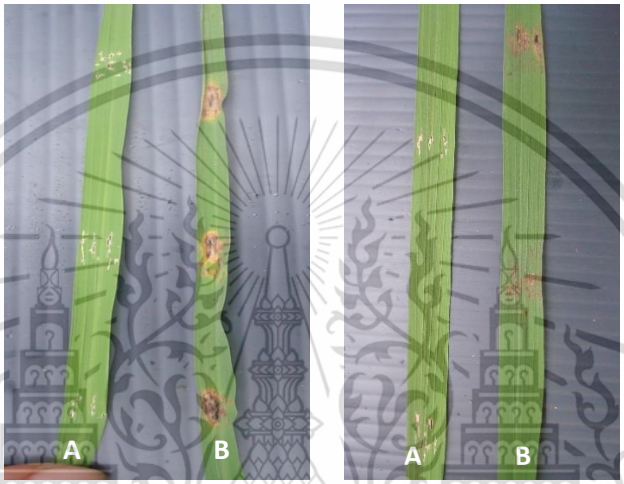


Figure 4.5 Pathogenicity test of *Curvulari lunata* in rice var. RD41 and Pitsanulok 2 (PL2). A=inoculated control and B= inoculated leaves after 5 days

Isolate of *Drechslera oryzae* (brown leaf spot on rice var. RD47 and RD57) was proved for pathogenicity within 7 days after inoculation.

The pathogenicity was confirmed that the symptoms appeared in from as small light brown spot or brownish red spots at first appeared, the lesions were shown spots with surrounding halo around lesion (Figure 4.6).

RD47 RD57

Figure 4.6 Pathogenicity test of *Drechslera oryzae* on rice var. RD47 and RD57. The inoculated control (left) and inoculated leaves (right) after 7 days

4.2 Investigation of Photosynthetic bacteria

The samples of waste water were isolated *Rhodospirillum centenum* strains SM41, SM61, SM72 and SM92. The tubes were incubated under anaerobic at 30-35 ^{o}C for 5-10 days under illumination with a 100 W incandescent lamp. A loopful of culture of pinkish, reddish or brownish culture both were streaked onto G-5 agar and placed in anaerobic jars. After 5 days incubation, the colony was streaked onto new G-5 agar to get pure culture. Result found that photosynthetic bacteria expressing red colonies (Figure 4.7 and 4.8) which was identified using Bergey's Manual of Systematic Bacteriology (Brenner *et al*., 2001).

Figure 4.7 Samples of waste water from rice fields in Samutprakan, Thailand. A= 1 day and B=10 days

4.2.1 Morphological and physiological characterization

Photosynthesizing bacteria was isolated by dilution plate on the soft-agar tubes G-5 medium to get pure culture. SM41, SM61, SM72 and SM92 isolates were found and morphologically identified as *Rhodospirillum centenum* strains SM41, SM61, SM72 and SM92. The colonies showed reddish color, shiny and convex colony, gram-negative, motile cells, spiral, 0.7-1.5 μm in width (Figure 4.8). The physiological characteristics of each strain were shown in Table 4.1.

Rhodospirillum centenum SM41, SM61, SM72 and SM92 are gram negative, mobile cell motility and negative of gelatin test. The Triple Sugar Iron (TSI) activity showed that *R. centenum* SM41 and SM 61 showed good activities of glucose , lactose and sucrose activity but isolate SM72 and SM 92 were non-fermented activity (Table 4.1).

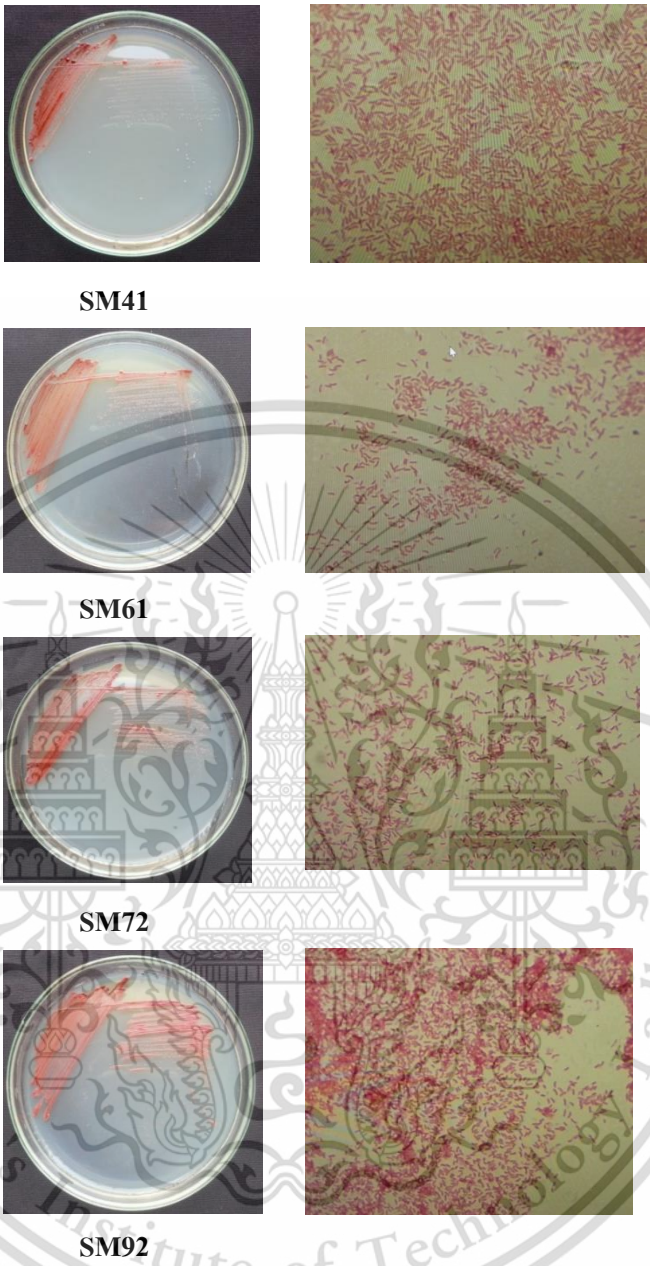


Figure 4.8 *Rhodospirillum centenum* strains SM41, SM61, SM72 and SM92 on NA (left) and gram stains (right) 1000X

Table 4.1 Physiological characteristics of *Rhodospirillum centenum* strains SM41, SM61, SM72 and SM92

Characteristic	SM41	SM61	SM72	SM92
Cell motility	Motile	Motile	Motile	Motile
Gram stain	Negative	Negative	Negative	Negative
TSI	A/A	A/A	NC/NC	NC/NC
Gelatin	Negative	Negative	Negative	Negative

A/A = Glucose and lactose and/or sucrose fermentation, NC/NC =No fermentation

Rhodospirillum centenum strains SM41, SM61, SM72 and SM92 were confirmed species level by phylogenic analysis. *pufM* gene sequence showed 99% similarity with *R. centenum*. The sequences of SM41, SM61, SM72 and SM92 were closely related to *R. centenum* SW in genbank No CP00613.2 using MEGA11 and phylogenetic analysis which aligned in the same clade of *R. centenum* SW (Figure 4.10). Scanning electron microscopy of *R. centenum* was presented in Figure 4.9.

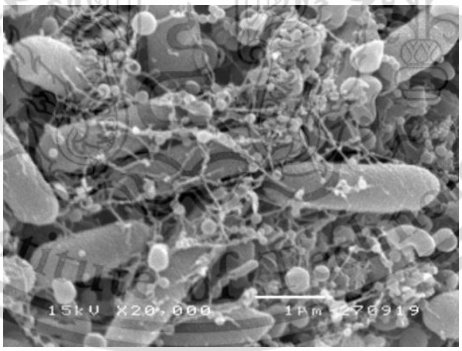


Figure 4.9 SEM image of *Rhodospirillum centenum*

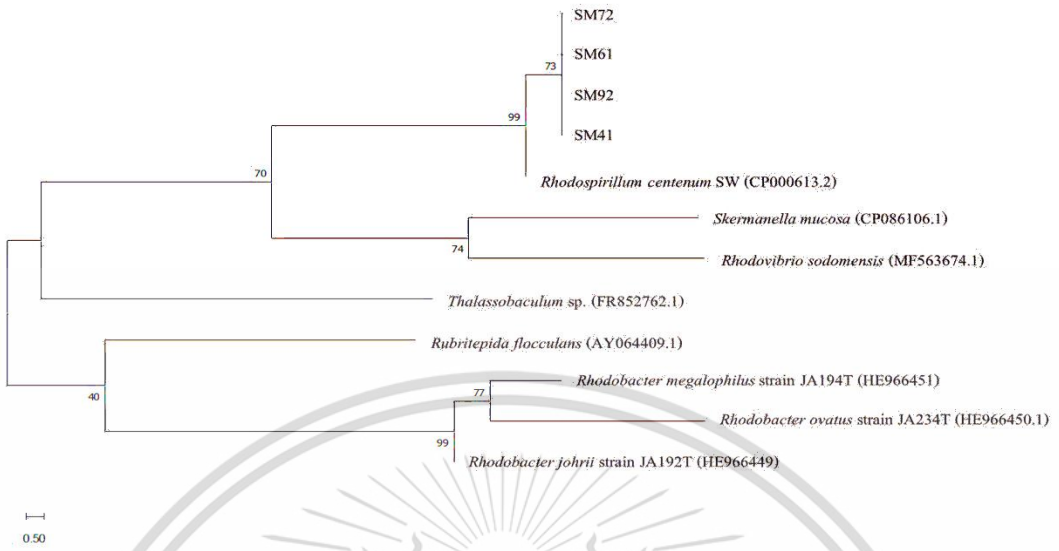
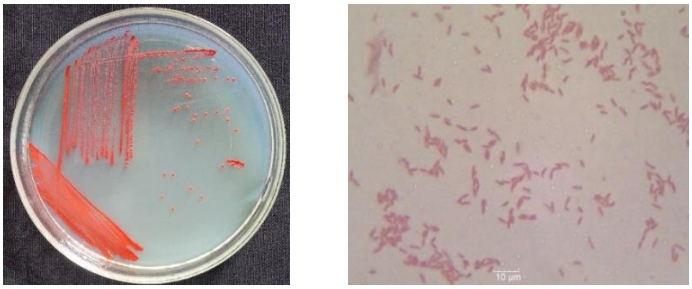
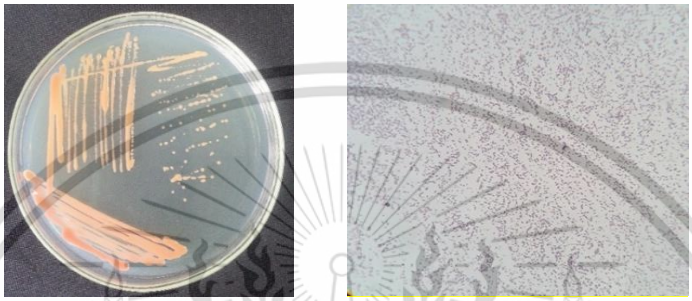


Figure 4.10 Amplified *pufM* gene fragment from strains *Rhodospirillum centenum* SM41, SM61, SM72 and SM92 sequenced and blast searched through NCBI database.

