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Efficacy of antifungal metabolites from some antagonistic fungi against *Pythium aphanidermatum*

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Antifungal metabolites from *Chaetomium aureum* MB601, *C bostrychodes* PR101, *C. cochliodes* RY301, *C. cupreum* NB201, *C. cupreum* RY202, *Gliocladium catenulatum* RY102, *G. catenulatum* RY111, *Trichoderma harzianum* RY 101, *T. harzianum* RY 104 and *T. harzianum* RY 112 extracted by hexane, EtOAc and MeOH were tested for inhibition of *Pythium aphanidermatum* RY803 causing pineapple root rot. Crude EtOAc extract of *C. cochliodes* strain RY301 showed the highest antifungal activity against *P. aphanidermatum* RY803. Treatment with crude EtOAc extract of *C. cochliodes* strain RY301 at 1,000 µg/ml inhibited mycelial growth and oospore formation against *P. aphanidermatum* RY803 by 71.00 and 88.95%, respectively. It also inhibited oospore formation with effective dose (ED_{50}) value of 64 µg/ml. Moreover, *P. aphanidermatum* RY803 on PDA added with crude EtOAc extract of *C. cochliodes* RY301 showed abnormal features of hyphae, oogonia and oospores. It was implied that the antagonistic mechanism of *C. cochliodes* RY301 was lysis and antibiosis.

Key words: antifungal metabolites, *Chaetomium cochliodes*, *Pythium aphanidermatum*

Introduction

Pineapple (*Ananas comosus* Merr.) is one of the most important economic plants in Thailand. It is grown mainly for fresh, canned fruits and juice, and is the only source of bromelain, an enzyme used in pharmaceuticals. The growth area of pineapple in Thailand has been expanded because the increased worldwide demand for pineapple products has greatly stimulated

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plantings. Root rot symptoms are commonly found in the fields and become major losses in pineapple plantations. The disease caused by *Pythium aphanidermatum* (Edson) Fitzp. It is a ubiquitous phytopathogen with a wide host range and an aggressive species of *Pythium*, causing serious root rot and damping-off in various economic plant crops (Parker, 2010).

The sustainable agricultural practices rely on the integration of biotechnology with traditional agricultural practices (Haggag and Mohamed, 2007). Most sustainable and environmentally acceptable control may be used biocontrol agents for reducing the use of agricultural chemicals and their residues in the environment. Biological control using antagonists to control root rot of several plants caused by pathogenic fungi has been extensively studied, and several examples of successful disease control exist. The used antagonistic fungi for controlling root rot have been reported such as *Aspergillus* spp., *Chaetomium cupreum*, *C. globosum*, *C. cochliodes*, *Gliocadium virens*, *Penicillium* spp., *Trichoderma harzianum*, *Trichoderma viride* (Ezziyyani *et al*., 2007; Abdelzaher, 2003; Soytong *et al*., 2001; Galland and Paul, 2001; Naseby *et al*., 2000; Ahmed *et al*., 1999).

Biological control of plant diseases by using antagonistic microorganisms involved in mechanisms as antibiosis, competition suppression, direct parasitism, induced resistance, hypovirulence and predation (Haggag and Mohamed, 2007). The antagonistic activity against plant pathogens has often been associated with secondary metabolite production (Aggarwal *et al*., 2003; Park *et al*., 2005; Eziashi *et al*., 2006; Kishore *et al*., 2007) This paper attempted to screen for promising antagonists producing antifungal metabolites and investigated efficacy of antifungal metabolites in inhibiting mycelial growth and oospore formation of *P. aphanidermatum* RY803.

