รายงานการวิจัย

การจำแนกชนิดของเชื้อ *Curvularia* sp. สาเหตุของโรคใบจุดของต้นกล้าปาล์มน้ำมันโดยเทคนิคทางชีวโมเลกุลและการควบคุมเชื้อสาเหตุโรคโดยชีวภัณฑ์ไคโตซาน

Molecular characterization of *Curvularia* sp. the causal agent of oil palm leaf spot and the control by chitosan, a bioactive compound

โดย

อนุรักษ์ สันป่าเป้า

ภาควิชาการจัดการศัตรูพืช **มหาวิทยาลัยสงขลานครินทร์**

คณะทรัพยากรธรรมชาติ **วิทยาเขตหาดใหญ่ จังหวัดสงขลา**

กิตติกรรมประกาศ

ขอขอบคุณมหาวิทยาลัยสงขลานครินทร์ สำหรับทุนวิจัยจากเงินรายได้มหาวิทยาลัย ประเภททั่วไป ที่ได้ให้การสนับสนุนทุนวิจัย และขอขอบคุณ ภาควิชาการจัดการศัตรูพืช คณะทรัพยากรธรรมชาติ มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่ จังหวัดสงขลา สำหรับสถานที่ทดลอง อุปกรณ์ เครื่องมือ เพื่อให้ดำเนินการวิจัยได้สำเร็จลุล่วงด้วยดี

อนุรักษ์ สันป่าเป้า

ตุลาคม 2558

การจำแนกชนิดของเชื้อ *Curvularia* sp. สาเหตุของโรคใบจุดของต้นกล้าปาล์มน้ำมัน โดยเทคนิคทางชีวโมเลกุล และการควบคุมเชื้อสาเหตุโรคโดยชีวภัณฑ์ไคโตซาน

บทคัดย่อ

คุณภาพของต้นกล้าปาล์มน้ำมันลดลงเนื่องจากโรคต่าง ๆ โดยเฉพาะโรคใบไหม้และโรคใบจุด ไคโตซานเป็นโพลิเมอรจากธรรมชาติถูกใช้กระตุ้นการเจริญเติบโตของพืชและควบคุมโรคจากเชื้อราสาเหตุโรคในพืชปลูกหลายชนิด จุดประสงค์ของงานวิจัยนี้คือจำแนกชนิดของเชื้อสาเหตุโรคใบจุดโดยเทคนิคทางชีวโมเลกุลและใช้ไคโตซานเพื่อควบคุมเชื้อสาเหตุของโรคใบจุดของต้นกล้าปาล์มน้ำมัน เชื้อสาเหตุโรคใบจุดถูกแยกจากใบที่แสดงอาการใบจุดและจัดจำแนกทางลักษณะทางสัณฐานวิทยาคล้ายคลึงกับเชื้อ *Curvularia oryzae* เทคนิคทางชีวโมเลกุลถูกนำมาใช้ยืนยันผลจากการจำแนกเชื้อสาเหตุโดยลักษณะทางสัณฐานวิทยา ผลของปฏิกิริยาลูกโซ่โพลีเมอเรสกับคู่ไพรเมอร ITS ได้ขนาดผลิตภัณฑ์ขนาด 1 kb การวิเคราะห์ลำดับนิวคลีโอไทด์ยืนยันว่าเชื้อสาเหตุโรคใบจุดของต้นกล้าปาล์มน้ำมันคือ *C. oryzae* ไอโซเลต PSU-NK1012 ไคโตซานในหลาย ๆ ความเข้มข้นถูกนำมาทดสอบกับการเจริญของเชื้อ *C. oryzae* ไอโซเลต PSU-NK1012 ในหลอดทดลองและในใบพืช เปอร์เซ็นต์การเจริญของเชื้อ *C. oryzae* ไอโซเลต PSU-NK1012 บนอาหาร PDA ผสมไคโตซานในหลายความเข้มข้น พบว่าไคซานที่ความเข้มข้น 2 mg/ml สามารถยับยั้งการเจริญของเชื้อราได้ 85 เปอร์เซ็น โดยความเข้มข้นของไคโตซานที่ระดับนี้ลดจำนวนแผลที่เกิดจากเชื้อราบนใบของต้นกล้าปาล์มน้ำมันด้วย นอกจากนี้กิจกรรมของเอนไซม์ β-1,3-glucanase ซึ่งเป็นเอนไซม์ในกลุ่มโปรตีน PR ยังเกี่ยวข้องกับการลดจำนวนของแผลบนใบในระยะเริ่มแรกของต้นกล้าปาล์มน้ำมันเมื่อให้ไคโตซาน

คำสำคัญ: ไคโตซาน, ITS, ใบจุด, ปาล์มน้ำมัน

Molecular characterization of *Curvularia* sp. the causal agent of oil palm leaf spot and the control by chitosan, a bioactive compound

Abstract

The quality of oil palm in nursery stage seedlings can be reduced by several diseases, particular leaf blight and leaf spot. Chitosan is a biological polymer used as a plant growth stimulator, and used against several phytopathogenic fungi on many crops. This research was conducted to identify the pathogen of leaf spot disease and to determine the effects of chitosan in controlling leaf spot disease. The fungus was isolated from leaves with disease symptoms, characterized by morphological properties as *Curvularia oryzae*. The identity of the phytopathogenic fungus was confirmed through polymerase chain reaction analysis using internal transcribed spacer (ITS) primers, which amplified about a 1 kb product. Sequencing this DNA product confirmed this pathogen was *C. oryzae* isolate PSU-NK1012. Various concentrations of chitosan were applied on *in vitro* and *in vivo* test against *C. oryzae* PSU-NK1012 infection. The growth rate of *C. oryzae* PSU-NK1012 on potato dextrose agar was determined with various chitosan concentrations, and a concentration of 2 mg/ml was sufficient to reduce the radial growth of *C. oryzae* PSU-NK1012 by about 85 % on agar plates. This concentration also reduced the number of spot on oil palm seedling leaves. Furthermore, the activities of β-1,3-glucanase, one of a pathogenesis related protein plays an important role in limiting the leaf spot on chitosan treated oil palm leaves samples at the early infection period.