Serratia marcescens (LB01) and *Dietzia cercidiphylli* (LB02) were cultured on NA and observed morphology under binocular microscope and scanning electron microscopy (SEM). The colonies of *S. marcescens* strain LB01 were red, smooth, gram-negative, rod, motility, gelatin positive. Colonies of *D. cercidiphylli* strain LB02 were orange, smooth, gram-positive, rod, non-motile, gelatin negative. The TSI activity showed that *S. marcescens* strain LB01 showed to be good activities of glucose and lactose and sucrose activity but *D. cercidiphylli* strain LB02 was non-fermented activity. (Figure 4.11, Table 4.2)



LB01



LB02

Figure 4.11 *Serratia marcescens* (LB01) and *Dietzia cercidiphylli* (LB02) on NA (left) and gram stains (right) 1000X

Table 4.2 Physiological characteristics of *Serratia marcescens* (LB01) *and Dietzia cercidiphylli* (LB02)

Characteristic	**LB01**	**LB02**
Cell motility	Motile	Non-motile
Gram stain	Negative	Positive
TSI	A/A	NC/NC
Gelatin	Positive	Negative

4.2.2 Enzyme production property of photosynthesizing bacteria

Rhodospirillum centenum SM41, SM61, SM72, SM92 and *Serratia marcescens* (LB01) and *Dietzia cercidiphylli* (LB02) produced amylase, protease and lipase.

Serratia marcescens LB01 produced amylase, protease and lipase which showed clear zones around colonies. (Figure 4.12, Table 4.3)

Table 4.3 Efficacy of the isolated bacteria to produce amylase, lipase and protease

	Enzyme production		
Isolates	**Amylase**	**Protease**	**Lipase**
SM41	+	-	+
SM61	+	+	-
SM72	-	-	+
SM92	-	+	+
LB01	+	+	+
LB02	+	+	-

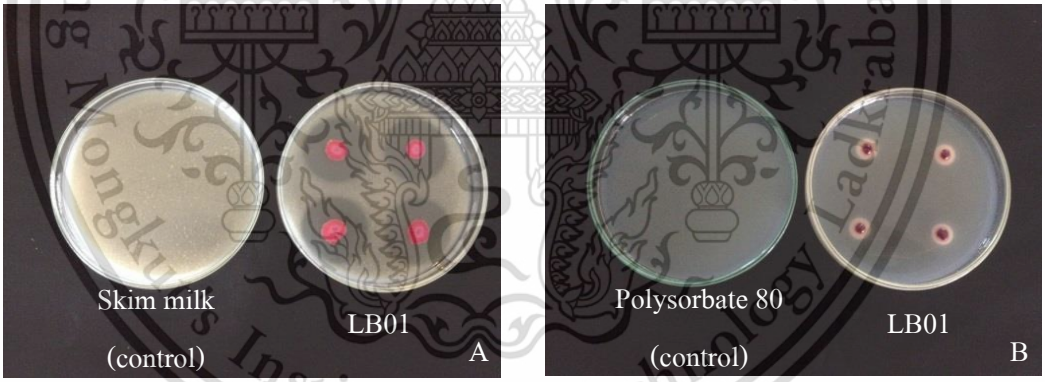


Figure 4.12 A = Protease production and B = Lipase production from *Serratia marcescens* (LB01)

4.2.3 Evaluation of photosynthetic bacteria, *Rhodospirillum centenum* to increase seed germination of rices var. RD41 and var. PL2

It showed that seed germination of rice var. RD41 in treated *R. centenum* SM41 was 45%, and followed by *R. centenum* strains SM92, SM61 and SM72 which were 35, 32 and 30%, respectively. Seed germination of rice var. PL2 found that *R. centenum* strains SM41, SM61 and SM72 were 12.5%

and followed by treated with *R. centenum* strain SM92 which was 10%. It found that *R. centenum* strain SM92 treated rice var. RD41 showed the highest seed germination, shoot length (5.34 cm) and root length (8.35 cm) and vigor index was 479.33. *Rhodospirillum centenum* strain SM61 treated to rice var. PL2 was highest seed germination of 12.5%, shoot length (3.61 cm), root length (4.62 cm) and vigor index was 102.97 (Figure 4.13, Table 4.4).

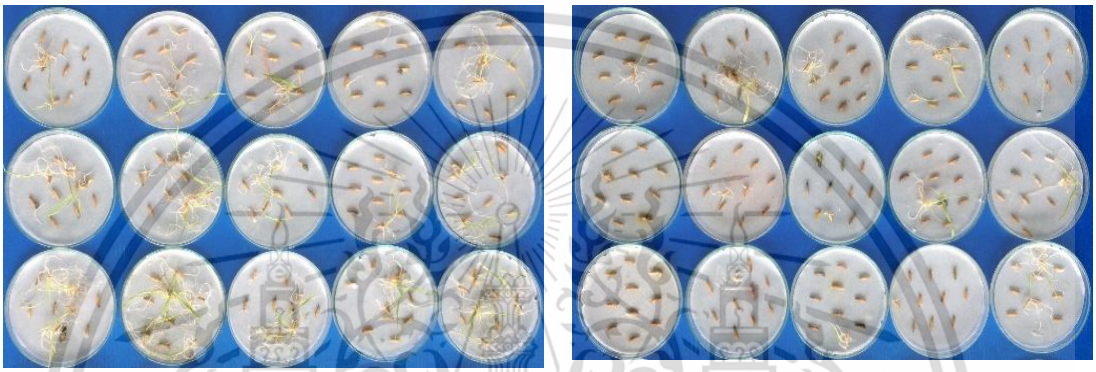


Figure 4.13 Seed germination of rice var. (A) RD41 and (B) PL2

Table 4.4 Effect of different Plant Growth promoting photosynthetic bacteria strains of *Rhodospirillum centenum* on seedling growth parameters under in vitro conditions

Rice variety	Treatment	Germination (%)	Shoot length (cm)	Root length (cm)	Vigour index
RD41	Control	35	4.63ab[1]	5.11bc	341.16
	SM41	45	4.45ab	7.27ab	527.63
	SM61	32	4.11b	4.96bc	290.48
	SM72	30	3.47b	4.317c	233.70
	SM92	35	5.34a	8.35a	479.33
PL2	Control	5	2.00ab	1.70ab	18.5
	SM41	12.5	2.61ab	5.38a	100
	SM61	12.5	3.61a	4.62a	102.97
	SM72	12.5	3.08a	4.58a	95.88
	SM92	10	2.83ab	4.10ab	69.38

[1]Values are mean of four replications. Means in a column followed by same superscript letters are not significantly different according to Duncan's multiple range test at P = 0.05.

Photosynthesizing bacteria for stimulating plant growth and control leaf spot caused by *Curvularia lunata* of rice var. RD41 and Pitsanulok 2 (PL2) by *Rhodospirillum centenum*

Result found that *R. centenum* strains SM41, SM61, SM72 and SM92 promoted seed germination on rice var. RD41 and var. PL2. They produced amylase, protease and lipase which act as control mechnism. SM41 and SM61 isolates reduced leaf spot and promoted plant growth. *R. centenum* is shown to be a new growth stimulant and antagonistic bacterium against leaf spot of rice (Table 4.5).

Table 4.5 Effect of *Rhodospirillum centenum* on plant growth and disease control

Rice variety	Treatment	Germination (%)	Shoot length (cm)	Root length (cm)	Disease index[2]
RD41	Control	28c[1]	3.63d	3.11d	5
	SM41	45a	4.65a	7.27a	2
	SM61	42a	4.11b	5.96ab	4
	SM72	40ab	3.70cd	5.31c	4
	SM92	42a	4.34c	6.35b	3
PL2	Control	25c	2.00d	1.70d	5
	SM41	44a	4.83a	7.28a	3
	SM61	42a	4.61a	5.62c	2
	SM72	42a	4.08b	6.58b	4
	SM92	35ab	4.63ab	5.10c	4

[1] Means of four replications, means followed by a common letter are not significant different by DMRT at P=0.01. Disease index, 1= 1-20% disease incidence, 2 = 21-40% disease incidence, 3 = 41-60% disease incidence, 4 = 61-80% disease incidence and 5 = 76 -100% disease incidence

4.2.4 Effects of *Serratia marcescens* strain LB01 and *Dietzia cercidiphylli* strasin LB02 on the growth of rice var. RD41

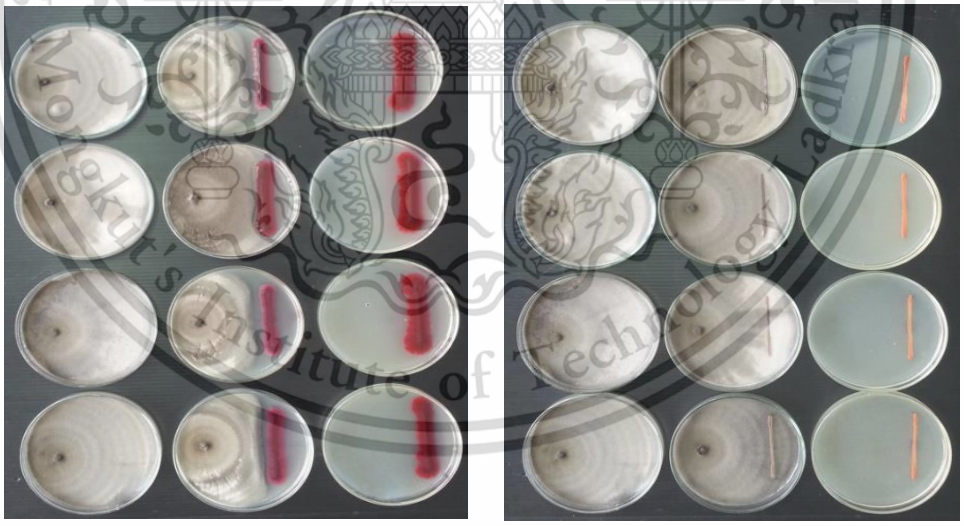
Curvularia lunatra causing leaf spot of rice var. RD41 proved to be aggressive isolate which inhibited by *S. marcescens* (LB01) and *Dietzia cercidiphylli* (LB02). Result showed *S. marcescens* LB01 inhibited mycelia growth of *C. lunata* in NRA in 15 days (Table 4.6, Figure 4.14).

Table 4.6 *Serratia marcescens* (LB01) and *Dietzia cercidiphylli* (LB02) against *Curvularia lunatra* in bi-culture tests

Antagonist bacteria	*Curvularia lunata*			
	Colony diameter (cm)	Inhibition of colony[2] (%)	Number of spore (10^4)	Spore inhibition (%)
Control	9.00a[1/]	-	17.66a[1/]	0c
S. marcescens	6.62b	26.38a	6.88b	61.29a
D. cercidiphylli	9.00a	0.00b	11.72b	34.28b
CV.%	0.35	3.64	18.33	13.09

[1]Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.01,

[2]Inhibition (%) = R1-R2/R1x100, where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in bi-culture plates.



Curvularia* sp. bi-cultue *S. marcescens ***Curvularia* sp. bi-culture *D. cercidiphylli***

Figure 4.14 Bi-culture test between *Serratia marcescens* strain LB01 *Dietzia cercidiphylli* strain LB02 and *Curvularia lunata* causing leaf spot of rice var. RD41

It was shown that at 90 days after treatment, all tested bacteria was significantly differed in plant height when compared to chemical fungicide (P=0.05). *Serratia marcescens* (LB01) was significantly promoted plant growth (52.61 cm.), followed by *Dietzia cercidiphylli* (LB02) and non-treated control where plant heights were 50.88 and 47.75 cm., respectively. The height of chemical fungicide was 24.29 cm. which significantly lower than other treatments (Table 4.7, Figure 4.15).

