Graduate Studies

Faculty of Agricultural Technology, King Mongkut's Institute Technology Ladkrabang, Bangkok, Thailand

PROGRESS REPORT

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The research project: *In vitro* test for evaluation the potent *Chaetomium spp.* as the antagonists and their bioactive substances to control *phytophthora sp.* a pathogen causing root rot on pomelo

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สำนักหอสมุดกลาง พระจอมเกล้าลาดกระบัง

Abstract

Phytophthora diseases have become a major impediment in the citrus production in Thailand. In this study, an isolate of *Phytophthora* denominated as PHY02 was proven to be causal pathogen of root rot of Pomelo (*Citrus maxima*) in Thailand. The isolate PHY02 was morphologically characterized and identified as *Phytophthora palmivora* based on molecular analysis of an internal transcribed spacer rDNA sequence. This work also presents *in vitro* evaluations of the capacities of *Chaetomium* spp. to control the *P. palmivora* PHY02. As antagonists, *Chaetomium globosum* CG05, *Chaetomium cupreum* CC3003, *Chaetomium lucknowense* CL01 inhibited 50 – 61% mycelial growth, degraded mycelia and reduced 92 – 99% sporangial production of *P. palmivora* PHY02 in bi-culture test after 30 days. Fungal metabolites from *Chaetomium* spp. were tested against PHY02. Results showed that, methanol extract of *C. globosum* CG05 expressed strongest inhibitory effects on mycelial growth and sporangium formation of *P. palmivora* PHY02 with effective dose ED50 values of 26.5 µg/mL and 2.3 µg/mL, respectively. It is interesting that *C. lucknowense* is reported for the first time as an effective antagonist against a species of *Phytophthora*.

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Introduction

Citrus are important fruit crops, being grown widely and commercially in Southeast Asia. However, under prevailing wet climatic conditions, *Phytophthora* is a major impediment in the citrus production and causes annual loss of about 6 – 12% of yield in Thailand alone [1].

Phytophthora palmivora (Butl.) is a ubiquitous and prominent plant pathogen with a wide host range, which infects various important crops in Southeast Asia [1, 2]. On citrus, this fungal-like organism has been reported to infect almost every part of the plant at any stage of its growth [3]. The spread and pathogencity were reported to be faster and more aggressive to roots of citrus than *P. parasitica* and *P. citrophthora* [4, 5]. Since *Phytophthora* and other oomycetous organisms have different biochemical pathways compared to the true fungi, they are insensitive to many fungicides [6]. In addition, the resistance of *Phytophthora* species to an important group of fungicides such as phenylamides (metalaxyl and related compounds) has become a serious problem in their chemical control [2]. In order to develop eco-friendly management of *Phytophthora* diseases and to reduce the costly applications and harm of fungicides, screening of bio-control agents against Citrus *Phytophthora* has become a vital research aspect and carried out all over the world [3].

Chaetomium Kunze is a large genus of saprophytic ascomycetes with more than 350 species [7]. Relying on lytic enzymes, they decompose cellulose and other organic material [8, 9]. Some *Chaetomium* species are reported to act as antagonists against various plant pathogens. Even commercial bio-product has also been developed from potent strains of *Chaetomium* spp. [10]. Furthermore, over 200 metabolites with a wide range of bioactivities have been found from the genus

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Root inoculation was done using the infested soil method of Ziko *et al.* [4]. Six-month old pomelo seedlings were thoroughly washed to be free of potting mix and then planted in plastic tubes (10 x 15 cm) containing sterilized clay soil-sand (1:1) with 5 chlamydospores of PHY02 per cubic centimeter. Controls were prepared by planting the seedlings in same size tubes, but containing sterilized clay soil-sand (1:1) only. All pots including the controls were maintained in the green house at temperature of about 25 – 30^0 C and flooded with water for 24 hr each week. After 6 wk, the plants were carefully removed from plastic tubes, the soil was washed out and rating was done based on degree of the root rot symptoms. The pathogen then was re-isolated from newly infected roots symptom and morphology characteristics were compared with the isolate PHY02.

Fungal isolates

C. globosum strain CG05, *C. cupreum* strain CC3003 and *C. lucknowense* strain CL01 (from collection of Dr. Soytong K., KMITL, Thailand) were used as antagonists and produced antagonistic substances. The isolate PHY02 was represented as the target control in this study.