Keywords: Chitosan, ITS, leaf spot, oil palm

Table of content

(ภาษาไทย) การจำแนกชนิดของเชื้อ *Curvularia* sp. สาเหตุของโรคใบจุดของต้นกล้าปาล์มน้ำมันโดยเทคนิคทางชีวโมเลกุล และการควบคุมเชื้อสาเหตุโรคโดยชีวภัณฑ์ไคโตซาน

(ภาษาอังกฤษ) Molecular characterization of *Curvularia* sp. the causal agent of oil palm leaf spot and the control by chitosan, a bioactive compound

Introduction

The oil palm (*Elaesis guineensis* Jacq.) is an economically important crop in southern Thailand. Major cultivation areas of oil palm are in the southern Thailand. Oil palm planting needs high rainfall to gain high yield production which favorable for some pathogen germination. Oil palms are infected with several diseases in all stages of growth. For instance, seed rot and brown germ caused by *Schizophyllum commune* have been severe in Malaysia (Dikin *et al*., 2003), whereas the basal stem rot caused by *Garnoderma boninense* is the most severe diseases in Indonesia and Malaysia (Susanto *et al*., 2005). The main diseases are caused by fungi, inhibiting the growth and reducing the yield (Turner, 1981; Tengoua and Bakoume, 2005; Flood, 2006). Leaf spot of oil palm is concerned to be a minor disease, but during the long period of high rainfall in the southern, it can spread rapidly. The disease primarily affects young seedling up to 3 months old or seedlings which have recently been transplanted (Turner, 1981). It has been reported that *Botryodiplodia palmarum*, *Melanconium* sp. and *Clomerella cingulate* caused anthracnose in Africa, while in southeast Asia *Curvularia eragrostidis* and *Leptosphaeria* spp. were common for leaf spot disease (Aderungboye, 1977)

Application of fungicide is effective method to control fungal diseases. However, the high cost of fungicide, fungicide-resistant strains of the pathogen, as well as effects on human health have raised concerns in many countries. Biological products are widely used in agricultural production and are considered environmentally friendly and sustainable. Chitosan, a natural polymer, and chitosan derivatives, have been widely used as growth stimulators to increase various crops, such as orchids (Nge *et al*., 2006), faba beans (El-sawy *et al*., 2010), cucumbers (Shehata *et al*., 2012), and corn (Boonlertnirun *et al*., 2011; Lizarraga-Paulin *et al*., 2011). Aside from its role as plant growth promoter, chitosan has also been effective in the biological control

against several fungi (Allan and Hadwiger, 1979; Hirano and Nagao, 1989; Kendra *et al*., 1989; Stossel and Leuba, 1984; Benhamou, 1996; Sunpapao and Pornsuriya, 2014).

Only few previous studies have been conducted on leaf spot disease of oil palm and the control by bioactive compound. The objective of this study was (i) to examine this disease pathogen by molecular method in nurseries stage of oil palm, and (ii) to control leaf spot disease by application of chitosan.

Objective

1. To identify oil palm leaf spot pathogen by molecular properties

2. To test effect of chitosan in controlling leaf spot disease on oil palm seedlings

3. To test enzyme activity response to fungal infection

Materials and methods

Sample collection, fungal isolation and identification (Sunpapao *et al*., 2014)

Leaf samples with leaf spot symptoms were collected from oil palm nurseries in Thailand during 2012–2013. Symptomatic leaves were collected from 11 provinces. The palms were 3–4-month-old nursery seedlings. Samples were placed in plastic bags and stored in a cooler box. Isolations were made within 24 h of cutting. Four or five symptomatic leaves were washed by distilled water and incubated in a moist chamber for 3–5 days to induce sporulation. Single spores were sampled directly under stereomicroscope from thallus on the diseased sample using a fine needle. Each single spore was placed onto potato dextrose agar (PDA). The isolates collected in this study are maintained in PDA slants. The single-spore isolates were cultured on corn meal agar (CMA) to induce spore production. The conidiophores and conidia of phytopathogenic fungi were observed under light microscope (Olympus, Japan), and characterized based on their morphology. The conidiophores and conidia were photographed.

DNA extraction (Sunpapao *et al*., 2014)

The PSU-NK1012 isolate of fungus from *E. guineensis* was selected and grown on PDA at room temperature for 1 week. The cetyl trimethyl ammonium bromide (CTAB) method was adopted to extract total DNA from the mycelia of the isolate (Manamgoda et al. 2012). The mycelium was scraped off the agar surface using a sterilized slide and transferred to a new 1.5 ml microtube. The mycelium was crushed by using a sterile micro pestle in CTAB solution (2.0 g CTAB, 10 ml of 1 M Tris pH 8.0, 4 ml of 0.5 M EDTA pH 8.0, 28 ml of 5 M NaCl, 40 ml of distilled water and made up to 100 ml with distilled water), and after incubation at 65°C the sample was mixed with chloroform isoamyl alcohol (24:1). After centrifugation, the supernatant was transferred to a fresh microtube and isopropanol was added. After centrifugation the pellet was collected and air dried, and finally dissolved in 20 µl of sterilized deionized water (DI). The presence of total DNA was checked by gel electrophoresis prior to use as template for PCR amplification.

PCR amplification, sequencing and analyses (Sunpapao *et al*., 2014)

Internal transcribed spacer (ITS) primers were used to confirm the identification of the fungus. The ITS genes were amplified using a BIO–RAD T100^{TM} Thermal Cycler (Bio-Rad, Hercules, CA, USA). A small portion of 18S rDNA, ITS1, 5.8S rDNA, ITS2 and a small portion of the 28S rDNA of *Curvularia* isolates was amplified by PCR, using PN3 forward: 5' CGTTGGTGAACCAGCGGAGGGATC 3' and PN16 reverse: 5' TCCCTTTCAACAATTTCACG 3' primers (Neuveglise *et al*.1994). PCR was performed in 50 µl of reaction mixture containing 10 pmol of each primer, 1.25 U Taq DNA polymerase (New England BioLabs, Ipswich, MA, USA), 10X Thermopol reaction buffer (New England BioLabs), 10mM dNTPs (New England BioLabs) and 50 ng of template DNA. An initial denaturation step for 2min at 94°C was followed by 35 cycles of denaturation for 40 s at 94°C, annealing for 40 s at 56°C and extension for 80 s at 72°C, with a final extension step of 10 min at 72°C. The PCR products were visualized by agarose gel electrophoresis.