Materials and methods

Promising antagonistic fungi and pathogen

Ten promising antagonistic fungi *Chaetomium aureum* MB601, *C bostrychodes* PR101, *C. cochliodes* RY301, *C. cupreum* NB201, *C. cupreum* RY202, *Gliocladium catenulatum* RY102, *G. catenulatum* RY111, *Trichoderma harzianum* RY 101, *T. harzianum* RY 104 and *T. harzianum* RY 112 were isolated from soil under pineapple plantation in Phatthalung and Rayong provinces. Fungal pathogen *Pythium aphanidermatum* RY803 used in this study was isolated from rhizosphere soil of pineapples showing root rot symptom. The isolate was proved to be the highest virulence causing root rot of pineapple according to previous report (Pornsuriya *et al*., 2009). All promising

antagonistic fungi were tested to be potential antagonists against *P. aphanidermatum* RY803 by bi-culture plate method. Cultures were maintained on potato dextrose agar slants.

Fungal growth and extraction of crude extracts

The mycelial plugs of each promising antagonist was transferred into potato dextrose broth (PDB) and incubated in static state at room temperature (28-30°C) for 4 weeks. Fungal mycelia were removed from liquid by cheesecloth filtration and dried over night at 28-32°C. Subsequently, the extraction of each promising antagonist was performed by the method described by Kanokmedhakul *et al*. (2006). Each air-dried mycelial mat was ground and extracted with hexane (1:1 vol/vol) and incubated by shaking for 24 hrs at room temperature. The solvent was separated out of the marc by filtration through filter paper (Whatman No.4). The marc from hexane extraction was further extracted with ethyl acetate (EtOAc) and followed with methanol (MeOH) using the same procedure as hexane. The solvents were separately evaporated in vacuo to yield crude hexane, EtOAc and MeOH extracts, respectively. Each crude extract was weighed (Table 1), and then kept in refrigerator (4°C) until use for testing antifungal metabolites against *P. aphanidermatum* RY803.

In vitro antifungal metabolites against Pythium aphanidermatum RY803

Each crude extract from antagonists was dissolved with 2% dimethyl sulfoxide (DMSO) and then tested for inhibitory activity against mycelial growth and oospore formation of *P. aphanidermatum* RY803 on potato dextrose agar (PDA) at concentrations of 0, 10, 50, 100, 500 and 1000 μg/ml. Agar plug (3 mm diameter) of *P. aphanidermatum* RY803 was cut from the margin of the 3-d-old colony and transferred to the middle of PDA containing each concentration of crude extract and incubated at room temperature for 2-10 days depended on its activity. The experiment was done by using Completely Randomized Design (CRD) with four replications. Data were collected as colony diameter (cm) and oospore formation. The statistical analysis of variance (ANOVA) was computed. Treatment means were compared using the Duncan's multiple range test (DMRT) at P=0.01. The effective dose of ED_{50} values was computed using probit analysis.

Results and discussion

Fungal growth and extraction of crude extracts

Yields of crude extracts from 10 antagonistic fungi were recorded as shown in Table 1. Crude MeOH extract from *Trichoderma harzianum* RY104 gave the highest yield (4.113 g) followed by crude MeOH extract from *Chaetomium bostrychodes* PR101, crude EtOAc extract from *C. aureum* MB601, crude EtOAc extract from *C. cupreum* NB201 and crude hexane extract from *T. harzianum* RY104 that gave yield of crude extract by 3.492, 3.004, 2.032 and 2.331 g, respectively. The results indicated that the yields of crude extracts varied according to species of fungi, number of mycelial mats and kind of solvents.

Table 1. Yields of mycelial mats and crude extracts from 10 antagonistic fungi.

Fungi	dried mycelial mats (g)	Yields of crude extracts (g)		
		hexane	EtOAc	MeOH
Chaetomium aureum MB601	49.93	1.335	3.004	1.892
Chaetomium bostrychodes PR101	36.49	0.625	1.098	3.492
Chaetomium cochliodes RY301	37.09	0.430	1.352	1.503
Chaetomium cupreum NB201	49.92	0.927	2.032	1.327
Chaetomium cupreum RY202	48.54	1.012	1.083	2.316
Gliocladium catenulatum RY102	45.38	0.343	0.257	1.322
Gliocladium catenulatum RY111	38.65	0.415	0.916	1.234
Trichoderma harzianum RY 101	40.20	0.996	0.450	1.207
Trichoderma harzianum RY 104	70.27	2.331	1.333	4.113
Trichoderma harzianum RY 112	42.37	1.172	0.453	1.365