Bi-culture test

A mycelial disc of PHY02 (5 mm diameter) was placed singly (as controls) or oppositely to a mycelial disc of each above antagonist on 9 cm – diameter Petri dishes, which contained PDA. After incubation at 25^0C for 30 days, data were collected as colony diameter and number of sporangia produced by PHY02 in both bi-culture and control plates. Numbers of sporangia were counted by using haemacytometer. Data were computed in a form of inhibition percentage of mycelial growth and sporangial production of the pathogen by using the formula below:

% inhibition = 100 x (A-B)/A.

Where: A = colony diameter or numbers of sporangia of PHY02 in control plates; B = colony diameter or numbers of sporangia of PHY02 in bi-culture plates.

Finally, variance and the treatment means were analyzed and compared by using Duncan's Multiple Range Test (DMRT) at 0.05.

***In vitro* test of crude extracts from *Chaetomium* spp. to inhibit *P. palmivora* PHY02**

C. globosum CG05, *C. lucknowense* CL01 and *C. cupreum* CC3003 were separately cultured in potato dextrose broth (500 plates per antagonist) at room temperature in 45 days. Fungal biomass of each antagonist was separately collected as fresh biomass, then was dried out at room temperature. Subsequently, the extraction of dried biomass from each antagonist was performed by the method described by Kanokmedhakul *et al* [13]. Each dried biomass was ground and extracted with hexane

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(1:1 v/v) and incubated by shaking for 72 hrs at room temperature. The filtrate was separated out the marc by filtration through filter paper (Whatman No.4). The hexane filtrate was performed through rotary vacuum evaporator to yield crude hexane extract. The marc from hexane extraction was further extracted with ethyl acetate and followed with methanol using the same procedure as hexane to yield crude ethyl acetate and methanol extract.

The three different crude extracts of each antagonist were conducted in a factorial experiment to test for inhibition of mycelial growth and sporangium formation of *P. palmivora* PHY02 by using poisonous food method. A mycelial disc (5 mm in diameter) of PHY02 placed on PDA plates (5 cm in diameter), which contained different concentrations of each crude extract (0, 10, 50, 100, 500 and 1,000 µg/mL). To obtain the desired crude extract concentrations, stock crude extract was weighted then dissolved in 2% dimethyl sulfoxide (DMSO) and added to molten PDA before autoclaving at 121°C (15 psi) for 20 minutes. After incubating at 25°C for 10 days, colony diameter and numbers of sporangia production of the pathogen were collected and then expressed as inhibition percentage using the same formula above. Effective dose ED50 values on mycelial growth and sporangial production were calculated by probit analysis using the software SPSS Statistics ver.19.0 (IBM Co., Armonk, NY, USA).

RESULTS

Pathogenicity of *Phytophthora* PHY02

After 6 weeks of inoculation with chlamydospores of PHY02 on roots, the pomelo seedlings had an average of 47.6% root tips rotted and produced very few new roots (data not shown). Cortex of feeder roots turned soft and was sloughed to leave only steles, which were similar to symptoms of root rot in the affected orchards in Chang chen Sao – Thailand (Fig.1). Meanwhile, non-inoculated seedlings produced abundantly new roots and no symptom of rot was observed. A single species of *Phytophthora* was re-isolated from disease symptoms on newly infected roots, which all had identical morphology characteristics with the isolated PHY02. As a result, PHY02 was proven to be the causal pathogen of the pomelo root rot in Chang chen Sao – Thailand.

Characteristics and identification of *Phytophthora* PHY02

Colonies of PHY02 showed stellate pattern, with aerial mycelia on V8A and PDA whereas nearly no aerial mycelium on CMA (Fig.2). The isolate PHY02 produced lumpy-branching hyphae with hyphal swellings. Zoospores were directly released from sporangia when flooded in distilled water. Sporangia produced abundantly when grown on PDA and V8A after 3 – 5 days, occurred in

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groups on sympodium or irregularly, were papillate and caducous with short pedicels up to 6 μm (mean 3.3) long. Sporangial shape varied from ellipsoid, ovoid, pyriform, obpyriform to near spherical, and mean of sizes were 53.8 x 33.3 μm with a length to breadth ratio of 1.2 – 2.2 (mean 1.6) (Table 1). Most of chlamydospores were globose in shape, produced abundantly from mycelia when incubated in dark. No sexual organ was observed in cultures of this isolate since it was a heterothallic species.