Phothosynthetic bacteria was significantly differed in plant height when compared to chemical fungicide. The treated rice with *S. marcescens* LB01 showed number of tillers was 5.18 which differed from other treatments and followed by *D. cercidiphylli* LB02 which the number of tiller was 4.37. The chemical fungicide and non-treated control were not significantly differed in root dry weights which were 3.43 and 2.93, respectively (Table 4.7)

Table 4.7 *Serratia marcescens* (LB01) and *Dietzia cercidiphylli* (LB02) on growth of rice var. RD41 in the pot experiment

Treatment	Plant height (cm)	No. of Tiller per plant	Fresh weight of plant (g)	Dry weight of plant (g)	Dry weight of root (g)	Dry grain yield(g)
Control	47.75c[1]	2.93c	48.39bc	14.31b	6.177b	3.81c
LB01	52.61a	5.18a	79.52a	25.64a	12.57a	8.48a
LB02	50.88b	4.37b	63.72ab	22.01a	11.03a	6.28b
Chemical	24.29d	3.43c	33.09c	11.14b	8.325ab	0.55d
CV.%	1.35	9.25	20.46	17.42	27.26	6.79
LSD(P=0.05)	0.95	0.58	18.39	5.09	4.15	0.52

[1]Means with the same letters are not significantly different by Duncan's multiple range test (DMRT) at $p<0.05$

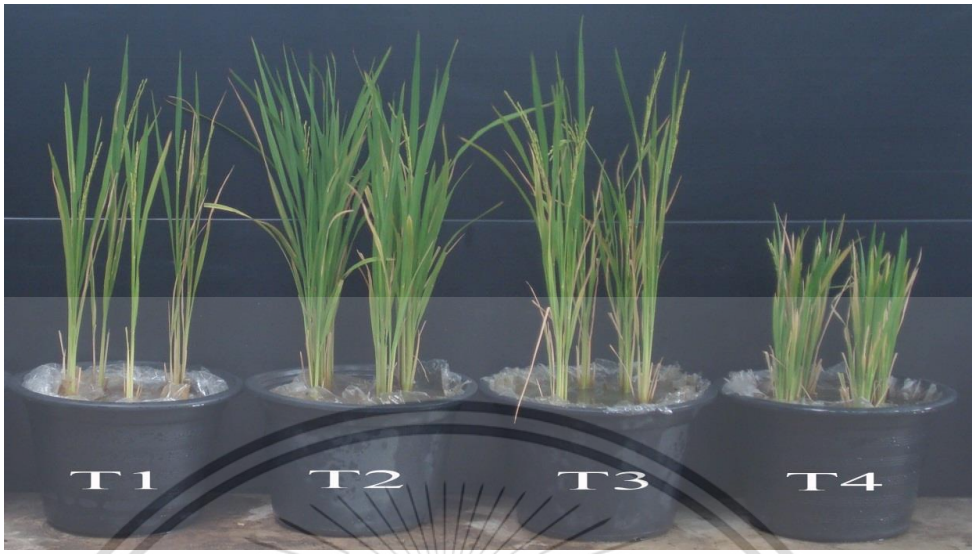


Figure 4.15 Growth of rice var. RD41 at 90 days after treated phothosynthetic bacteria, *Serratia marcescens* (LB01) and *Dietzia cercidiphylli* (LB02). T1= Non-treated control, T2= Treated with LB01, T3= Treated with LB02, T4=Treated with Tebuconazole

Fresh and dry weights revealed that all phothosynthetic bacteria were significantly differed in plant fresh and dry weights when compared to chemical fungicide (P=0.05). The fresh weight after treated with *S. marcescens* (LB01) was 79.52 g which was significantly differed from other treatments and followed by *D. cercidiphylli* (LB02) and non-treated control which the root fresh weight was 63.72 and 48.39 g., respectively. Root fresh weight of chemical control was 33.09 g which gave the lowest root fresh weight and differed from phothosynthetic bacteria treatments. The dry weight after treated with *S. marcescens* (LB01) and *D. cercidiphylli* (LB02) were not significantly differed in root dry weights of 25.64 and 22.01 g., respectively. The chemical fungicide and non- treated control were not significantly differed in root dry weights which were 11.14 and 14.31 g., respectively (Table 4.7, Figure 4.16).

Figure 4.16 Growth of rice var. RD41 after treated phothosynthetic bacteria, *Serratia marcescens* LB01 and *Dietzia cercidiphylli* LB02. T1= Non-treated control, T2=Treated with LB01, T3= Treated with LB02, T4=Treated with Tebuconazole

Serratia marcescens (LB01) and *Dietzia cercidiphylli* (LB02) were significantly differed in root dry weights when compared to non-treated control (P=0.05). The root dry weight after treated with *S, marcescens* (LB01) and *D. cercidiphylli* (LB02) gave non- significantly different in root dry weights were 11.03 and 12.57 g., respectively (Table 4.7).

The grain dry weight showed that *S. marcescens* (LB01) and *D. cercidiphylli* (LB02) were significantly differed in grain dry weights when compared to chemical fungicide (P=0.05). The grain dry weight after treated with *S. marcescens* (LB01) was 8.48 g, followed by *D. cercidiphylli* (LB02) and non-treated control where the plant height was 6.28 and 3.41 g, respectively. The chemical fungicide was 0.55 g which was significantly lower than other treatments (Table 4.7, Figure 4.17).

Figure 4.17 Dry grain of rice var. RD41 after treated phothosynthetic bacteria, *Serratia marcescens* LB01 and *Dietzia cercidiphylli* LB02. T1= Non-treated control, T2=Treated with LB01, T3= Treated with LB02, T4=Treated with Tebuconazole

4.3 Antagonistic *Chaetomium brasiliense* (CB01) and *Neosartorya hiratsukae* (EU06) against brown leaf spot (*D. oryzae)* of rice var. RD47

Chaetomium brasiliense (CB01) and *Neosartorya hiratsukae* (EU06) were cultured on PDA and observed the growing the structures under binocular compound microscope and scanning electron microscopy (SEM).

Neosartorya hiratsukae (EU06) is grown in PDA showing creamy-white colony, cleistothecia are globose (45.97x51.33 μm), ascus is globose and ascospores were broadly ellipsoidal with two equatorial crests, ascospores are averaged 4.90x5.77 μm (Figure 4.18).

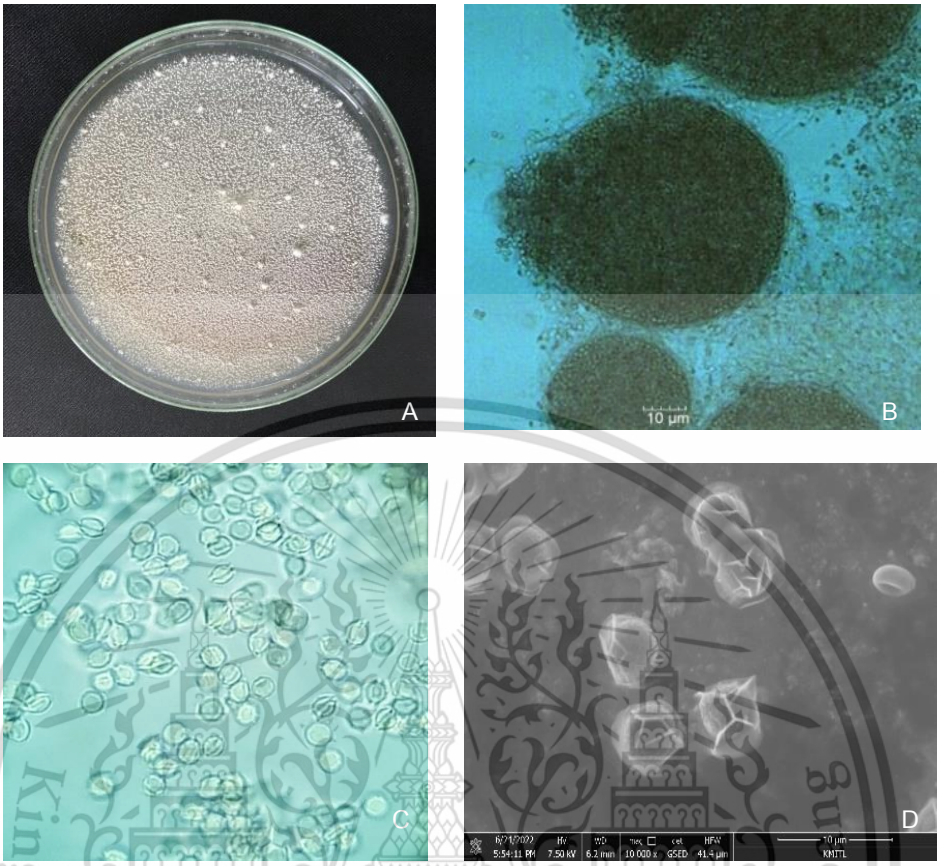


Figure 4.18 Morphological characters of *Neosartorya hiratsukae* (EU06); A = Colonies on PDA, B = Cleistothecia (400x), C = Ascospores (400x) and D = Ascispores (SEM photograph 10000X)

Chaetomium brasiliense (CB01) was dark grey colour, perithecia globose 33.01x40.58 µm brown to black ascocarp, cloth with grey hairs, cyclindrical asci with 8 ascospores, ascospores are averaged 5.77x7.08 µm, dark olive-brown and lemon shaped (Figure 4.19).

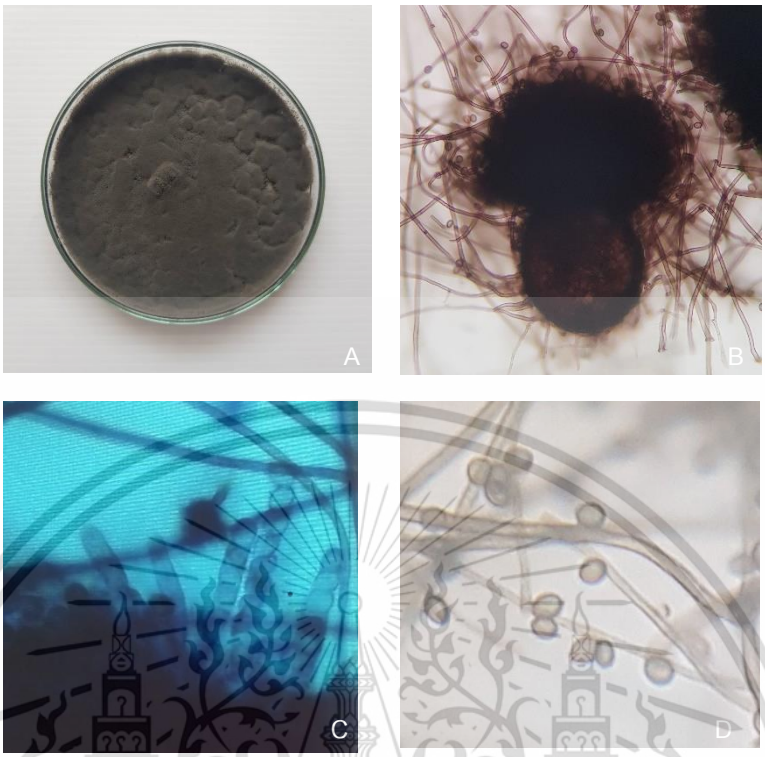
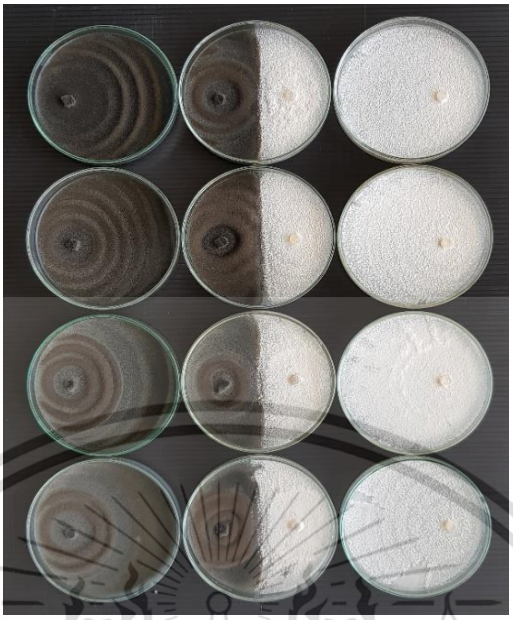


Figure 4.19 *Chaetomium brasiliense* (CB01); a = colony, b = perithecium (100x), c = asci (400x), d = ascospores (400x)

4.3.1 Bi-culture antagonistic test between *N. hiratsukae* EU06 against *D. oryzae*

N. hiratsukae (EU06) inhibited the growth of *D. oryzae* causing brown leaf spot of rice var. RD47and inhibited mycelia growth which averaged colony of 6.15 cm when compared to control plate of 9.00 cm. It inhibited mycelial growth of 31.67% in 10 days. *N. hiratsukae* (EU06) inhibited conidial production of *D. oryzae* of 33.56% (Table 4.8, Figure 4.20).