In vitro antifungal metabolites against Pythium aphanidermatum RY803

Antifungal activities of crude extracts on mycelial growth of *P. aphanidermatum* RY803 were recorded at 2 days (Fig. 1 and Table 2). Crude EtOAc extract from *Chaetomium cochliodes* RY301 at concentration of 100, 500 and 1,000 µg/ml and *Trichoderma harzianum* RY112 at concentration of 500 and 1,000 µg/ml gave colony diameter of *P. aphanidermatum* RY803 by 3.23, 1.83, 1.45, 2.75 and 1.48 cm, respectively (Table 2). The data of colony diameter were transformed into percent of mycelial growth inhibition. The results indicated that crude EtOAc extract from *C. cochliodes* RY301 gave the greatest inhibition on mycelial growth of *P. aphanidermatum* RY803 by 63.40 and 71% at concentration of 500 and 1,000 µg/ml, respectively (Fig. 1) while crude EtOAc extract from *T. harzianum* RY112 could inhibit the mycelial growth of *P. aphanidermatum* RY803 by 70.04% at concentration of 1,000 µg/ml, and the other crude extracts inhibited mycelial growth of *P. aphanidermatum* RY803 less than 50% at all tested concentrations.

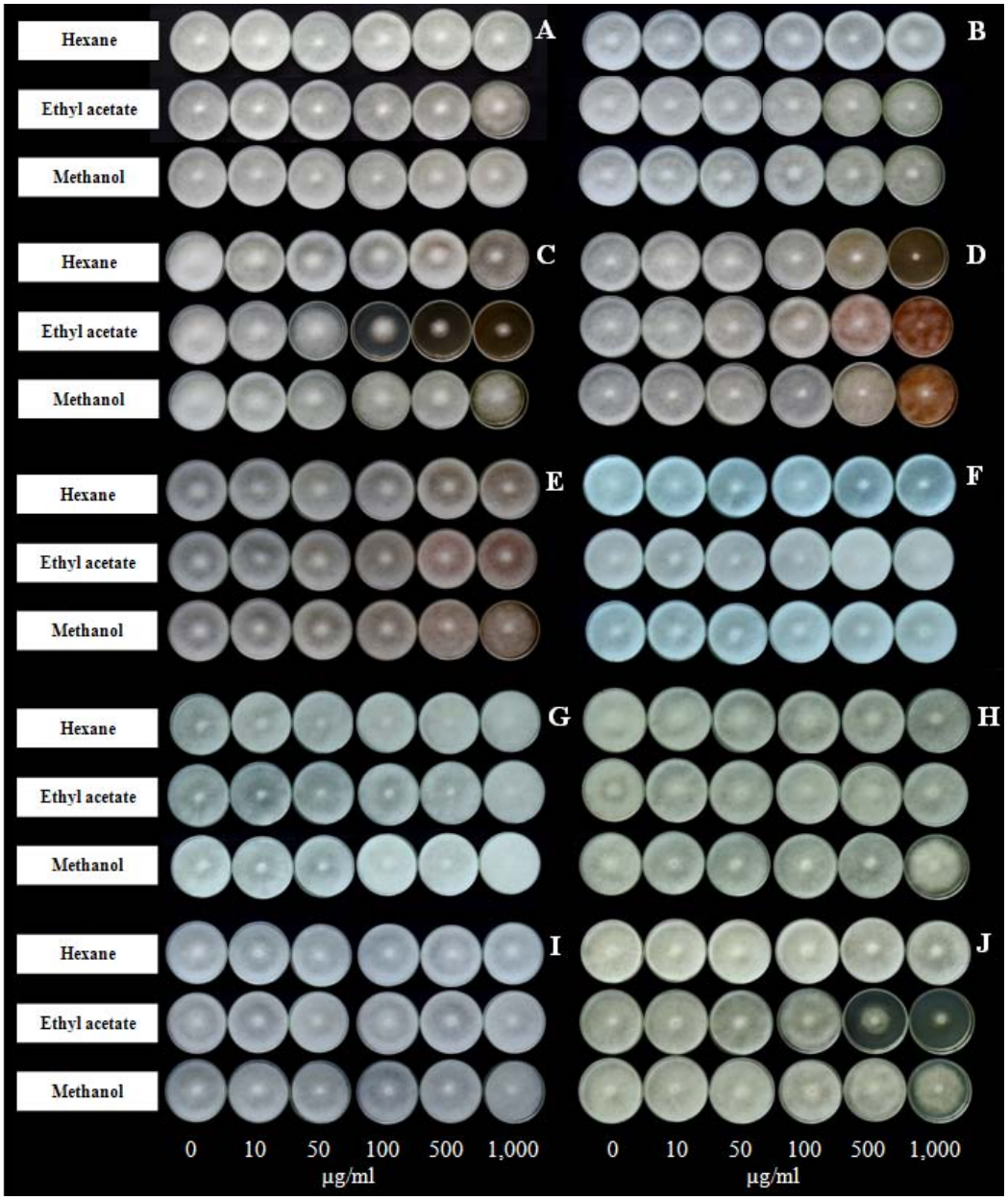

Fig.1. Colony growth of *Pythium aphanidermatum* RY803 on PDA containing crude hexane, EtOAc and MeOH extracts from promising antagonistic fungi at 0, 10, 50, 100, 500 and 1,000 µg/ml concentrations. A. *Chaetomium aureum* MB601, B. *Chaetomium bostrychodes* PR101, C. *Chaetomium cochliodes* RY301, D. *Chaetomium cupreum* NB201, E. *Chaetomium cupreum* RY202, F. *Gliocladium catenulatum* RY102, G. *Gliocladium catenulatum* RY111, H. *Trichoderma harzianum* RY101, I. *Trichoderma harzianum* RY104 and J. *Trichoderma harzianum* RY112.

Table 2. Colony diameter of *Pythium aphanidermatum* RY803.

Crude extracts of promising antagonistic fungi	Colony diameter of *Pythium aphanidermatum* RY803 at each concentration (cm)					
	0	**10**	**50**	**100**	**500**	**1,000**
Chaetomium aureum MB601						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Chaetomium bostrychodes PR101						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Chaetomium cochliodes RY301						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	3.23 b	1.83 c	1.45 d