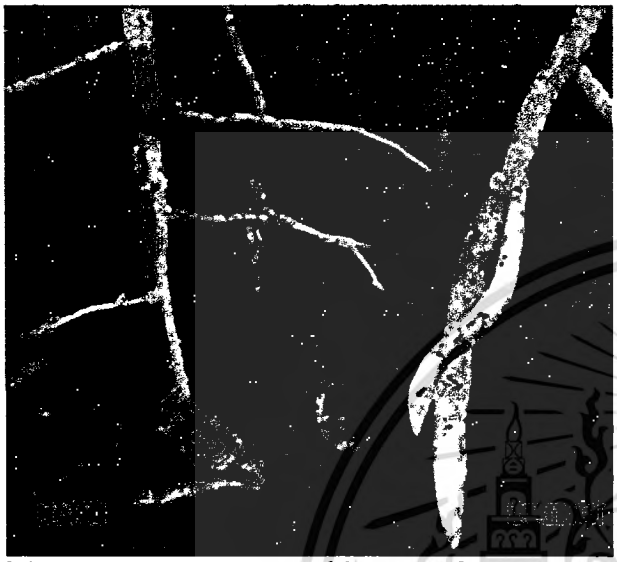

Fig. 1. Symptoms caused by *P. palmivora* PHY02 on root of pomelo in pathogenicty test (INO: inoculated; N-INO: non-inoculated).

Table 1. Characteristics of *P. palmivora* PHY02 from *citrus maxima*

Structure	Mean	Max	Min
Sporangia[1]			
Length (μm)	53.8	76.7	34.2
Breadth (μm)	33.3	45.6	23.6
L/B (μm)	1.6	2.2	1.2
Pedicel length(μm)	3.2	5.6	0.9
Papilla length (μm)	6.4	9.8	3.1
Chlamydospore[2] *diameter* (μm)	36.9	54.7	25.3

[1]Data collected from 200 separate sporangia
[2]Data collected from 150 separate chlamydospores

The sequence with 951 bases of the ITS rDNA PCR product of PHY02 was determined and used as a query to search the Genbank DNA database of the PhytophthoraDatabase using the BLAST search. It was found that there was 99.75% (809/811), 99.87% (782/783) to identity with *Phytophthora palmivora* accession number PD_00627 and PD_02505, respectively. The phylogenetic tree, which illustrated relationships between PHY02 and related taxa was constructed as shown in Fig. 3. The isolate PHY02 was identified as *Phytophthora palmivora* (Butl.), based on its morphology and the molecular analysis.

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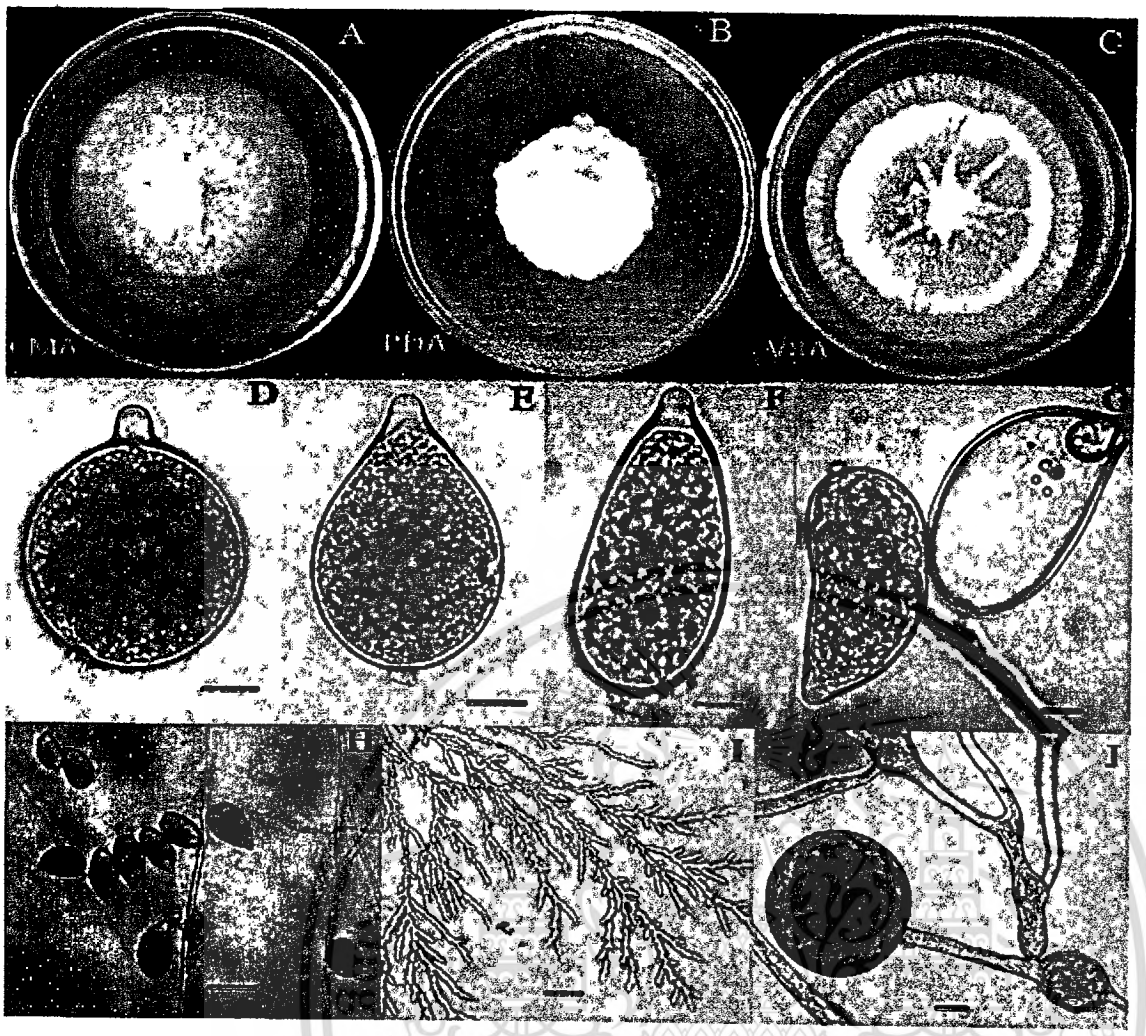