D. oryzae **Bi-culture** ***N. hiratsukae***

Figure 4.20 Bi-culture antagonistic test between *Drechslera oryzae* causing brown leaf spot of rice var. RD47 and *N. hiratsukae* (EU06)

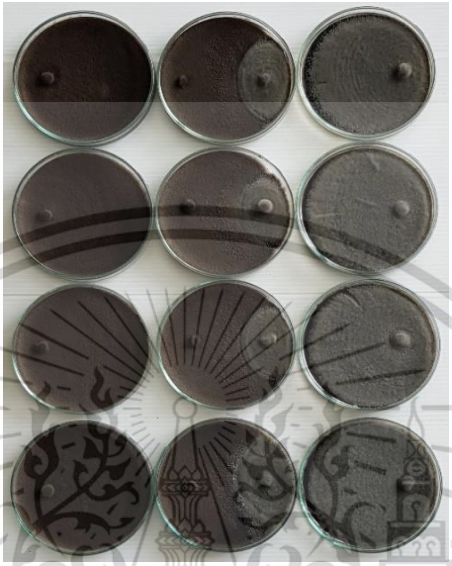
Table 4.8 Inhibition of mycelial growth and sporulation of *Drechslera oryzae* causing brown leaf spot of rice var. RD47 in bi-culture test

	Colony diameter (cm.)[1/]	**Growth inhibition (%)**	**Spore number ($\times 10^7$ /ml)**[1/]	**Spore inhibition (%)**
Control	9.00a	0.00	4.48a	0.00
Bi-culture	6.15b	31.67	2.92a	33.56
CV (%)	1.00		35.14	

[1/]Means four repeated experiments and followed by the same letter are not significantly differed by DMRT at P=0.05.

Ch. brasiliense (CB01) inhibited mycelial growth of *D. oryzae* causing browh leaf spot of rice var. RD47 which colony was 6.62 cm. when compared to control plate . It was significantly inhibited

the mycelia growth of 26.38%. *Ch. brasiliense* (CB01) significantly spore inhibited spore production of *D. oryzae* at 4.80% as shown in Figure 4.21 and Table 4.9.



D. oryzae **Bi-culture** ***Ch. brasiliense***

Figure 4.21 Bi-culture antagonistic test between *Drechslera oryzae* causing brown leaf spot of rice var. RD47 and *Chaetomium Brasiliense* (CB01)

Table 4.9 *Chaetomium brasiliense* CB01 against *Drechslera oryzae* causing brown leaf spot of rice var. RD47 in bi-culture tests

	Colony diameter (cm.)[1/]	**Growth inhibition (%)**	**Spore number (×10^7 /ml)**[1/]	**Spore inhibition (%)**
Control	9.00a	0.00	3.85a	0.00
Bi-culture	6.62b	26.38	3.66a	4.80
CV (%)	0.45		8.93	

[1/]Means four repeated experiments and followed by the same letter are not significantly differed by DMRT at P=0.01.

4.3.2 Active metabolites from *Chaetomium brasiliense* (CB01) and *Neosartorya hiratsukae* (EU06) testing for inhibition of brown leaf spot of rice var. RD47 caused by *Drechslera oryzae*

N. hiratsukae EU06 which extracted with hexane showing greyed-orange in color, crude ethyl acetate showed brown and crude methanol was greyed-purple, respectively. Crude extracts from *Ch. brasiliense* CB01 was extracted with hexane, ethyl acetate and methanol to yield crude hexane as an orange, crude ethyl acetate as an orange-red in color and crude methanol as red-gray, respectively. (Figure 4.22) According to R.H.S colour chart, The Royal Horticultural Society, London.

Figure 4.22 Crude extracts of *Ch. brasiliense* (CB01) and *Neosartorya hiratsukae* (EU06)

***In vitro* testing crude extracts from *Neosartorya hiratsukae* (EU06) against *Drechslera oryzae* causing brown leaf spot of rice var. RD47**

Crude metabolite of *Neosartorya hiratsukae* (EU06) at 0, 10, 50, 100, 500, and 1000 ppm. inhibited *D. oryzae* (brown leaf spot of rice var. RD47). Ethyl acetate and methanol crude metabolites gave significantly highest inhibition of 42.50% for the colony growth of *D. oryzae* at 1,000 ppm when compared to the control. Crude hexane at 1,000 ppm. showed significantly highest conidial inhibition of 83.92%, and followed by crude methanol (83.03%) and ethyl acetate (73.57%). Crude ethyl acetate gave highest inhibition of *D. oryzae* which the ED_{50} of 12.18 ppm., and followed by crude hexane and methanol which the ED_{50} of 29.16 and 43.90 ppm., respectively. It observed that conidia of pathogen showed abnormal cells (Table 4.10, Figure 4.23 and 4.24).

Table 4.10 Growth inhibition of crude extracts of *N. hiratsukae* (EU06) testing to inhibit *D. oryzae* causing brown leaf spot of rice var. RD47

Crude extracts	Concen-tration (ppm)	Colony-diameter (cm)[1/]	Growth inhibition (%)[1/]	Number of spore ($\times 10^5$)[1/]	Spore inhibition (%)[1/]	ED_{50} (ppm)
Crude Hexane	0	5.00a	0.00f	15.75a	0g	
	10	4.96a	0.75f	8.12c	48.92e	
	50	4.68b	6.25e	7.92c	50.00e	29.16
	100	4.48c	10.25d	7.87c	50.17e	
	500	4.46c	10.75d	4.12de	73.92abc	
	1000	4.23d	15.25c	2.62e	83.92a	
Crude	0	5.00a	0.00f	15.75a	0g	
Ethyl acetate	10	4.54c	9.00d	7.62c	52.85e	
	50	4.54c	9.00d	7.25cd	53.92e	12.18
	100	4.47c	10.50d	6.87cd	56.60e	
	500	3.69e	26.00b	4.50de	71.96bc	
	1000	2.87f	42.50a	4.25de	73.57abc	
Crude	0	5.00a	0.00f			
Methanol				15.75a	0g	
	10	4.58bc	8.25de	12.50b	20.53f	
	50	4.47c	10.50d	7.87c	51.25e	
	100	4.47c	10.50d	6.62cd	58.57de	43.90
	500	3.63e	27.25b	5.00cde	69.10cd	
	1000	2.87f	42.50a	2.75e	83.03ab	
	C.V. (%)	2.02	13.20	24.34	15.02	

1/: Means four repeated experiments and followed by the same letter are not significantly differed by DMRT at P=0.05.

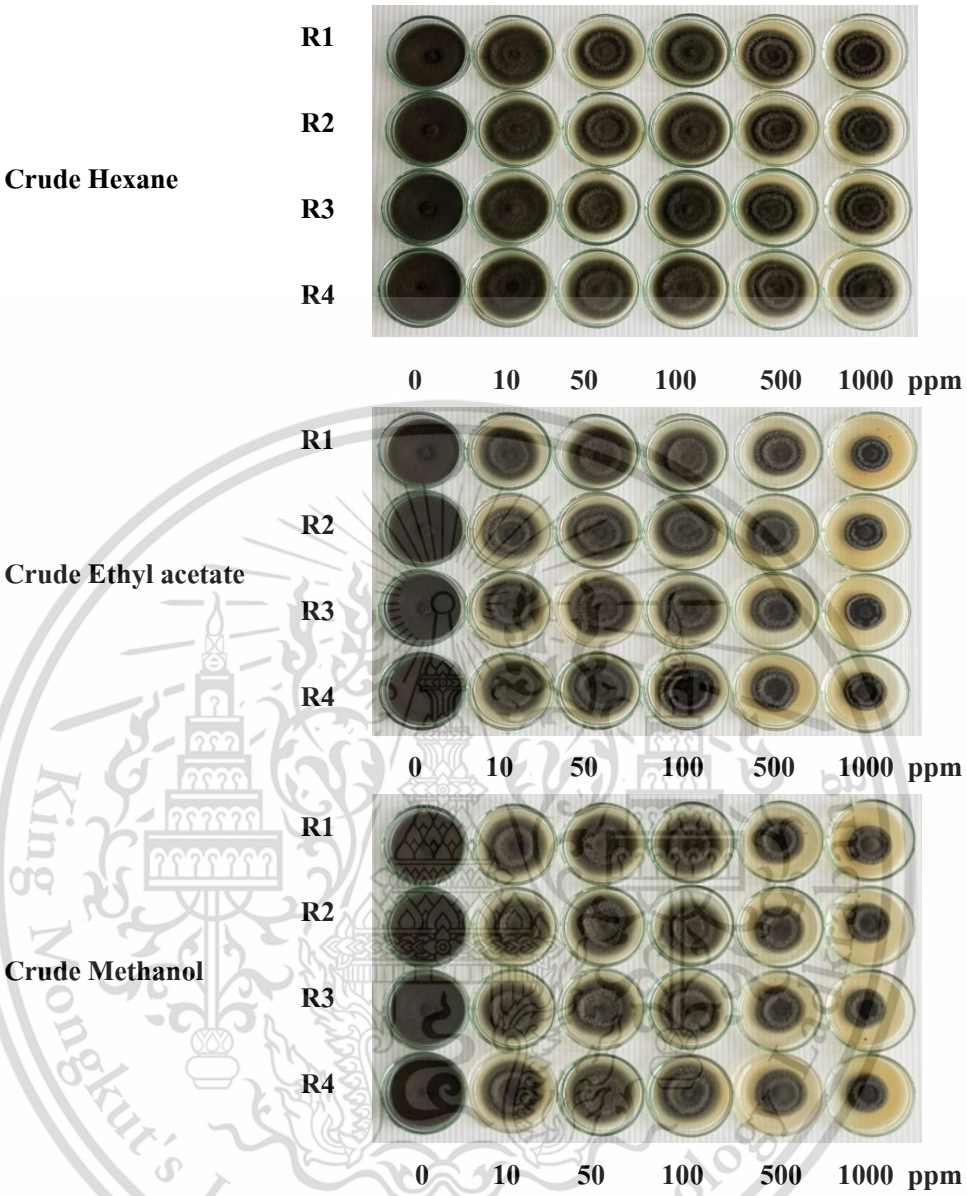
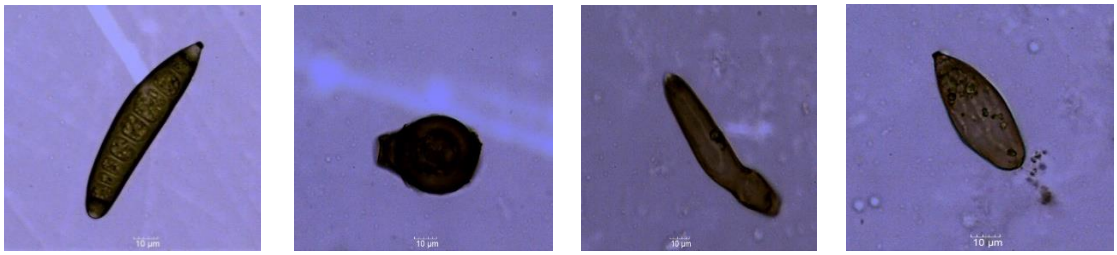


Figure 4.23 Testing crude extracts of *N. hiratsukae* (EU06) against *D. oryzae* causing brown leaf spot of rice var. RD47



Control	Crude hexane	Crude EtOAc	Crude methanol
20.73x88.75 µm	33.69x51.37 µm	18.37x84.57 µm	20.4x52.35 µm

Figure 4.24 Abnormal conidia of *D. oryzae* affected by crude extracts from *N. hiratsukae* EU06 (100x)

***In vitro* test of crude extracts from *Ch. brasiliense* (CB01) against *Drechslera oryzae* causing brown spot of rice var. RD47**

Crude ethyl acetate of *Chaetomium brasiliense* (CB01) gave significantly highest inhibition of *Drechslera oryzae* (brown leaf spot of rice var. RD47) which the ED_{50} of 9.64 ppm, and followed by crude hexane and methanol which the ED_{50} of 14.35 and 23.35 ppm., respectively. Crude methanol at 1,000 ppm. showed significantly highest spore inhibition of 99.15%, and followed by crude hexane (99.11%) and ethyl acetate (98.42%). Crude methanol significantly inhibited colony growth by 83.50 %, and followed by the crude ethyl acetate (82%) and hexane (74.50%) as shown in Table 4.11 and Figure 4.25 and 4.26.