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Chaetomium cupreum NB201						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Chaetomium cupreum RY202						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Gliocladium catenulatum RY102						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Gliocladium catenulatum RY111						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Trichoderma harzianum RY101						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Trichoderma harzianum RY104						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Trichoderma harzianum RY112						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	2.75 b	1.48 c
MeOH	5 a	5 a	5 a	5 a	5 a	5 a

[1/] Average of four replications. Means followed by a common letter in each promising antagonistic fungus are not significantly different at P=0.01 by Duncan's Multiple Range Test.

Antifungal activities of crude extracts on oospore formation of *P. aphanidermatum* RY803 were recorded at 10 days (Table 3). All tested crude extracts significantly inhibited oospore formation of *P. aphanidermatum* RY803. Particularly, crude EtOAc extract of *C. cochliodes* RY301 at 1,000 µg/ml gave the most inhibition effect on oospore formation of *P. aphanidermatum* RY803 by an average of 88.95%. The data of oospore inhibition were computed into effective dose (ED_{50}) values at each crude extract. Crude EtOAc, MeOH and hexane extract from *C. cochliodes* RY301 gave the greatest ED_{50} at 64, 84 and

178 µg/ml, respectively followed by crude EtOAc extract from *C. aureum* MB601, crude EtOAc extract from *T. harzianum* RY101 gave the greatest ED_{50} at 368 and 473 µg/ml, respectively.

Besides, mycelial growth and oospore formation of *P. aphanidermatum* RY803 on PDA added with crude EtOAc extract from *C. cochliodes* RY301 showed abnormal features. Hyphae, oogonia and oospores formed abnormal protoplasm in cell and demonstrated uncommon shape (Fig. 2).

Table 3. Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803.