Fig. 2. Morphological characteristics of *P. palmivora* PHY02: A-C, colony types of PHY02 at 7 days grown on different media; D - F, different shapes of zoosporangia; G, zoospore release directly from sporangium; H, occurrence of sporangia on sympodium; I, lumpy-branching mycelia; J, chlamydospore and swelling hyphae (scale bars: H and I = 50 μm, the others = 10 μm).

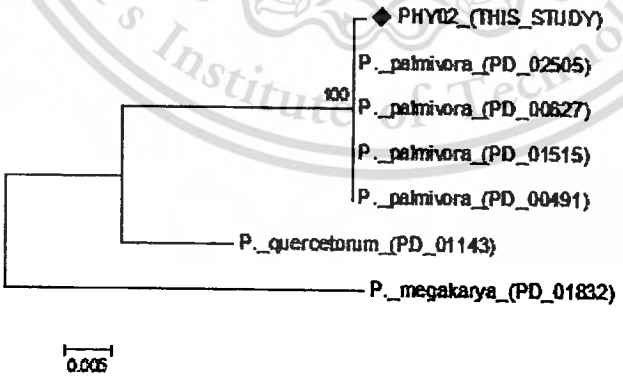

Fig. 3. Phylogenetic relationship between *P. palmivora* PHY02 and related taxa inferred using a neighbor-joining method with internal transcribed spacer (ITS) rDNA sequences. Bootstrap value based on 1,000 replications is shown above the branch.

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Bi-culture test

As shown in Table 2, the tested antagonists led to 50 – 61% growth inhibition and reduced 92 – 99% sporangial production of *P. palmivora* PHY02 in bi-culture plates at 30 days, when compared to the controls. Moreover, CG05 and CL01 were seen to grow rapidly over PHY02 colony after 10 – 15 days. In all bi-culture plates, both antagonists degraded mycelia of the pathogen, resulting in change of color from white to light yellow-brown and a part or entire colony death (Fig. 4). Differently, *C. cupreum* CC3003 was a slow growing fungus, as it grew over gradually and resulting in degradation of colony of PHY02 in some cases. Mycelia of all the tested antagonists were observed to penetrate mycelia of PHY02 in some cases. In both inhibitions of mycelial growth and sporangial production, CC3003 was significantly less efficient than CG05 and CL01.

Table 2. Inhibition of mycelial growth and sporagial production of *P. palmivora* PHY02 in bi-culture test with *Chaetomium* spp.

Treatments	Colony inhibition (%)	Sporagium inhibition (%)
CG05 vs PHY02	61. 0 ± 1.0 a[1]	99.0 ± 0.4 a
CL01 vs PHY02	59.2 ± 1.2 a	97.5 ± 0.2 a
CC3003 vs PHY02	49.7 ± 1.2 b	92.2 ± 0.9 b

[1]Average of four replications, the same letter represents no significant difference between treatments, based on the Duncan's multiple range test at $p = 0.05$

***In –vitro* tests of crude extracts from *Chaetiomium spp.* to inhibit *P. palmivora* PHY02**

Total nine crude extracts from CG05, CL01 and CC3003 were tested at different concentrations to evaluate their capacities to inhibit mycelial growth and sporangium formation of *P. palmivora* (PHY02). The effective doses (ED_{50}) of each crude extract on mycelial growth and sporangial production were also examined to determine their fungicidal spectrum.