Table 4.11 Crude extracts of *Chaetomium brasiliense* (CB01) testing to inhibit *Drechslera oryzae* causing brown leaf spot of rice var. RD47

Crude extracts	Concentration on (ppm)	Colony diameter (cm.)[/1]	Growth inhibition	ED_{50} (ppm)	Number of spore[/1] ($x10^7$)	Spore inhibition (%)	ED_{50} (ppm)
Crude	0	5.00a	-		46.26a	-	
Hexane	10	3.50c	30.00k		22.78c	50.62f	
	50	3.30de	34.00ij	115.69	20.35cd	55.95e	14.35
	100	2.80h	44.00h		12.23e	73.37c	
	500	1.53j	69.50d		0.76g	98.34a	
	1000	1.27k	74.50c		0.40g	99.11a	
Crude	0	5.00a	-		46.26a	-	
Ethyl	10	3.22e	35.50i		20.56cd	55.26ef	
Acetate	50	2.47g	50.50g	43.02	18.13d	60.71d	9.64
	100	2.12i	57.50e		12.76e	72.35c	
	500	1.05l	79.00b		1.16g	97.45a	
	1000	0.90m	82.00a		0.73g	98.42a	
Crude	0	5.00a	-		46.26a	-	
Methanol	10	4.07b	18.50l		29.32b	36.52g	
	50	3.38cd	32.50jk	101.36	21.43c	53.51ef	23.35
	100	2.32h	53.50f		8.02f	82.66b	
	500	1.27k	74.50c		2.18g	95.19a	
	1000	0.83m	83.50a		0.38g	99.15a	
	C.V. (%)	3.25	3.89		11.79	5.20	

1/: Means four repeated experiments and followed by the same letter are not significantly differed by DMRT at P=0.05.

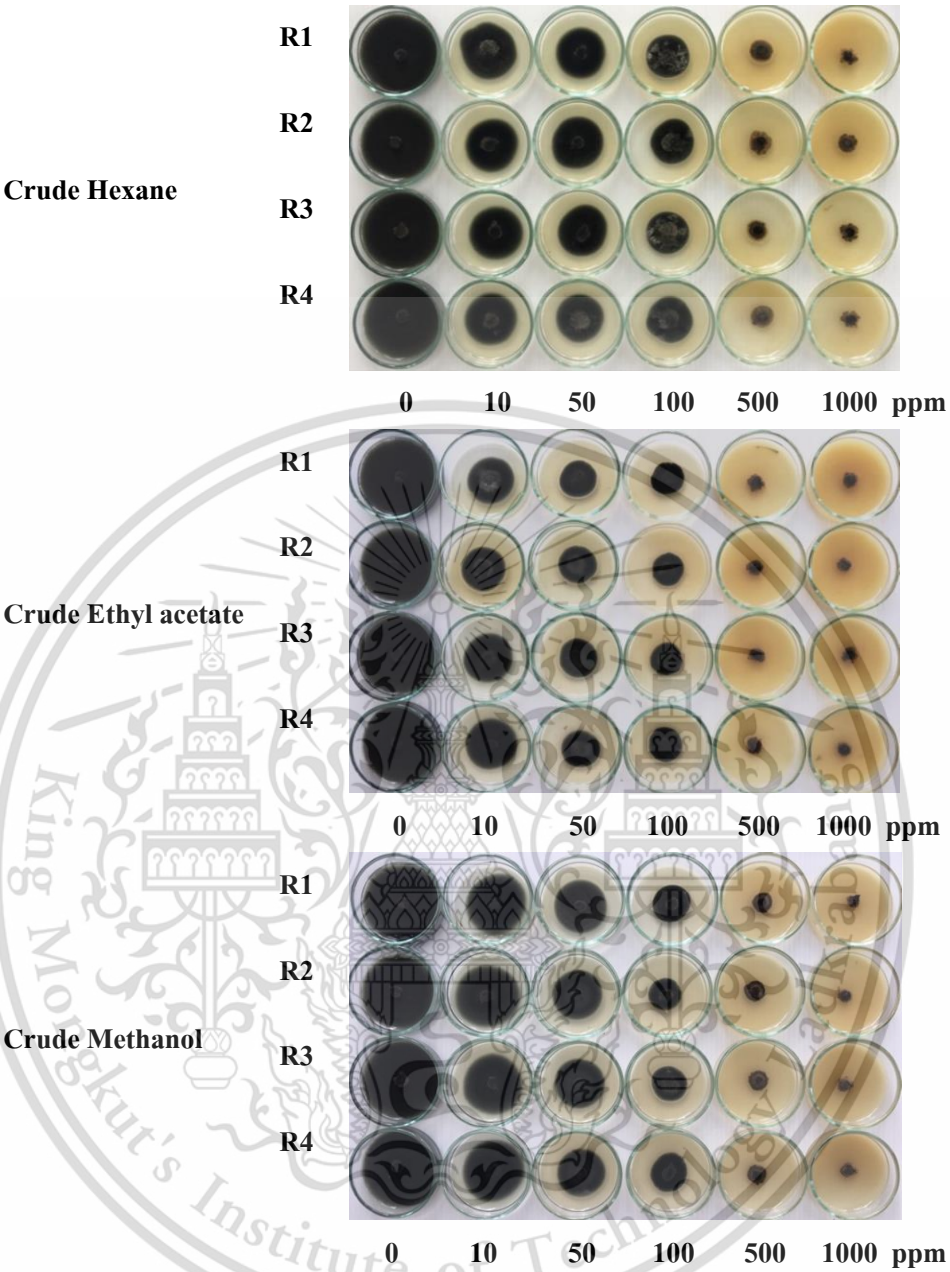
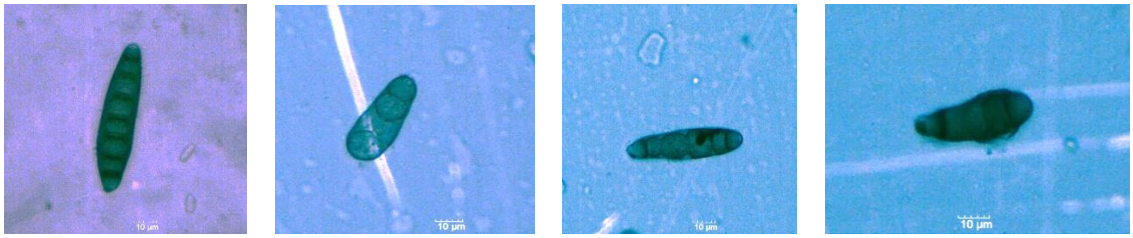


Figure 4.25 Testing crude extract of *Chaetomium brasiliense* (CB01) against *D. oryzae* causing brown leaf spot of rice var. RD47

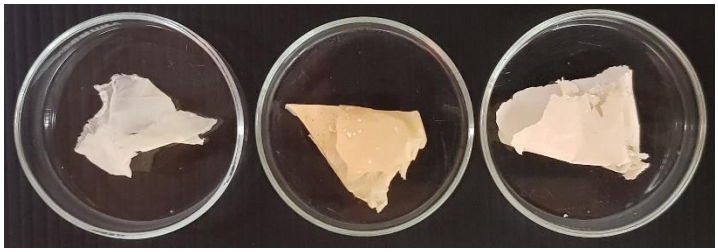


Control	Crude hexane	Crude EtOAc	Crude methanol
16.57x67.72 µm	14.03x38.32 µm	13.68x52.64 µm	14.38x38.31 µm

Figure 4.26 Abnormal conidia of *D. oryzae* causing brown leaf spot ofv rice var. RD47 affected by crude extracts from *Chaetomium brasiliense* (CB01) (100x)

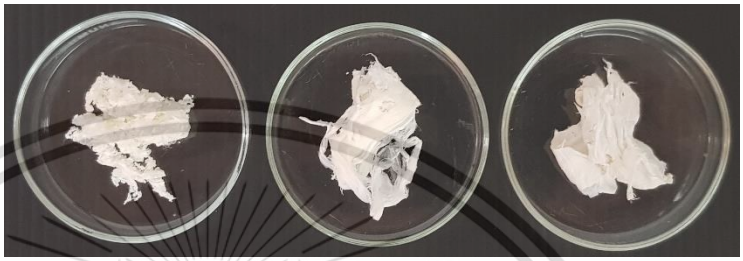
4.3.3 Testing nano-particles from *Chaetomium brasiliense* strain CB01 and *Neosartorya hiratsukae* strain EU06 to control brown leaf spot of rice var. RD47 caused by *D. oryzae*

Nanoparticles constructed with crude extracts were shown in Figure 4.27. Nano-particles d from *Ch. brasiliense* (CB01) coded as nano-CBH, nano-CBE and nano-CBM were white color. Nanoparticle from *N. hiratsukae* (EU06) coded as nano-NHH was white color, nano-NHE was brown color and nano-NHM was white color according to R.H.S colour chart, The Royal Horticultural Society, London. Interestingly, the scanning electron images found the range of particle size of nano-CBH, nano-CBE and nano-CBM were ranged between 244-298 nm, 43.19-69.52 nm and 51.48-56.13 nm, respectively. The range of particle size of nano-NHH, nano-NHE and nano-NHM were ranged between 30.16-37.70 nm, 43.97-61.91 nm and 48.29-53.32 nm, respectively (Figure 4.28).