The ITS region was sequenced at Scientific Equipment Center, Prince of Songkla University, Songkhla, Thailand, by automated DNA sequencing with ABI Prism 377 (Applied Biosystems, Foster City, CA, USA) using the same primers used in the PCR reaction. Using BLAST analyses, the sequences obtained were compared with sequences of *C. oryzae* available in GenBank (The National Center of Biological Information).

Culture and growth condition of Curvularia oryzae

Curvularia oryzae isolate PSU-NK1012 (Sunpapao *et al*., 2014) was provided from Culture Collection of Pest Management, Faculty of Natural Resources, Prince of Songkla

University, Thailand. The fungi were stored in potato dextrose agar (PDA) slants at 4°C. The fungal mycelia were routinely maintained in PDA at 25°C before testing with chitosan.

Effect of chitosan on radial growth of Curvularia oryzae

Effect of chitosan on fungal growth was evaluated by culture *C. oryzae* PSU-NK1012 on PDA amended with 0.125, 0.25, 0.5, 1.0 and 2.0 mg/ml (Laflamme et al., 2000). Unamended PDA plate with 0.05% acetic acid (pH 5.6) was used as negative control, comparison of this exclude effect of acidic condition. The leaf spot disease pathogen *C. oryzae* PSU-NK1012 was assessed for its growth inhibition. Five replicate plates at each chitosan concentration were inoculated in the center with a plug (5 mm diameter) form the edge of *C. oryzae* PSU-NK1012 colonies. The colony radii were measured 7 days after inoculation. The experiments were repeated twice. Percent inhibition of diameter growth (PIDG) was calculated using following formula:

$$\text{PIDG} = \frac{\mathbf{A-B}}{\mathbf{A}} \times \mathbf{100}$$

Where: A = Diameter of control colonies, B = Diameter of treated colonies

Effect of chitosan in vivo

To test chitosan in controlling leaf spot disease caused by *Curvularia*, the leaves of oil palm seedling were sprayed with chitosan 1 hr prior to inoculate the pathogen. Spore suspension of *C. oryzae* PSU-NK1012 in concentration 1×10^6 was inoculated to chitosan treated oil palm leaves by detached leaf method. Leaf treated with 0.05% acetic acid was served as negative control. The inoculated leaf samples then were incubated in moist chamber for 3 days, number of leaf spot were measured.

Protein extraction and enzyme activity assay

Oil palm leaf samples were ground with sodium acetate buffer (50 mM CH_3COONa buffer, pH 5.0) for β-glucanase assay. The extract were kept at 4°C overnight and then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was used in the enzyme assay. Enzyme activities of β-1,3-glucanase were conducted according to DNS methods (Miller, 1959). For β-1,3-glucanase assay, crude enzyme were mixed with 1 % laminarin (Sigma-Aldrich, USA) in 50 mM sodium acetate buffer pH 5.0 as substrate. Reaction mixture was incubated at 37°C for 1 hr, added with DNS (3,5 dinitrosalicylic acid) solution and boiled 15

min. Then H_2O was added and kept on ice. The reaction mixture was measured at A550. Glucose solution was prepared for standard of β-1,3-glucanase activities.

Results and discussion

Chapter I, Identification of leaf spot disease pathogen on oil palm seedlings in nurseries stage

Field inspection and disease samples were conducted to diagnose oil palm diseases in southern Thailand. The location of observed symptom was noted. The typical symptoms of disease or signs of pathogen were observed to determine that the disease was caused by a pathogen or an environmental factor. Oil palm trees were surveyed in several nurseries and private oil palm plantations in eleven provinces of southern Thailand. Infected leaf tissues and seeds samples were collected and brought to laboratory, where identification was done. The main diseases noticed in seed during germination process were brown germ and seed rot. *Curvularia* leaf spot had the highest incidence in the nurseries stage, whereas the basal stem rot was found to be the most destructive disease at the fruiting stage, causing decline and death of oil palm trees. Summaries of the diseases found in oil palms, from seed, nurseries and field stage, are shown in Fig. 1. The incidence rate of diseases and disorders are located in Table 1.

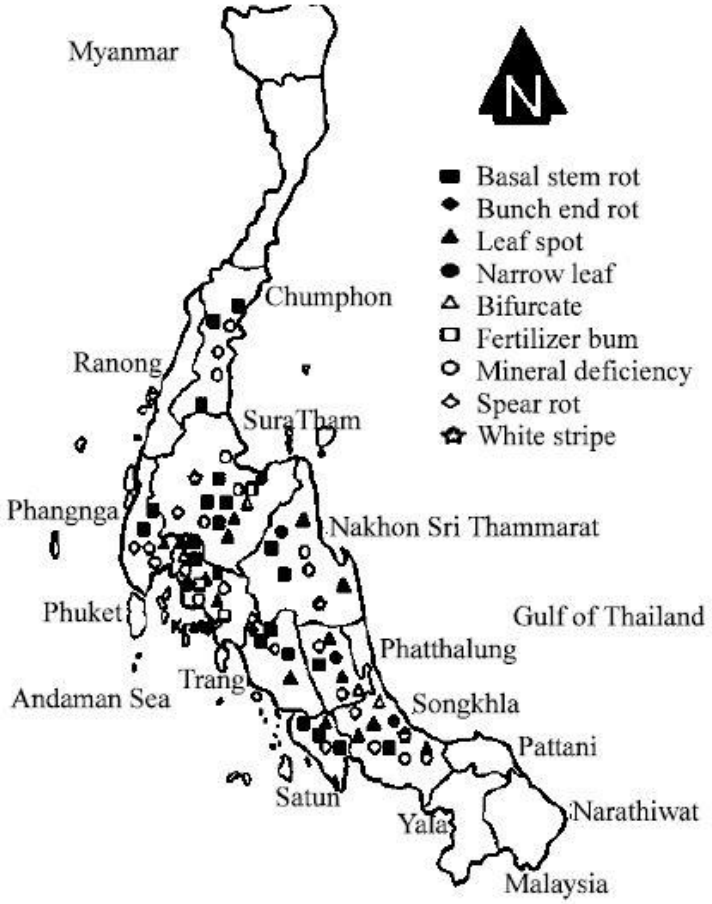

Fig.1 Survey sites of oil palm nurseries and private plantations in southern Thailand (Source: Pornsuriya *et al*., 2013)