As shown in Table 3, the three crude extract of CG05 exhibited more antifungal activities against mycelial growth of PHY02 as compared to crude extracts of CL01 and CC3003. Particularly, the tested pathogen did not grow at all in the presence of higher 500 μg/mL methanol extract and 1,000

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μg/mL ethyl acetate extract of CG05. At tested concentrations from 10 – 500 μg/mL, the methanol extract was more effective than the others (Fig. 4 and Fig. 5).

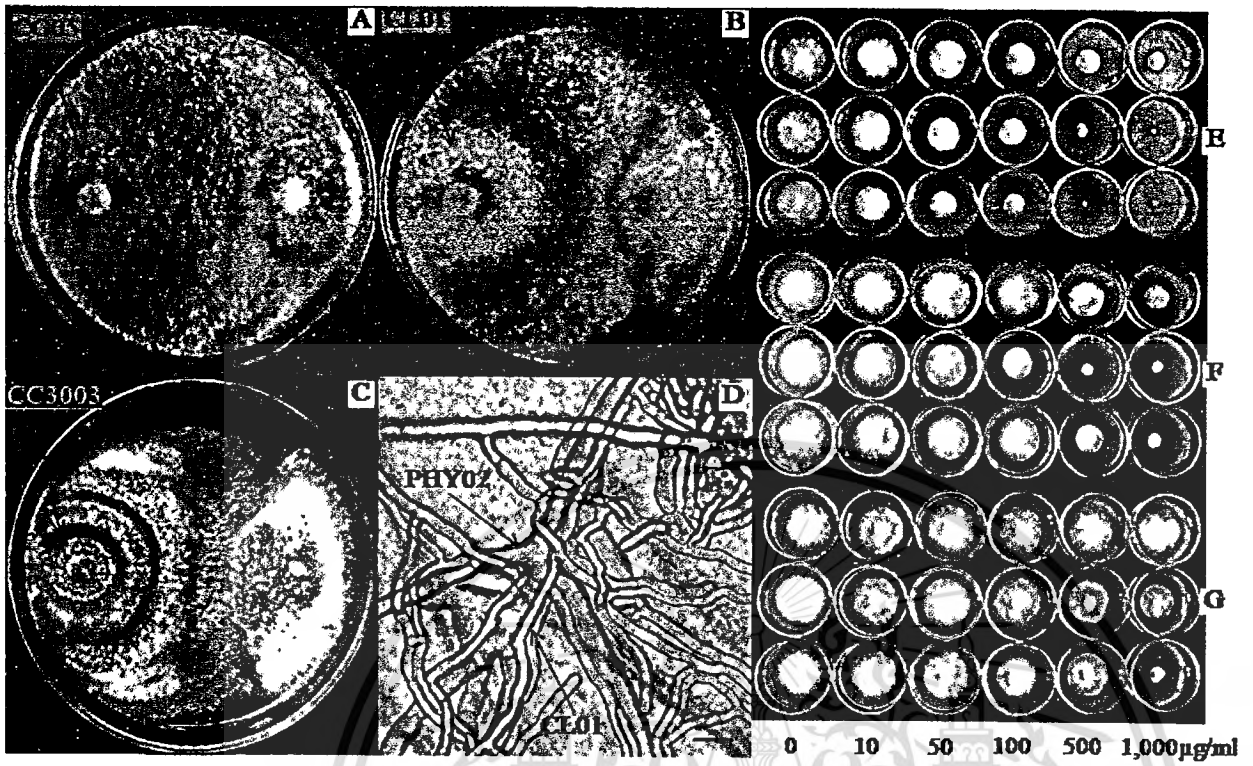

Fig. 4. Growth of *P. palmivora* PHY02 in bi-culture tests (at 30 days) and in crude extracts (at 10 days) of *Chaetomium* spp.: A-C, PHY02 and *Chaetomium* spp in bi-culture plates; D, interaction between mycelia of CL01 and PHY02 in bi-culture test (scale bar = 10 μm); E, F, G, growth of PHY02 at different concentrations of crude extracts of CG05, CL01 and CC3003, respectively (Upper row: hexane extracts; middle row: ethyl acetate extracts; lower row: methanol extracts).