N. hiratsukae (EU06)

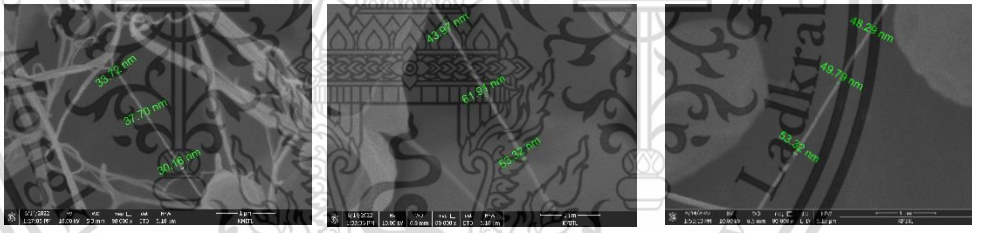
Nano-NHH Nano-NHE Nano-NHM



Ch. brasiliense (CB01)

Nano-CBH Nano-CBE Nano-CBM

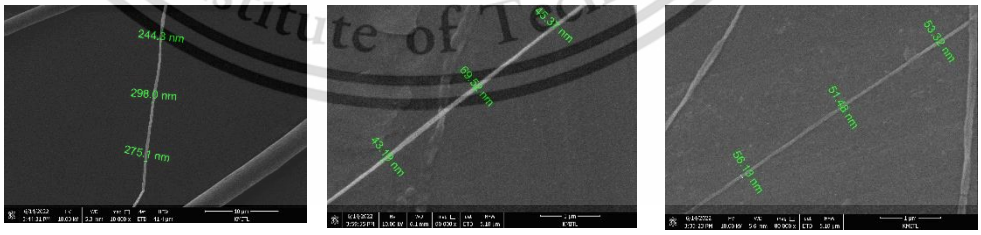
Figure 4.27 Characteristic of nanoparticles from *Chaetomium brasiliense* (CB01) and *Neosartorya hiratsukae* (EU06).



EU06

Nano-NHH Nano-NHE Nano-NHM

30.16-37.70 nm 43.97-61.91 nm 48.29-53.32 nm



CB01

Nano-CBH Nano-CBE Nano-CBM

244.3-298.0 nm 43.19-69.52 nm 51.48-56.13 nm

Figure 4.28 The scaning electron microscope showed the range of particles size of nanoparticles from *Neosartorya hiratsukae* (EU06) and *Chaetomium brasiliense* (CB01) (80000X)

Nano-NHM from *N. hiratsukae* (EU06) gave significantly highest inhibition of 64.00% for the colony growth of *D. oryzae* (brown leaf spot of rice var. RD47) at 10 ppm. when compared to the control (Table 4.12). Nano-NHM at 10 ppm. inhibited spore production of pathogen by 94.87%, and followed by nano-NHE (93.31%) and nano-NHH (88.73%), respectively. These nano-particles showed antifungal activity against *D. oryzae* with ED_{50} values of 3.93, 3.97 and 4.46, respectively (Table 4.12, Figure 4.29).

Table 4.12 Nano particles of *N. hiratsukae* (EU06) testing to inhibit *Drechslera oryzae* causing brown leaf spot of rice var. RD47

Nano particle	Concen-tration (ppm)	Colony diameter (cm)[1/]	Growth inhibition (%)[1/]	ED_{50} (ppm)	Number of spore ($\times 10^5$)[1/]	Spore inhibition (%)[1/]	ED_{50} (ppm)
Nano-NHH	0	5.00a	0.00i		4.22a	0.00g	
	1	3.57b	28.50g		3.55b	15.70f	
	3	3.42c	31.50g	8.83	3.06c	27.11def	4.46
	5	2.97d	40.50f		2.79d	33.78d	
	7	2.52f	49.50d		1.30f	68.89b	
	10	2.27g	54.50c		0.47g	88.73a	
Nano-NHE	0	5.00a	0.00i		4.22a	0.00g	
	1	2.97d	40.50f		3.34b	20.31ef	
	3	2.92de	41.50ef	5.27	3.34b	20.50ef	3.97
	5	2.92de	41.50ef		2.31e	44.83c	
	7	2.22g	55.50c		1.23f	70.66b	
	10	1.99h	60.00b		0.28g	93.31a	
Nano-NHM	0	5.00a	0.00i		4.22a	0.00g	
	1	3.00d	40.00f		3.32b	20.94ef	
	3	2.97d	40.50f	4.71	3.00cd	28.57de	3.93
	5	2.80e	44.00e		2.40e	42.96c	
	7	2.29g	54.00c		1.43f	65.69b	
	10	1.79i	64.00a		0.21g	94.87a	
	C.V. (%)	3.09	5.02		7.85	14.14	

[1]Means four repeated experiments and followed by the same letter are not significantly differed by DMRT at P=0.05.

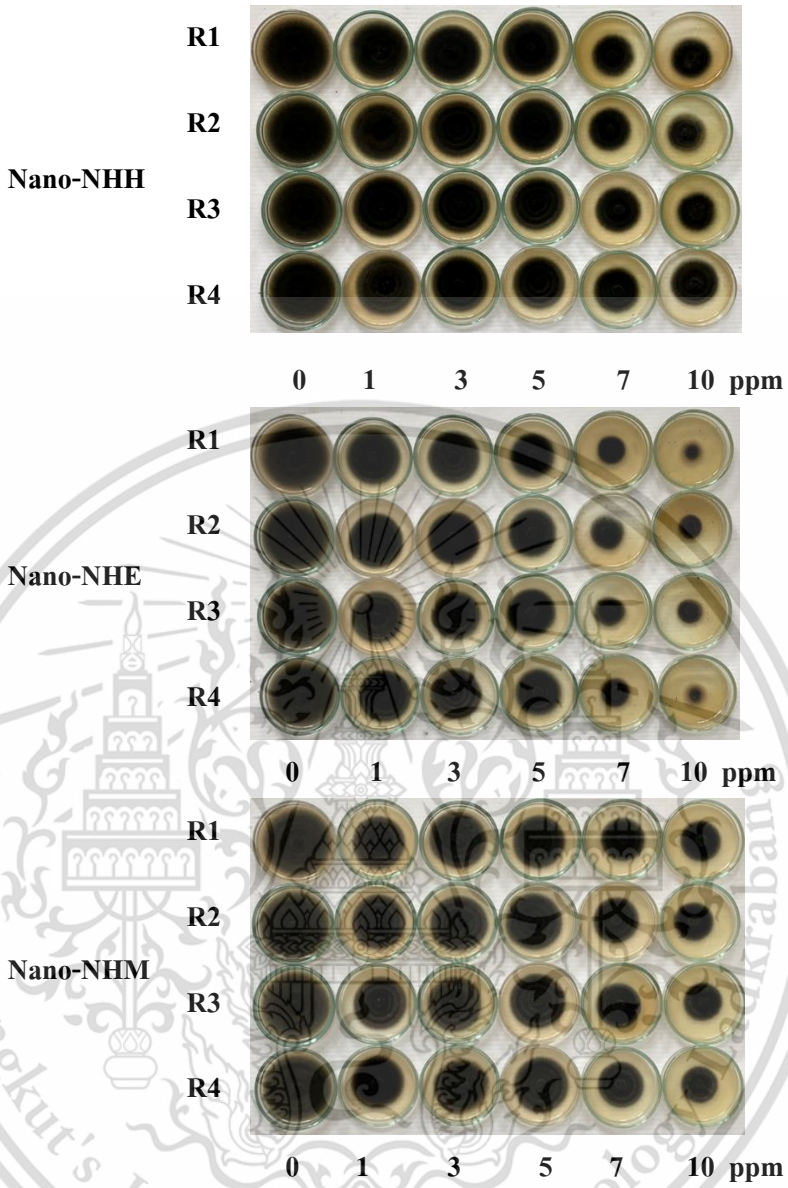


Figure 4.29 Testing nanoparticle of *N. hiratsukae* (EU06) against *D. oryzae* causing brown leaf spot of rice var. RD47

Testing nano-particles from *Chaetomium brasiliense* (CB01) to control brown leaf spot caused by *D. oryzae* of rice var. RD47

Nano-CBH, nano-CBE, and nano-CBM from *Chaetomium brasiliense* (CB01) at the 10 ppm inhibited spore production by 85.23%, 77.84%, and 79.92%, respectively. These nanoparticles showed

antifungal activity against *D. oryzae* (brown leaf spot of rice var RD47) with ED_{50} values of 0.83, 1.70 and 2.59 ppm., respectively (Table 4.13, Figure 4.30).

Table 4.13 Nano-particles of *Chaetomium brasiliense* (CB01) testing to inhibit *Drechslera oryzae* causing brown leaf spot of rice var. RD47

Nano particles	Concen-tration (ppm)	Colony diameter (cm)[1/]	Growth inhibition (%)[1/]	ED_{50} (ppm)	Number of spore ($\times 10^7$)[1/]	Spore inhibition (%)[1/]	ED_{50} (ppm)
Nano-CBH	0	5.00a	-		5.44a	-	
	1	2.32b	53.50h		2.37cde	55.66de	
	3	2.15c	57.00g		1.61efg	69.49bc	
	5	2.07cd	58.50fg	0.51	1.27fgh	76.34ab	0.83
	7	1.97cde	60.50cde		0.98gh	81.72ab	
	10	1.79fg	64.00cd		0.77h	85.23a	
Nano-CBE	0	5.00a	-		5.44a	-	
	1	2.13c	57.25g		2.88c	46.69e	
	3	2.12cd	57.50fg		2.57cd	52.35de	
	5	1.94def	61.00def	0.34	2.02def	62.49cd	1.70
	7	1.82efg	63.50cde		1.99def	62.78cd	
	10	1.53hi	69.50ab		1.20gh	77.84ab	
Nano-CBM	0	5.00a	-		5.44a	-	
	1	2.02cd	59.50fg		3.96b	25.59f	
	3	2.00cd	60.00fg		2.35cde	56.83cde	
	5	1.67gh	66.50bc	0.21	2.01def	63.31cd	2.59
	7	1.52hi	69.50ab		1.25fgh	77.12ab	
	10	1.50i	70.00a		1.12gh	79.92ab	
	C.V. (%)	4.68	4.40		19.39	15.22	

1/: Means four repeated experiments and followed by the same letter are not significantly differed by DMRT at P=0.05.

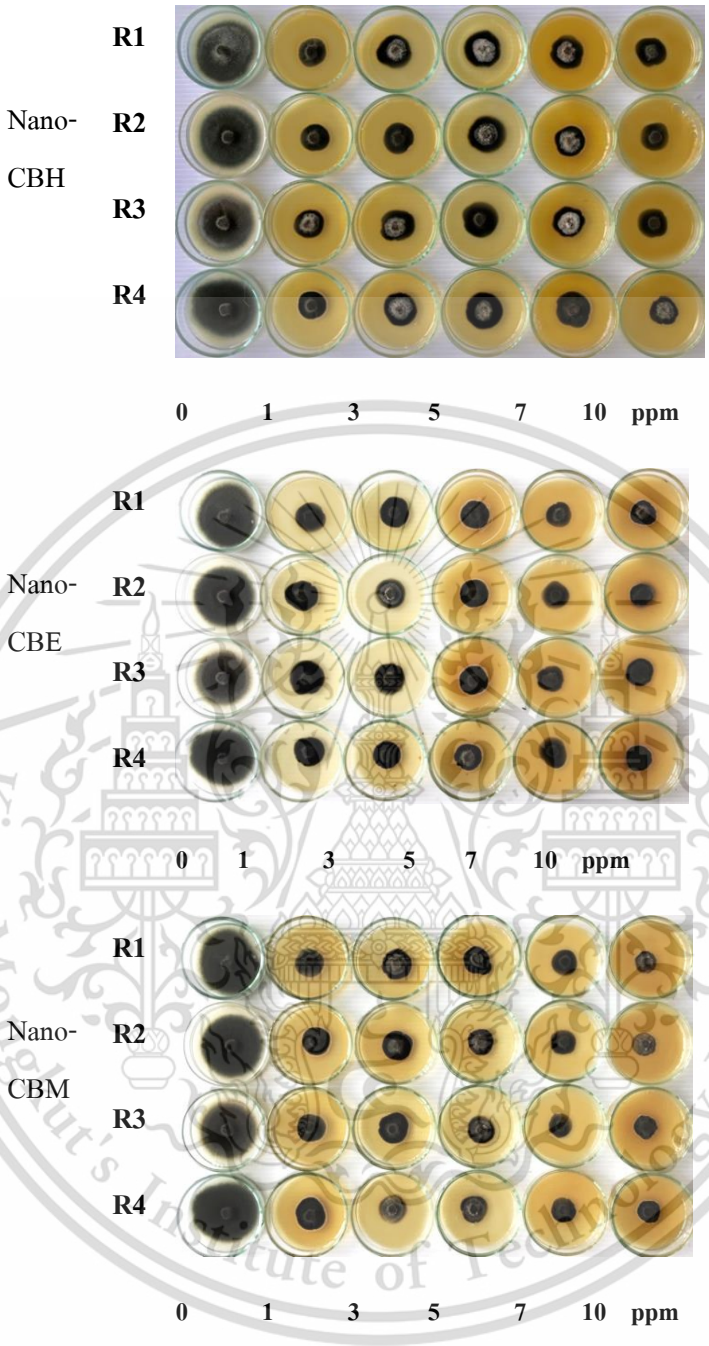


Figure 4.30 Testing nanoparticle of *Chaetomium brasiliense* (CB01) against *D. oryzae* causing brown leaf spot of rice var. RD47

4.4 Nanoparticles derived from *Chaetomium brasiliense* (CB01) testing nanoparticles to induce plant immunity for brown leaf spot caused by *D. oryzae* of rice var. RD47

Nanoparticles of CBH, CBE and CBM from *Ch. brasiliense* (CB01) treated to the inoculated rice seedings var. RD47 which compared to the inoculated with *D. oryzae* (control) causing brown spot of rice var. RD47 and Propiconazole were significantly differed whivch shown in Figure 4.31.