Table 1. Severity and incidence of diseases in different growth stage of oil palm (Source: Pornsuriya *et al*., 2013)

Growth stage	Diseases	Disease incidence (%)
Seed	Brown germs	13.12
	Seed rot	4.20
Nurseries	Leaf spot*	11.26
	Genetic disorder	6.85
Field	Algae	24.92
	Basal stem rot	1.53
	Bunch end rot	1.16
	Sooty mold	51.30
	Spear rot	1.24
	B deficiency	19.15
	K deficiency	6.53
	Mg deficiency	9.54
	N deficiency	13.46
	P deficiency	7.44

The disease of oil palm in nurseries stage were conducted. A total 277 samples of symptomatic leaf blight and leaf spot were collected from nurseries in eleven provinces of southern Thailand. The leaf blight showed as small circular and translucent yellow to brown necrotic tissues diffusely scattered on leaves (Fig.2d), while leaf spot was demonstrated by dark brown pin point on the leaves (Fig. 2c). The collected samples were subjected to single spore isolation on CMA and maintained in Potato Dextrose Agar (PDA). The leaf blight and leaf spot causal agents were examined by compound microscope and were identified as a genera *Curvularia* and *Colletotrichum* leaf spot based on morphology. The most common fungal isolates found in this study in decreasing order were *Curvularia* 149 isolates and *Colltetotrichum* 48 isolates.

Fig. 2 (a-h): Nursery diseases and disorders of oil palm, (a) Anthracnose, (b) *Cercospora* leaf spot, (c) *Curvularia* leaf spot, (d) *Pestalotiopsis* leaf blight, (e-h) genetically abnormal seedlings, (f) fertilizer burn at leaf tips, (g) narrow leaf (grass leaf) and (h) white leaf. (Source: Pornsuriya *et al*., 2013)

In order to characterize the leaf spot disease causal agent, field inspection and sample collection were conducted. The most prevalent symptom patterns of oil palm seedlings observed in this study in nurseries of southern Thailand were leaf spot and leaf blight. The primary symptom was dark brown pin points in most parts of the leaves. The disease progressed by developing dark brown lesions surrounded by yellowish halos, and finally became diffuse leaf blight (Fig. 3a–c). After incubation, single spores were obtained from the symptomatic leaf samples, and the spores cultured initially on PDA. Characteristics of conidiophores and conidia were observed with a compound microscope. The conidiophores of this fungus were single with one conidium, straight or flexuous and brown to dark brown. Conidia of this fungus were 3-distoseptate, ovoid, obclavate, with the second cell from the base the largest; intermediate cells were brown or dark brown, and the largest cell was often also the darkest. The spore size was approximately 24–40 × 12–22 μm (Fig. 3e). Based on these morphological characteristics (Sivanesan 1987), the fungus was preliminarily identified as *Curvularia oryzae*. Two cultures of the fungus (PSU-NK1012 and 1013) were deposited in the Culture Collection of Pest Management Department, Faculty of Natural Resources, Prince of Songkla University (PSU), Thailand.

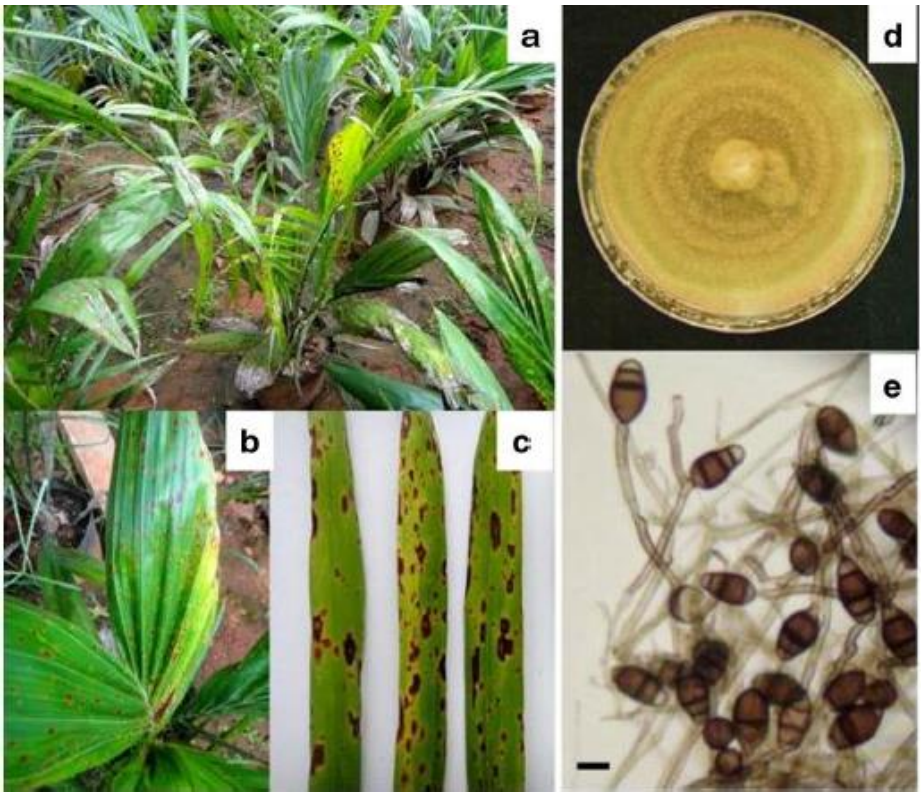

Fig. 3 Leaf blight and leaf spot symptoms caused by *Curvularia oryzae*. Disease symptoms of oil palm seedlings at nursery stage (a), leaf spot on bifid leaves (b), leaf spot and leaf blight on spear leaves (c), colony of *C. oryzae* (d), conidiophore and conidia of *C. oryzae* (e). Bar = 20 μm. (Source: Sunpapao *et al*., 2014)