Conversely, all crude extracts of CC3003 were less effective on mycelial growth of PHY02 with higher ED_{50} values (596.8 - 2,495 μg/mL). The methanol extract of CC3003 was more effective than the hexane and ethyl acetate at concentrations from 100 – 1,000 μg/mL. However, none of them showed significant inhibition of mycelial growth of PHY02 at 10 μg/mL. Whereas, mycelial growth of PHY02 was significantly inhibited by all crude extracts of CL01 at 10 μg/mL. Of which, the ethyl acetate extract was more significantly effective than the others at concentrations from 100 – 1,000 μg/mL, with ED_{50} value of 77.03 μg/mL and exhibited 83.5% growth inhibition of PHY02 at 1,000 μg/mL. Meanwhile, ED_{50} values of the methanol, hexane extract were 143.9 and 919.1 μg/mL, respectively. Unlike in bi-culture test, mycelia of PHY02 were not degraded by crude extracts of the antagonistic fungi.

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All the tested crude extracts exhibited stronger inhibitory effects on sporangium formation than on mycelial growth of PHY02 with much lower ED_{50} values. The sporangium formation of PHY02 was most sensitive to crude extracts of CG05 with ED_{50} values of as 5.1, 3.0 and 2.3μg/mL for the hexane, ethyl acetate and methanol extract, respectively.

Meanwhile, all three crude extracts of CC3003 were least effective on sporangial production of PHY02 with higher ED_{50} values. Of which, the methanol extract was more significantly effective than the others with ED_{50} value of 93.6 μg/mL, and gave an inhibitory rate of 96.5% at 1000 μg/mL. The hexane and ethyl acetate extract gave the highest ED_{50} values among nine tested crudes, which were 307.9 and 145 μg/mL, respectively.

Table 3. Effective dose values (ED50) of different crude extracts of *Chaetomium* spp. on mycelial growth and sporangial production of *P. palmivora* PHY02

Crude extracts		ED50 (μg/mL) on mycelial growth	ED50 (μg/mL) on sporangial production
CG05	Hexane	85.6	5.1
	EtOAc	33.4	3.0
	MeOH	26.5	2.3
CL01	Hexane	919.0	16.7
	EtOAc	77.0	3.5
	MeOH	143.9	4.0
CC3003	Hexane	>2,495	307.9
	EtOAc	>1,730	145.7
	MeOH	596.8	93.6

Hexane: Hexane extract; EtOAc: Ethyl acetate extract; MeOH: Methanol extract

To reduce 50% sporangial production of PHY02, the hexane, ethyl acetate and methanol extract of CL01 required concentrations of 16.7, 3.5 and 4.0 μg/mL, respectively. However, none of them could reduce 90% sporangial production of PHY02 at 50 μg/mL. At concentrations from 10 – 500 μg/mL, the ethyl acetate extract of CL01 was more significantly effective than the others, showing an inhibitory rate of 94.0% at 100 μg/mL. While at concentration of 500 μg/mL, the hexane and methanol extract reduced 82.3 and 93.8% sporangial production of PHY02, respectively.

All the hexane extracts of tested antagonists were less effective than the ethyl acetate and methanol in both inhibitions of mycelia growth and sporangium formation of *P. palmivora* PHY02.