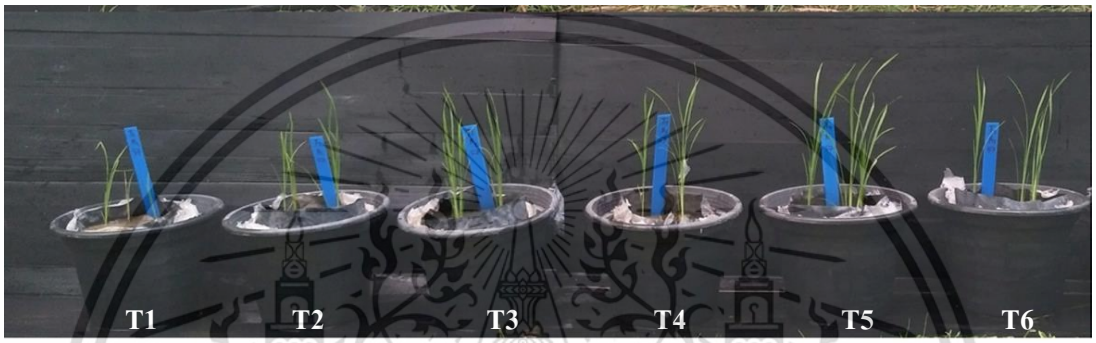


Figure 4.31 Testing nanaparticles derived from *Chaetomium brasiliense* (CB01). The inoculated control with *D. oryzae* (T1), treated with phytoalexin standard (T2), treated with nano-CBH (T3), inoculated with *D. oryzae* and treated with nano-CBE (T4), inoculated with *D. oryzae* and treated with nano-CBM (T5) and inoculated with *D. oryzae* and treated with Propiconazole (T6)

Rrice leaves var. RD47 inoculated with *D. oryzae* and treated with nano-CBH, nano-CBE, nano-CBM and Propiconazole were evaluated phytoalexin production by TLC. The treated phytoalexin standard, nano-CBH, nano-CBE, nano-CBM and Propiconazole were found the spots on TLC plates in benzene : ethyl acetate at the ratio of 10:1 in 365 nm UV light that revealed spots on TLC plates which R_f of 0.61 as standard Oryzalexin C (Figure 4.32).

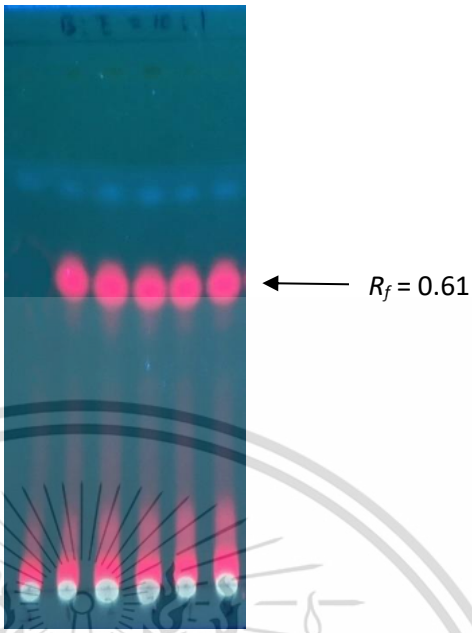


Figure 4.32 The presence of phytoalexin of Oryzalexin C at R_f value 0.61 on TLC plates when using mixture of benzene: ethyl acetate (10:1) under 365 nm UV light. The inoculated control with pathogen (T1), treated with phytoalexin standard (T2), treated with nano-CBH (T3), inoculated with pathogen and treated with nano-CBE (T4), inoculated with pathogen and treated with nano-CBM (T5) and inoculated with pathogen and treated with Propiconazole (T6)

CHAPTER 5

DISCUSSION

5.1. Isolation, identification of pathogen from leaf spot and brown leaf spot of rice var. RD41, RD47, RD57 and Pitsanulok 2 (PL2)

Curvularia lunata caused leaf spot of rice var. RD41 and PL2 and proved to be aggressive pathogenic isolate as similar to report of Keereerat *et al.* (2021) who isolated brown spot disease of rice.

Drechslera oryzae caused brown leaf spot of rice var. RD47 and RD57. Similar reports were confirmed by Manamgoda *et al.* (2014), Jittikornkul *et al.* (2021) and Domsch *et al.*, 1980). Marin-Felix *et al.* (2017) who found new species and recorded as *Bipolaris* and *Curvularia* sp. causing leaf spot of rice in Thailand.

5.1.1 Pathogenicity tests

Curvularia lunata (leaf spot of rice var. RD41 and Pitsanulok 2) were proved to be aggressive pathogenic isolates. It is confirmed by Keereerat *et al.* (2021). *Drechslera oryzae* was proved to be pathogenic isolate causing brown leaf spot on rice var. RD47 and RD57. The brown spot of rice caused by *D. oryzae* was isolated and proved pathogenicity as similar report of Chaijuckam *et al.* (2019) and Valarmathi and Ladhalakshmi (2018) and Marin-Felix *et al.* (2017).

5.2 Investigation of Photosynthetic bacteria

Rhodospirillum centenum strains SM41, SM61, SM72 and SM92 were isolated from waste-water samples from rice fields. Elbadry and Elbanna (1999) reported that phototrophic purple nonsulphur bacterium (PPNSB) was identified as *Rhodospirillum* sp. which stated as nitrogen-fixing bacteria which can be isolated from paddy rice.

5.2.1 Morphological and physiological characterization

Rhodospirillum centenum strains SM41, SM61, SM72 and SM92 were isolated and all isolates showed reddish color, shiny and convex colony. These stranis were shown to be similar reports of Montano *et al.* (2009) who collected from rice paddy soil within a rice field in San Jose del Monte, Bulacan, Philippines.

They are gram-negative, motile cells are spiral, 0.7-1.5 μm in wide which also reported by Imhoff and Trüper (1989) and Montano *et al*., 2009).

R. centenum strain SM41, SM61, SM72 and SM92 are gram negative, mobile cell motility and negative of gelatin activity test. It is reported as anoxygenic photosynthetic bacterium that capable to differentiate several cell types. When it grows in phototrophically condition in liquid media, cells exhibit a vibrioid shape and a single polar flagellum. But it grows on a solid surface, *R. centenum* is differentiated to rod-shaped, swarm cells that appear many lateral flagella (Berleman and Bauer, 2004).

R. centenum strain SM41 and SM61 showed good activities of glucose, lactose and sucrose fermentation activities. and isolates SM72 and SM92 found to be non-fermented activity as reported by Imhoff and Trüper (1989).

Phylogenetic confirmation and scanning electron microscopy

Rhodospirillum centenum strains SM41, SM61, SM72 and SM92 were molecular phylogenic confirmed by *PufM* gene sequence analysis with related to *R. centenum* SW in genbank No CP00613.2 using MEGA11. Berleman and Bauer (2004) reported that *R. centenum* is an anoxygenic photosynthetic bacterium. It can grow in phototrophically liquid, a single polar flagellum expressed heat tolerance and drought resistance.

Serratia marcescens strain (LB01) are red culture appearance, smooth, gram-negative, rod, motility, gelatin positive. It is similar reported by Giri *et al*. (2004) and Purkayastha *et al.* (2018).

Colonies of *Dietzia cercidiphylli* (LB02) is orange in color, smooth, gram-positive, rod, non-motile, gelatin negative which similar reported of Li *et al*. (2008). It was isolated from a surface-sterilized root sample of *Cercidiphyllum japonicum* which collected from Yunnan Province, south-west China.

5.2.2 Enzyme production property of photosynthesizing bacteria

Rhodospirillum centenum strains SM41, SM61, SM72, SM92 and *Serratia marcescens* (LB01) and *Dietzia cercidiphylli* (LB02) produced amylase, protease and lipase with similar reported by Anderson and Fuller (1969). *Serratia marcescens* (LB01) produced amylase and lipase as reported by Purkayastha *et al.* (2018).

5.2.3 Photosynthetic bacteria, *Rhodospirillum centenum* to increase seed germination of rices var. RD41 and var. PL2

Rice var. RD41 in treated *Rhodospirillum centenum* SM41 was 45% seed germination, and followed by *R. centenum* strains SM92, SM 61 and SM 72. Rice var. PL2 showed that treated *R. centenum* strains SM41, SM61 and SM 72 were 12.5% seed germination and *R. centenum* strain SM92 was 10% seed germination. *R. centenum* strain SM92 treated to rice var. RD41 showed the highest seed germination, shoot length and root length and vigor index., *R. centenum* strain SM61 treated to rice var. PL2 showed the highest seed germination, shoot length, root length and vigor index. Photosynthetic bacteria treated to rice showed good root development and increased yield (Kobayashi and Kobayashi, 2000).

R. centenum strains SM41, SM61, SM72 and SM92 stimulated seed germination on rice var. RD41 and var. PL2. Withthis Harada *et al*. (2005) reported that inoculation of *R. palustris* can be increased the grain yield of rice while *Rhodobacter capsulatus* enhanced seedling growth, i.e. increasing shoot height of rice seedlings, regardless of rice variety (Elbadry and Elbanna, 1999) and Serdyuk *et al*. (1993).

The finding of *R. centenum* strains SM41 and SM61 decreased leaf spot and stimulated plant growth of rice. Rana *et al.* (2016) stated that *Rhodospirillum rubrum* is a good mineral solubilizing and plant growth promoting activities on fly-ash. Rana *et al.* (2016) stated that some isolate of *Rhodospirillum rubrum* decreased disease incidence in rice. *R. centenum* and controlled leaf spot of rice caused by *Curvularia lunata* which is also reported by Vareeket and Soytong (2017).

5.2.4 Effects of *Serratia marcescens* strain (LB01) and *Dietzia cercidiphylli* strasin (LB02) for stimulating plant growth and control leaf spot caused by *Curvularia lunata* of rice var. RD41 and Pitsanulok 2 (PL2) by *Rhodospirillum centenum*

Curvularia lunatra causing leaf spot of rice var. RD41 is inhibited by *Serratia marcescens* (LB01) and *Dietzia cercidiphylli* (LB02). The research findings were also similar reported by Jaiganesh *et al.* (2007), Parani *et al*. (2011), Kamensky *et al*. (2003), Queiroz and Melo (2006) and El Khaldi *et al*. (2015).