The identity of the fungus was confirmed through PCR using PN3 and PN16 primers to amplify the ITS region. BLAST searches in GenBank indicated that the present fungus was grouped within *C. oryzae* with 99% identity. The present fungus most closely matched *C. oryzae* with accession numbers AF163083, EU272519 and JQ936251 (Fig. 4). The sequences of the amplified products were then deposited in the GenBank database and assigned accession number AB860213. This supports the identification by morphological observations. Therefore, the fungus that caused leaf spot disease on oil palm seedlings was characterized and identified as *C. oryzae*, based on both morphological and molecular properties.

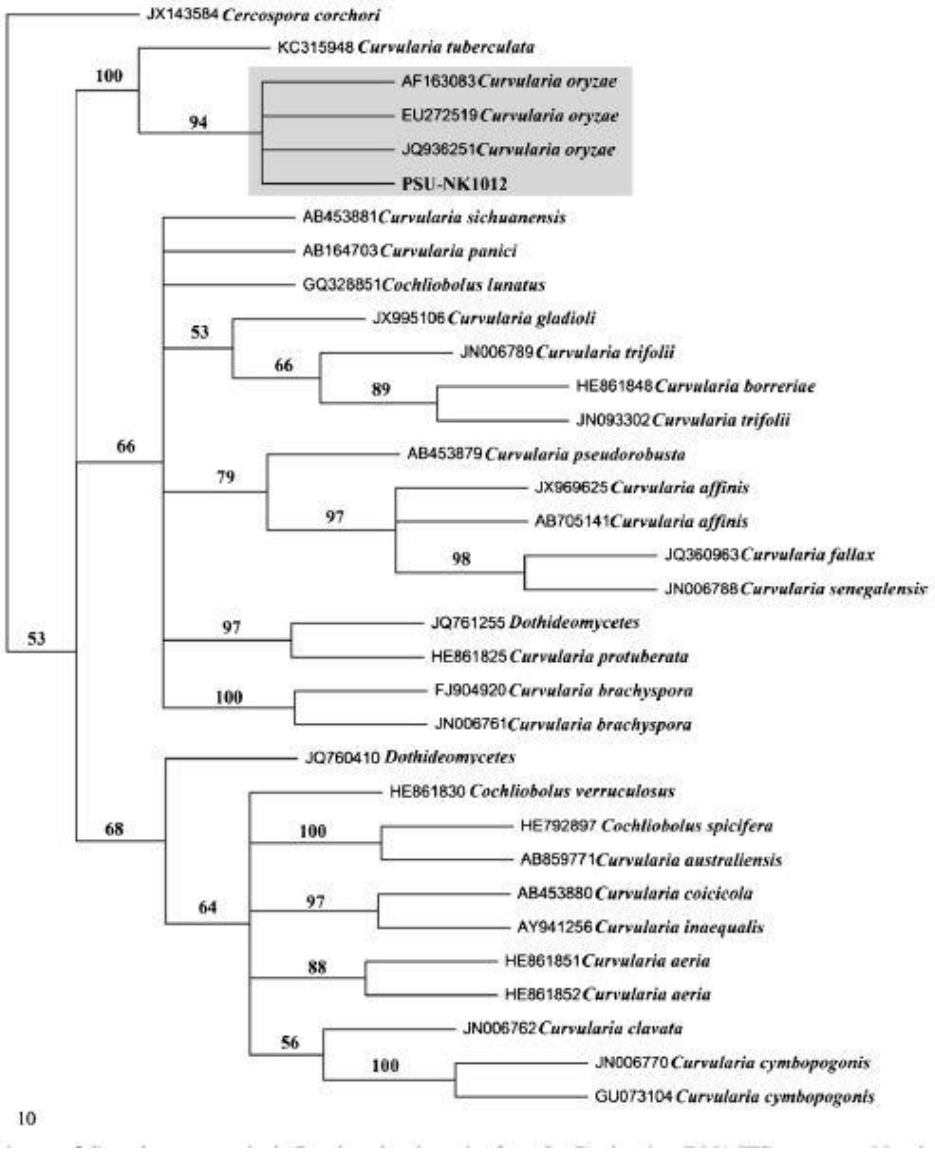

Fig. 4 Phylogenetic tree of *Curvularia oryzae* PSU-NK1012 including the related species from GenBank using rDNA ITS sequences. Numbers of the branches indicate the percentage of bootstrap value after 1,000 replications. *Cercospora corchori* illustrates the lack of similarity outside of this group.

Chapter II, Efficacy of chitosan in controlling leaf spot disease of oil palm seedlings stage

Low molecular weight (10253 kDa) chitosan was purchased from Aldrich, China. Purified chitosan was prepared as described by Benhamou et al. (1998). The stock solution (1%, w/v) of chitosan was prepared by dissolving purified chitosan in 0.5% (v/v) glacial acetic acid under

continuous stirring and the pH was adjusted to 5.6 using 1 N NaOH (Du et al., 1998). The chitosan solution was autoclaved (120°C, 20 min) prior to use in assays. Chitosan solution in five concentrations (0.125, 0.25, 0.5, 1 and 2 mg/ml) were tested for their inhibitory effect against *C. oryzae* PSU-NK1012 linear growth. All concentrations reduced the growth of *C. oryzae* PSU-NK1012 at 7 days post inoculation and the growth inhibition consistently increased with concentration (Fig. 5 & 6). Effective inhibition was obtained with 2 mg/ml (84.27 ± 1.78, linear growth reduction (Fig. 5f).