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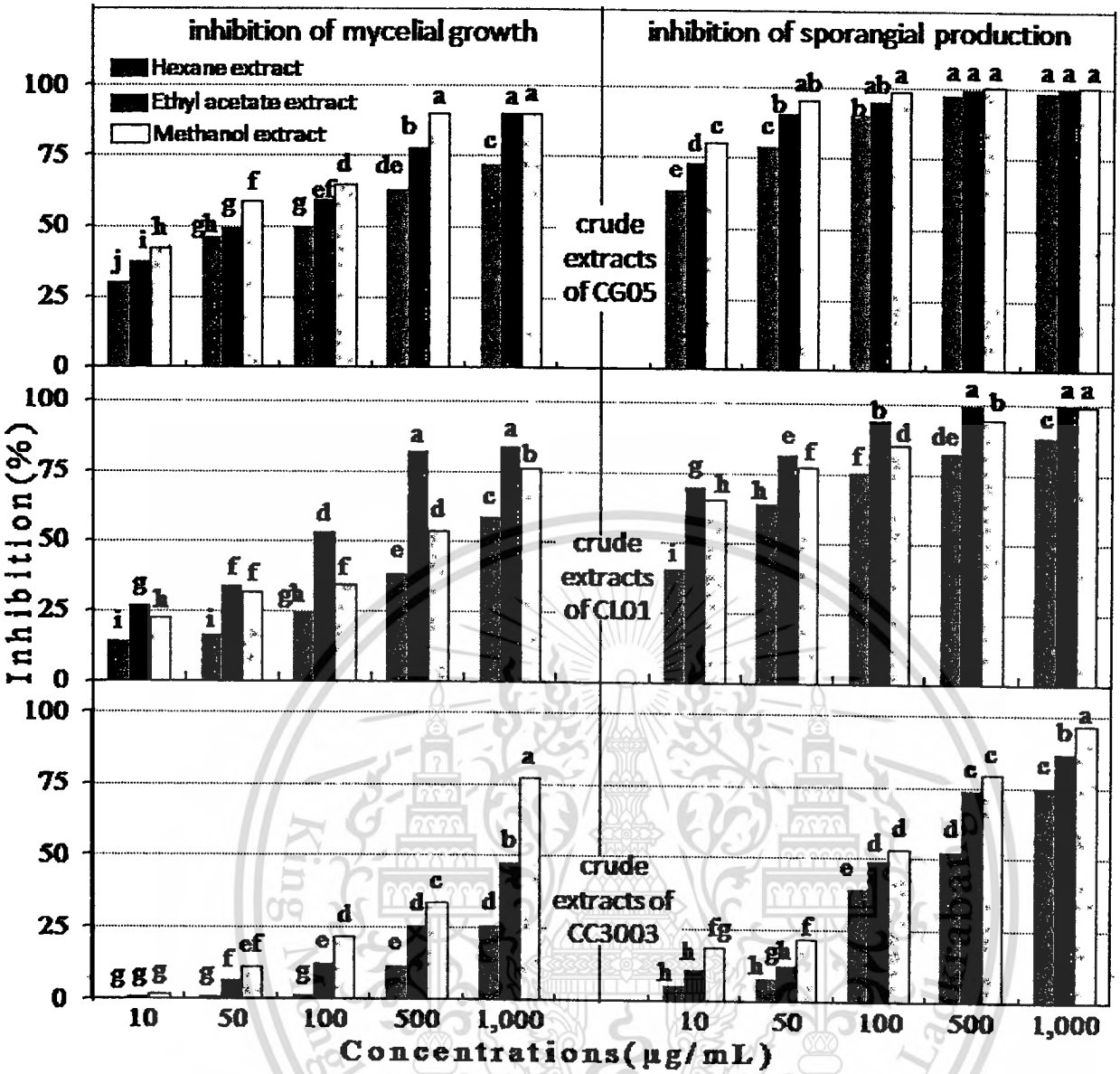

Fig. 5. Inhibitory effects of crude extracts of *Chaetomium spp.* on mycelial growth (the charts on the left site) and sporangial production (the charts on the right site) of *P. palmivora* PHY02 at different concentrations. The same letter above columns in each chart represents no significant difference between treatments, based on the Duncan's multiple range test at $p = 0.01$.

DISCUSSION

We have designated the species isolated from root rot symptoms of pomelo (*Citrus maxima*) in Thailand as *Phytophthora palmivora* (Butl.). The highly pathogenic of *P. palmivora* PHY02 to roots of pomelo here is supported by previous studies which demonstrated that this species is more aggressive and damages even larger root than *P. parasitica* on citrus [4, 5]. Serious root rot disease of citrus caused by *P. palmivora* has been recorded in India, America [3, 14].

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The antagonistic activity of bio-control microorganisms is often demonstrated by the inhibition of growth, infection or reproduction of pathogen [15]. The tested strains of *Chaetomium* spp. as antagonists here not only inhibited colony growth and degraded mycelia but also reduced 92 – 99% sporangial production of *P. palmivora* PHY02. The degradation of mycelia of tested pathogen probably resulted from the lytic enzymes which are commonly secreted by *Chaetomium* species [8, 9]. On other hand, every tested antagonist in this study was demonstrated to produce antibiotics exhibiting antifungal activities against different plant pathogens [10, 13, 16]. It refers that the inhibitions of *P. palmivora* here was resulted from both antibiotics and lytic enzymes secreted by the antagonists. *C. globosum* and *C. cupreum* have ever been reported to control *Phytophthora* spp. in dual culture test [17, 18]. *C. lucknowense* was concluded to control *Fusarium oxysporum* causing tomato wilt [16]. However, to best our knowledge, this is the first report of *C. lucknowense* as an effective antagonist against a species of *Phytophthora*.