Serratia marcescens (LB01) and *Dietzia cercidiphylli* (LB02) promoted plant growth (52.61 cm) of rice var. RD41. de Queiroz and de Molo (2006), Purkayastha *et al.* (2018) and Someya *et al*. (2005) who recorded similar results of these microorganism can be promoted plant growth.

5.3 Antagonistic *Chaetomium brasiliense* (CB01) and *Neosartorya hiratsukae* (EU06) against brown leaf spot (*D. oryzae)* of rice var. RD47

Neosartorya hiratsukae (EU06) is a creamy-white culture, produce cleistothecia in globose shape, 8 ascospores/ascus. It is also similar to the work of Poeaim *et al.* (2016). *Chaetomium brasiliense* (CB01) is dark grey colour, perithecia globose, cylindrical asci with 8 acospores as similar recorded by Abdullah and Zora (1993).

5.3.1 Bi-culture antagonistic test between *N. hiratsukae* (EU06) against *D. oryzae*

N. hiratsukae (EU06) inhibited the growth of *D. oryzae* (brown leaf spot of rice var. RD47). In this study, *N. hiratsukae* inhibited mycelial growth of *D. oryzae* of 31.67% with similar reported to *Neosartorya* sp. (KUFC 6301) inhibited 30% mycelium growth of *Bipolaris maydis*, *Colletotrichum capsici* and *C. gloeosporioides* (Eamvijarn *et al.*, 2009).

Ch. brasiliense (CB01) inhibited mycelial growth of *D. oryzae* (brown leaf spot of rice var. RD47). It inhibited the mycelia growth of 26.38%. *Ch. brasiliense* (CB01) inhibited spore production of *D. oryzae* by 4.80% with similar to Tathan *et al*.(2012) who found *Chaetomium* spp. inhibited *D. oryzae* causing leaf spot of rice at 35.83% and 75.13%.

5.3.2 Active metabolites from *Chaetomium brasiliense* (CB01) and *Neosartorya hiratsukae* (EU06) testing for inhibition of brown leaf spot of rice var. RD47 caused by *Drechslera oryzae*

***In vitro* testing crude extracts from *Neosartorya hiratsukae* (EU06) against *Drechslera oryzae* causing brown leaf spot of rice var. RD47**

Crude ethyl acetate of *N. hiratsukae* (EU06) inhibited *Drechslera oryzae* (brown leaf spot of rice var. RD47) at low concentration which the ED_{50} of 12.18 ppm., and followed by crude hexane and methanol which the ED_{50} of 29.16 and 43.90 ppm. Ethyl acetate and methanol crude extract from *N. hiratsukae* was significantly highest inhibited 42.50% for the colony growth of *D. oryzae* at 1,000 ppm. It was similar report that *N. pseudofischeri* KUFA0060 and *N. quadricincta* KUFA0064 crude extracts inhibited the mycelial growth of *P. palmivora* and *C. capsici* at 100 ppm (Boonsang *et al*., 2014). Jantasorn *et al*. (2016) reported that *N. fischeri* Bodhi004 crude extract recorded antifungal activity of 100% growth inhibition against *Phytophthora palmivora*, *Penicillium grisea, Alternaria* sp. and *Rhizoctonia solani* at 10,000 ppm.

Drechslera oryzae expressed abnormal cells due to crude extract of antagonist which similar report of Poeaim *et al.* (2016).

Crude ethyl acetate extract of *Ch. brasiliense* (CB01) inhibited *Drechslera oryzae* causing brown leaf spot of rice var. RD47 which the ED_{50} of 9.64 ppm, and followed by crude hexane and methanol extracts which the ED_{50} of 14.35 and 23.35 ppm. However, Tan and Soytong (2017) reported that crudec extrtacts from *Chaetomium cupreum* (CC3003) reduced leaf spot of rice var. Sen Pidao in Cambodia.

5.3.3 Testing nano-particles from *Chaetomium brasiliense* strain (CB01) and *Neosartorya hiratsukae* strain (EU06) to control brown leaf spot of rice var. RD47 caused by *D. oryzae*

Nano-NHM from *N. hiratsukae* (EU06) inhibited *D. oryzae* (brown leaf spot of rice var. RD47) The nano-NHM, nano-NHE and nano-NHH at 10 ppm inhibited spore production of 94.87%, 93.31%, and 88.73%, and ED_{50} values of 3.93, 3.97, and 4.46 ppm., respectively. The research finding is similar

to Paluka *et al.* (2020) that EtOAc extract from *Neosartorya hiratsukae* showed antibacterial activity against *Bacillus cereus* ATCC 11778 with MIC 40 ppm.

Nano-CBH, nano-CBE and nano-CBM from *Chaetomium brasiliense* CB01 at 10 ppm inhibited sporulation of *Drechslera oryzae* (brown leaf spot of rice var. RD47) with ED_{50} of 0.83, 1.70, and 2.59 ppm., respectively. It is similar result from the works of Khumkomkhet *et al.* (2009) and Song *et al*. (2020) who reported that *Chaetomium elatum*, *Chaetomium lucknowense* and *Chaetomium brasiliense* were antagonized *M. oryzae*.

5.4 Nanoparticles derived from *Chaetomium brasiliense* (CB01) testing nanoparticles to induce plant immunity for brown leaf spot caused by *D. oryzae* of rice var. RD47

Rice var. RD47 was inoculated with *D. oryzae* (brown leaf spot of rice var. RD47), nano-CBH, nano-CBE, nano-CBM accumualted Oryzalexin C and showed R_f of 0.61 which it is Oryzalexin C. *Chaetomium brasiliense* CB01 is produced mollicellins B (1), C (2), E (3), F (4) and mollicellins K-N (5-8) that compounds 1-3 and 5-7 inhibited *Plasmodium falciparum* and compound 30 inhibited *Candida albicans* and *Mycobacterium tuberculosis*. However, compounds 1-8 inhibited KB, BC1, NCI-H187 and cholangiocarcinoma cell lines (Khumkomkhet *et al.*, 2009). These compounds may act as control mechanism against the tested plant pathogens. Withthis, the research finding is similary reported by Song *et al.* (2020) that nano-particles from *Ch. brasiliense* inhibited rice blast pathogen in rice var. PL2. It is possible these bioactive substances act as control mechanism to controlt brown leaf spot of rice.

CHAPTER 6

CONCLUSIONS AND SUGGESTION

The research findings concluded that *Curvularia lunata* causing leaf spot of rice var. RD41 and PL2 and *Drechslera oryzae* causing brown leaf spot of rice var. RD47 and RD57. *C. lunata* and *D. oryzae* were confirmed to be pathogenic isolates. The photosynthetic bacteria, *Rhodospirillum centenum* strains SM41, SM61, SM72 and SM92 are reddish color, shiny convex colony, gram-negative, motile cells, gram negative, mobile cell motility and negative of gelatin activity test. *R. centenum* strains SM41 and SM61 revealed good activities of glucose, lactose and sucrose fermentation and isolates SM72 and SM92 were non-fermented activity.

The isolated antagonistic bacteria, *Serratia marcescens* strain LB01 showed red culture appearance, smooth, gram-negative, rod, motility, gelatin positive. *Dietzia cercidiphylli* strain LB02 showed orange in color, smooth, gram-negative, rod, motility, and gelatin negative. *Rhodospirillum centenum* SM41, SM61, SM72, SM92, *S. marcescens* (LB01) and *D. cercidiphylli* (LB02) produced amylase, protease and lipase which possible proved to be control mechanism for inhibitiong thec tested pathgogens. *Rhodospirillum centenum* SM41 treated to rice var RD41 stimulated seed germination of rice var. PL2 and followed by *R. centenum* strains SM92, SM61 and SM72. *R. centenum* strain SM92 gave a good plant strand of rice var. RD41 and *R. centenum* strains SM41, SM61, SM72 and SM92 stimulated germination of rice seeds var. RD41 and var. PL2. The antagonistic activities of *R. centenum* strains SM41 and SM61 decreased leaf spot and stimulated plant growth of rice. Morover, *S. marcescens* (LB01) and *D. cercidiphylli* (LB02) antagonize *C. lunatra* causing leaf spot of rice var. RD41 and stimulated plant growth of rice var. RD41.

The fungal antagonist, *N. hiratsukae* (EU06) inhibited *D. oryzae* causing brown leaf spot of rice var. RD47. *Ch. brasiliense* (CB01) is proved to be antagonized *D. oryzae* causing brown leaf spot of rice var. RD47. Crude metabolites from of *Ch. brasiliense* (CB01) controlled the growth of *D. oryzae* causing brown leaf spot of rice var. RD47. Crude ethyl acetate of *N. hiratsukae* (EU06)

inhibited *D. oryzae* causing brown leaf spot of rice var. RD47. Crude ethyl acetate extract of *Ch. brasiliense* (CB01) inhibited *D. oryzae* causing brown leaf spot of rice var. RD47.

Nanotechnological investigation found that nano-NHM from *N. hiratsukae* (EU06) inhibited *D. oryzae* causing brown leaf spot of rice var. RD47. Nano-NHM, nano-NHE and nano NHM completely inhibited sporulation. Nano-CBH, nano-CBE, and nano-CBM from *Ch. brasiliense* (CB01) revealed to be inhibited sporulation of *D. oryzae* causing brown leaf spot of rice var. RD47. Nano-CBH, nano-CBE nano-CBM were separately treated to the inoculated rice var RD 47 with *D. oryzae* causing brown leaf spot produced phytoalexin namely Oryzalexin C with the R_f of 0.61.

It is suggested that both antagonistic bacteria and fungi can be further to develop to be biolofical fungicides to control leaf spot of rice caused by *C. lunata* and brown spot of rice caused by *D. oryzae.* Interestingly investigation for research finding on nanoparticles derived from active metabolites of *N. hiratsukae* (EU06) and *Ch. brasiliense* (CB01) will be further studied for feasibility to develop to be nanoelicitor to induce plant immunity in rice.

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APPENDIX

PUBLICATION

Publications:

1. Vareeket, R. and Soytong, K. 2015. "Evaluation of photosynthesizing bacteria for the growth of rice var. RD41." **Journal of Agricultural Technology.** 11(8) : 2257-2261
2. Vareekat, R. and Soytong, K. 2016. "Screening of Photosynthetic Bacteria, *Rhodospirllum centenum* for Stimulation of Rice Seed Germination." **International Journal of Agricultural Technology**. 12(7.1) : 1449-1453.
3. Vareeket, R., Soytong, K., Kanokmedhakul, S. and Kanokmedhakul, K. 2018. Nano-particles from *Cheatomium brasiliense* against brown spot of rice. International Journal of Agricultural Technology. Vol. 14(7): 2207-2214. (SJR, Q4)
4. Vareeket, R. and Soytong, K. 2020. "*Rhodospirillum centenum*, A New Growth Stimulant and Antagonistic Bacteria against Leaf Spot of Rice Caused by *Curvularia lunata*." **AGRIVITA, Journal of Agricultural Science.** 42(1) : 160-167. (ISI)
5. Vareeket, R. Song, J. J. and Soytong, K. 2023. "New discovery of natural product nanoparticles constructed from active metabolites from *Chaetomium brasiliense* for immunity to brown spot of rice var. RD 47." **International Journal of Agricultural Technology**. 19(1) : 291-300. (SJR, Q4)

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Publication

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Poster

Vareekat, R. and Soytong, K. 2017. "Screening of Antagonistic Bacteria for Biological Control of Rice Diseases." **International Journal of Agricultural Technology.** 13(7.3) : 2569-2573.