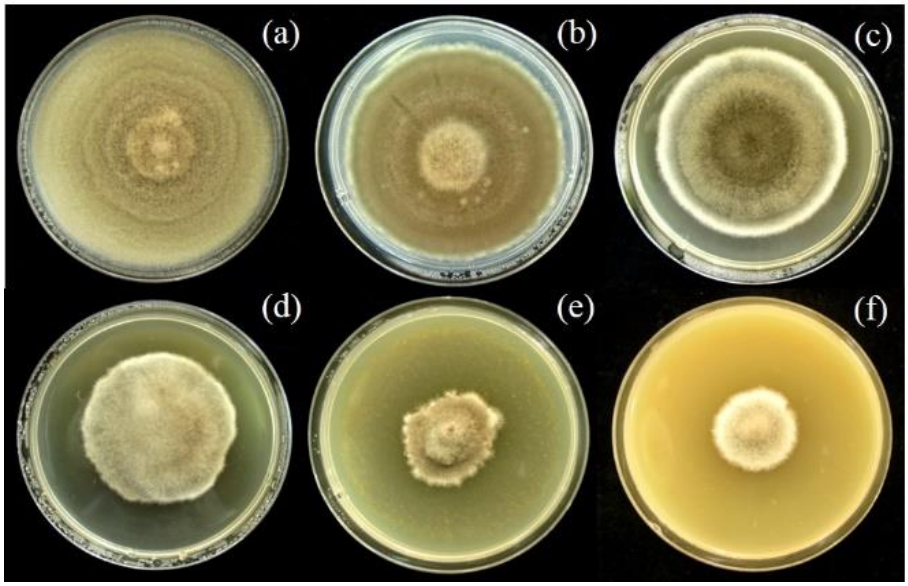

Fig. 5 Effect of chitosan concentration on radial growth of *Curvularia oryzae* PSU-NK1012, 0.05% acetic acid (a), 0.125 mg/ml (b), 0.25 mg/ml (c), 0.5 mg/ml (d), 1 mg/ml (e) and 2 mg/ml (f).

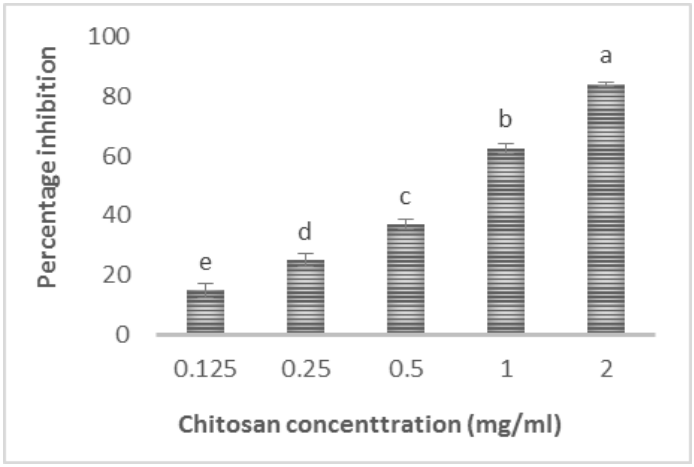

Fig. 6 Effect of chitosan on radial growth of *Curvularia oryzae* PSU-NK1012. Bars with the same letters are not significantly different from one another according to Turkey's test.

For *in vivo* test of chitosan, the leaves of oil palm seedling were sprayed with chitosan concentration 1, 2 and 3 mg/ml for 1 hr prior to inoculation of spore suspension of *C. oryzae* PSU-NK1012, spray with 0.05% acetic acid was served as control. The spot lesion on leaves demonstrated the germination of spore into plant tissues, therefore number of spot on oil palm leaves were measured. The results showed that number of spots were found in control reached maximum to about 150 spots, while treated with chitosan in all concentrations (1, 2 and 3 mg/ml) significantly reduced number of spot on oil palm seedling leaves (Fig. 6).

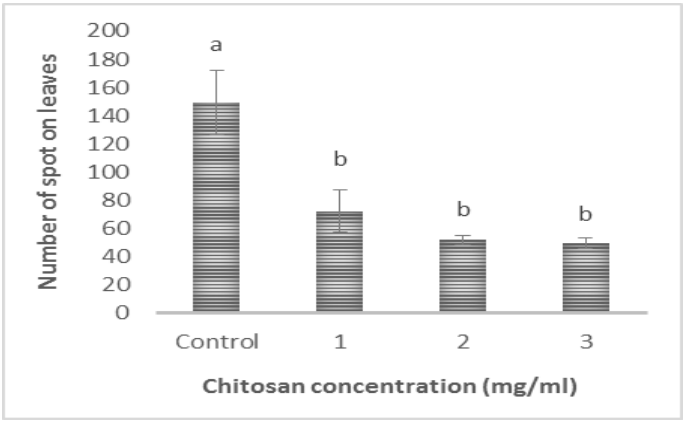

Fig. 6 Effect of chitosan on leaf spot symptom development by *Curvularia oryzae* PSU-NK1012 inoculation. Bars with the same letters are not significantly different from one another according to Turkey's test.

Plants have a broad range of defense mechanisms against pathogen infections. The responses to pathogen invasion include oxidative burst of cells, leading to programmed cell death, and synthesis of compounds like phytoalexin and pathogenesis-related proteins (PR). The PR proteins are the main ones conferring pathogen-specific resistance to the plant (Leiter *et al*., 2005; Adrienne & Barbara, 2006). To date, more than 17 different PR proteins are catalogued along with their properties and functions. Among these are proteins PR2 and PR3 with functions alike β-1,3-glucanase (β-glu) and chitinase, respectively, and they play an important role in many plant species against various pathogens. The protein β-glu in plants belongs to the PR2 family, and is an important component in the defense mechanism against pathogens. These proteins are hydrolytic enzymes that can cleave 1,3-β-D linkages in β-1,3-glucans, that are cell wall components in several pathogenic fungi (Simmons, 1994; Hoj & Fincher, 1995) and often the major component of cell wall.