Chaetomium species are known as producers of many different bioactive metabolites which play important roles in their biological control activities [7, 9]. One *Chaetomium* species may produce many metabolites with different bioactivities and molecular weight, this lead to differences in antifungal activity of its crude extracts [16, 19]. That explains why there were variations in responses of *P. palmivora* PHY02 to different crude extracts in this study. The less efficiency of hexane extracts of *Chaetomium spp.* in comparison with the ethyl acetate and methanol against fungal pathogens were also confirmed by previous authors [16, 20].

C. globosum CG05 has been known to produce many bioactive compounds such chaetoglobosin (A, G, C, V… *etc.*) and chaetoviridins (A, B) [10]. Of which, the chaetoglobosin C is usually referred as antifungal principle of this strain and was reported to inhibit colony growth, sporangium formation of *P. parasitisca* and other fungal pathogens [10, 19]. However, the chaetoviridins A produced from *C. globosum* F0142 also has strong antifungal activities against *P. infestans, P. capsici* [21]. Thus, it suggests that chaetoglobosin C and also other bioactive compounds involved in crude extracts of *C. globosum* CG05 caused the high inhibitions of *P. palmivora* PHY02 in this study. *C. cupreum* CC3003 has been known to produce compound rubrorotiorin which inhibited growth of *Candida albicans* at low concentration (EC_{50} = 0.6 μg/mL) [13]. However, crude extracts of *C. cupreum* CC3003 here showed least inhibitory effects on *P. palmivora* with high ED_{50} values. Similarly, crude extracts of *C. cupreum* have been known to be less effective against *Pythium aphanidermatum* [20]. Both the crude extracts and chaetoglobosin C produced by *C. lucknowense* CL01 have been reported to inhibit colony growth and spore formation of *Fusarium oxysporum* causing tomato wilt [16].

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However, this is the first time the inhibitory effects of metabolites from *C. lucknowense* on a species of *Phytophthora* are reported.

Sporangium formation is the most sensitive stage in life cycle of *Phytophthora* species [2]. This study demonstrated that sporangium formation of *P. palmivora* PHY02 was much more sensitive than mycelial growth to all tested crude extracts. The high inhibitory effects of crude extracts from *Chaetomium* spp. on spore formation of *Fusarium oxysporum* and *P. parasitisca* have been noted by previous authors [16, 19].

It is clear that only metabolites, which were not decomposed through the extraction and autoclaving of the crude extracts resulted in the inhibitions of *P. palmivora* PHY02. All enzymes and many antibiotics will be decomposed because of heat and pressure when autoclaving [2]. That why mycelia of the pathogen were not degraded when growing in crude extracts. Therefore, the antifungal activities of crude extracts here probably did not account for all bio-control activities of the tested antagonists against *P. palmivora* PHY02. Beside the known metabolites, degrading enzymes should be considered as effective factors involved in bio-control activities of the tested antagonists against the pathogen. The roles and secretion of lytic enzymes of *Chaetomium* species under antagonism conditions with *Phytophthora* need to be investigated in the future. The effective crude extracts may possible develop to be microbial elicitors to induce immunity in citrus plants against *P. palmivora*.

ACKNOWLEDGMENTS

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Record 1 of 1

Title: Biological Control of Phytophthora palmivora Causing Root Rot of Pomelo Using Chaetomium spp.

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Abstract: Phytophthora diseases have become a major impediment in the citrus production in Thailand. In this study, an isolate of Phytophthora denominated as PHY02 was proven to be causal pathogen of root rot of Pomelo (Citrus maxima) in Thailand. The isolate PHY02 was morphologically characterized and identified as Phytophthora palmivora based on molecular analysis of an internal transcribed spacer rDNA sequence. This work also presents in vitro evaluations of the capacities of Chaetomium spp. to control the P. palmivora PHY02. As antagonists, Chaetomium globosum CG05, Chaetomium cupreum CC3003, Chaetomium lucknowense CL01 inhibited 50 similar to 61% mycelial growth, degraded mycelia and reduced 92 similar to 99% sporangial production of P. palmivora PHY02 in bi-culture test after 30 days. Fungal metabolites from Chaetomium spp. were tested against PHY02. Results showed that, methanol extract of C. globosum CG05 expressed strongest inhibitory effects on mycelial growth and sporangium formation of P. palmivora PHY02 with effective dose ED50 values of 26.5 mu g/mL and 2.3 mu g/mL, respectively. It is interesting that C. lucknowense is reported for the first time as an effective antagonist against a species of Phytophthora.

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