Enzyme activities of β-1,3-glucanase were conducted on oil palm seedlings. Leaves without chitosan and inoculation were served as control. Oil palm leave treated with chitosan, chitosan prior to inoculation (chitosan + inoc) and inoculation with *C. oryzae* PSU-NK1012 spore suspension gave enzymes activity higher than those on control at 1 day after inoculation (Fig. 7). At the early infection stage treated with chitosan and fungal inoculation may resulted in β-1,3-glucanase production in oil palm seedling to defense themselves from the infection. The activities of β-1,3-glucanase has decreased in the *C. oryzae* PSU-NK1012 inoculation without chitosan at 5 days after inoculation (Fig. 8), this probably due to pathogen overcome host defense and showed the leaf spot symptom on oil palm leaves. For treatment with chitosan prior to inoculation with *C. oryzae* PSU-NK1012, they reduced the leaf spot symptom via release of β-1,3-glucanase for defense mechanism against fungal invasion.

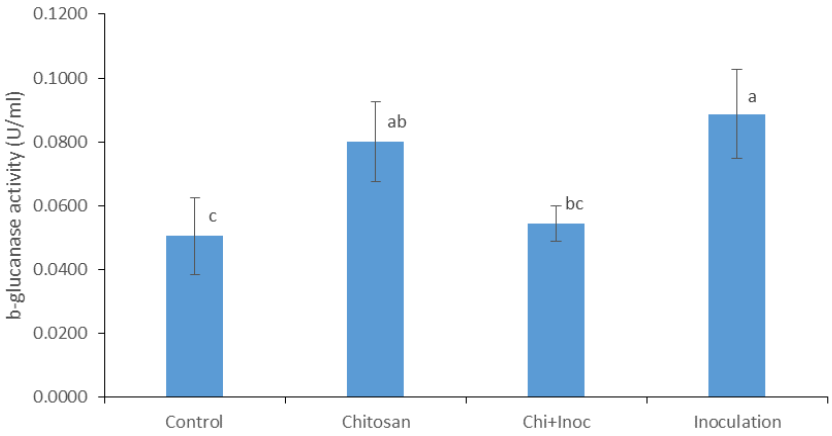

Fig.7 β-1,3-glucanase activities (U/ml) of oil palm seedlings inoculated with *C. oryzae* PSU-NK1012 at 1 day after inoculation. Bars with the same letters are not significantly different from one another according to Turkey's test.

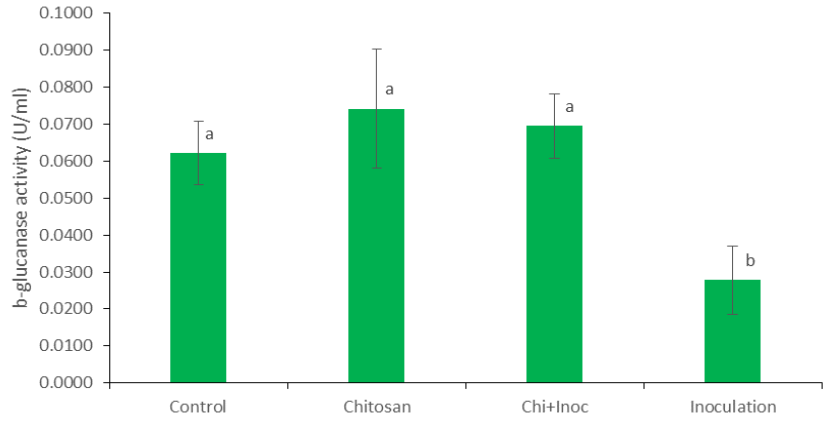

Fig.8 β-1,3-glucanase activities (U/ml) of oil palm seedlings inoculated with *C. oryzae* PSU-NK1012 at 5 days after inoculation. Bars with the same letters are not significantly different from one another according to Turkey's test.

Summary

The current study is the first to definitively associate *C. oryzae* with leaf spot disease of young oil palm seedlings in Thailand. Identifying the cause of disease in oil palm seedlings might help reduce its incidence, but further studies are needed to determine the incidence rate and evaluate options for controlling it by biological and chemical means. As a summary,

chitosan inhibits the growth of *C. oryzae*, and reduces disease severity in oil palm seedling leaves in early infection period, but not completely inhibits the disease on nursery stage.

References

Aderungboye, F.O. 1977. Disease of the oil palm. Trop. Pest Manage. 23: 305 – 326.

Adrienne, C.S. and Barbara, J.H. 2006. Parallels in fungal pathogenesis on plant and animal host: Eukaryotic Cell, 5(12): 1941-1949.

Allan, C.R. and Hadwiger, L.A. 1979. The fungicidal effect of chitosan on fungi of varying cell wall composition. Experimental Mycology, 3: 285-287

Benhamou, N. 1996. Elicitor-induced plant defense pathways, Trends. Plant Sciences, 1: 233-240.

Boonlertnirun, S., Suvannasara, R., Promsomboon, P. and Boonlertnirun, K. 2011. Application of chitosan for reducing chemical fertilizer uses in waxy corn growing. Thai Journal of Agricultural Science, 44: 22-28.

Dikin, A., Sijam, K., Zainal Abidin, M.A. and Seman, I.A. 2003. Biological control of seed borne pathogen of oil palm, Schizophyllum commune Fr. with antagonistic bacteria. International Journal of Agricultural Biology, 5: 507 – 512.

Du, J., Gemma, H., Iwahori, S. 1998. Effects of chitosan coating on the storability and on the ultrastructural changes of Jonagold apple fruit in storage. Food Preservation Science, 24: 23 – 29.

El-Sawy, N.M., El-Rehim, H.A.A., Elbarbary, A.M. and Hegazy, E.A. 2010. Radiation-induced degradation of chitosan for possible use as a growth promoter in agricultural purposes. Carbohydrate Polymer, 79: 555-562.

Flood, J. 2006. A review of Fusarium wilt of oil palm caused by *Fusarium oxysporum* F. sp. elaeidis. Phytopathology, 96: 660 – 662.

Hirano, A. and Nagao, N. 1989. Effects of chitosan, pectic acid, lysozyme and chitinase on the growth of several phytopathogens. Agricultural and Biological Chemistry, 53: 3065-3066.

Hoj, P.B. and Fincher, G.B. 1995. Molecular evolution of plant â-glucan endohydrolases. Plant Journal, 7: 367-369.

Kendra, D.F., Christian, D. and Hadwiger, L.A. 1989. Chitosan oligomers from *Fusarium solani*/pea interactions, chitinase/â-glucanase digestion ofsporelings and from fungal wall chitin actively inhibit fungal growth and enhance disease resistance. Physiological and Molecular Plant Pathology, 35: 215-230.

Laflamme, P., Benhamou, N., Bussiere, N. and Dessureault, M. 2000. Differential effect of chitosan on root rot fungal pathogens in forest nurseries. Canadian Journal of Botany, 77: 1460 – 1468.

Leiter, E., Szappanos, H. Oberparleiter, C., Kaiserer, L., Sernoch, L., Pusztahelyi, T., Emri, T., Po'csi, I, Salvenmoser, W. and Marx, F. 2005. Antifungal protein PAF severely affects the integrity of the plasma membrane of *Aspergillus nidulans* and induces and apoptosis-like phenotype. Antimicrobial Agents and Chemotherapy, 49(6): 2445-2453.

Lizarraga-Paulin, E. Torres-Pacheco, G., Moreno-Martinez, E. and Miranda-Castro, S.P. 2011. Chitosan application in maize (*Zea mays*) to counteract the effects of abiotic stress at seedling level. African Journal of Biotechnology, 10: 6439-6446.

Manamgoda, D. S., Cai, L., McKenzie, E. H. C., Crous, P. W., Madrid, H., Chukeatirote, E., et al. (2012). A phylogenetic and taxonomic re-evaluation of the Bipolaris-Cochilobolus-Curvularia complex. Fungal Divers, 56: 131–144.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31: 426-428

Neuveglise, C. Y., Brygoo, Y., & Riba, G. (1994). Comparative analysis of molecular and biological characteristics of strains of *Beauveria brongniartii* isolated from insects. Mycological Research, 98: 322–328.

Nge, K.L., New, N., Chandrkrachang, S. and Stevens, W.F. 2006. Chitosan as a growth stimulator in orchid tissue culture. Plant Sciences, 170: 1185-1190.

Pornsuriya, C., Sunpapao, A., Srihanant, N., Worapattamasri, K., Kittimorakul, J., Phithakkit S. and Petcharat, V. 2013. A survey of disease and disorders in oil palms of southern Thailand. Plant Pathology Journal 12: 169 – 175

Shehata, S.A., Fawzy, Z.F. and El-Ramady, H.R. 2012. Response of cucumber plants to foliar application of chitosan and yeast under greenhouse conditions. Australian Journal of Basic and Applied Sciences, 6: 63-71.

Simmons, C.R. 1994. The physiology and molecular biology of plant 1,3-â-D-glucanase and 1,3;14-β-D-glucanase. Crit. Rev. Plant Science, 13: 325-387.

Sivanesan, A. 1987. Graminicolous species of Bipolaris, Curvularia, Drechslera, Exserohilum and their teleomorphs. Wallingford, UK: CAB International.

Stossel, P. and Leuba, J.L. 1984. Effect of chitosan, chitin and some aminosugars on growth of various soilborne phytopathogenic fungi. Journal of Phytopathology, 111: 82-90.

Sunpapao, A. and Pornsuriya, C. 2014. Effects of chitosan treatments on para rubber leaf fall disease caused by *Phytophthora palmivora* Butler - a laboratory study. Songklanakarin Journal of Science Technology, 36(5): 507 – 512.

Sunpapao, A., Kittimorakul, J. and Pornsuriya, C. 2014. Disease Note: Identification of *Curvularia oryzae* as cause of leaf spot disease on oil palm seedlings in nurseries of Thailand. Phytoparasitica 42: 529 – 533. (ISI index, IF = 0.901)

Susanto, A., Sudharto, P.S. and Purba, R.Y. 2005. Enhancing biological control of basal stem rot disease (*Gaenoderma boninense*) in oil palm plantations. Mycopathologia, 159: 153 – 157.

Tengoua, F.F. and Bakoume, C. 2005. Basal stem rot and vascular wilt, two treats for the oil palm sector in Cameroon. Planter, 81: 97 – 105.

Turner, P.D. 1981. Oil Palm Diseases and Disorders. Oxford University Press, Oxford, pp: 88 – 110.

Output

Sunpapao, A., Kittimorakul, J. and Pornsuriya, C. 2014. Disease Note: Identification of *Curvularia oryzae* as cause of leaf spot disease on oil palm seedlings in nurseries of Thailand. Phytoparasitica 42: 529 – 533. (ISI index, IF = 0.901)

Kittimorakul, J., Pornsuriya, C., **Sunpapao, A.** and Petcharat, V. 2013. Survey and incidence of leaf blight and leaf spot diseases of oil palm seedling in southern Thailand. Plant Pathology Journal 12(3): 149 – 153. (AGRIS, CABI index)

Attachments

[1] **Sunpapao, A.,** Kittimorakul, J. and Pornsuriya, C. 2014. Disease Note: Identification of *Curvularia oryzae* as cause of leaf spot disease on oil palm seedlings in nurseries of Thailand. Phytoparasitica 42: 529 – 533. (ISI index, IF = 0.901)

[2] Kittimorakul, J., Pornsuriya, C., **Sunpapao, A.** and Petcharat, V. 2013. Survey and incidence of leaf blight and leaf spot diseases of oil palm seedling in southern Thailand. Plant Pathology Journal 12(3): 149 – 153. (AGRIS, CABI index)

[3] Pornsuriya, C., **Sunpapao, A.,** Srihanant, N., Worapattamasri, K., Kittimorakul, J., Phithakkit S. and Petcharat, V. 2013. A survey of disease and disorders in oil palms of southern Thailand. Plant Pathology Journal 12: 169 – 175. (AGRIS, CABI index)