Crude extracts of promising antagonistic fungi	Inhibition of oospore formation[1/] (%) of *Pythium aphanidermatum* RY803 at each concentration (µg/ml)						ED_{50}
	0	10	50	100	500	1,000	(µg/ml)
Chaetomium aureum MB601							
Hexane	0 k	8.35 j	9.71 i	12.54 h	12.83 h	12.83 h	NF
EtOAc	0 k	17.85 g	33.92 d	41.59 c	49.33 b	58.27 a	368
MeOH	0 k	8.13 j	8.53 j	29.66 f	30.59 ef	31.51 e	6,582
Chaetomium bostrychodes PR101							
Hexane	0 e	0.72 e	5.12 d	26.27 c	29.46 b	31.40 a	2,744
EtOAc	0 d	9.50 c	34.88 b	35.13 b	36.51 b	55.04 a	753
MeOH	0 e	15.20 d	36.16 c	37.19 b	37.23 b	57.98 a	662
Chaetomium cochliodes RY301							
Hexane	0 j	26.33 h	38.77 f	56.83 e	57.12 e	57.69 e	178
EtOAc	0 j	25.11 h	32.96 g	61.93 d	82.72 b	88.95 a	64
MeOH	0 j	20.38 i	27.63 h	57.72 e	61.91 d	70.10 c	84
Chaetomium cupreum NB201							
Hexane	0 j	1.90 j	19.00 h	35.00 e	37.08 e	61.93 a	575
EtOAc	0 j	1.34 j	26.28 f	50.60 c	58.17 b	58.20 b	297
MeOH	0 j	8.32 i	23.08 g	45.61 d	52.21 c	57.86 b	543
Chaetomium cupreum RY202							
Hexane	0 d	0.66 d	8.43 c	28.49 b	33.21 b	47.50 a	976
EtOAc	0 f	10.09 e	18.95 d	28.09 c	33.55 b	56.49 a	994
MeOH	0 d	13.58 c	21.78 c	36.09 b	37.13 b	58.22 a	762
Gliocladium catenulatum RY102							
Hexane	0 k	7.78 ij	10.96 hi	13.92 fgh	21.04 e	25.14 d	45,615
EtOAc	0 k	8.67 ij	11.66 ghi	18.18 f	34.66 ab	36.94 ab	3,515
MeOH	0 k	5.03 j	15.05 fg	29.70 c	33.87 b	38.08 a	2,218
Gliocladium catenulatum RY111							
Hexane	0 j	8.28 hi	11.32 gh	13.56 fgh	20.70 d	27.32 c	40,815
EtOAc	0 j	12.60 gh	14.18 efgh	19.60 def	33.84 ab	38.73 a	4,888
MeOH	0 j	3.66 ij	14.55 defgh	16.10 defg	20.27 de	31.90 bc	8,701
Trichoderma harzianum RY101							
Hexane	0 m	5.29 l	9.24 k	11.43 j	13.75 i	15.83 h	NF
EtOAc	0 m	18.44 g	33.73 e	43.46 c	49.75 b	54.07 a	473
MeOH	0 m	8.61 k	16.72 h	30.52 f	33.12 e	37.46 d	3,422
Trichoderma harzianum RY104							
Hexane	0 d	8.91 c	11.74 c	14.12 bc	21.56 ab	24.58 a	NF
EtOAc	0 d	11.15 c	13.90 bc	19.53 b	35.77 a	38.23 a	3,515
MeOH	0 h	6.24 g	14.47 ef	29.89 bc	34.06 ab	38.47 a	2136
Trichoderma harzianum RY112							
Hexane	0 j	7.05 hi	10.97 ghi	13.47 fgh	20.50 de	24.41 d	NF
EtOAc	0 j	10.03 ghi	13.18 fgh	18.94 def	45.18 b	61.87 e	617
MeOH	0 j	5.77 ij	14.64 efg	29.88 c	37.99 b	40.19 b	2,034

[1/]Average of four replications. Means followed by a common letter in each promising antagonistic fungus were not significantly different at P=0.01 by Duncan's Multiple Range Test. NF = no effect.

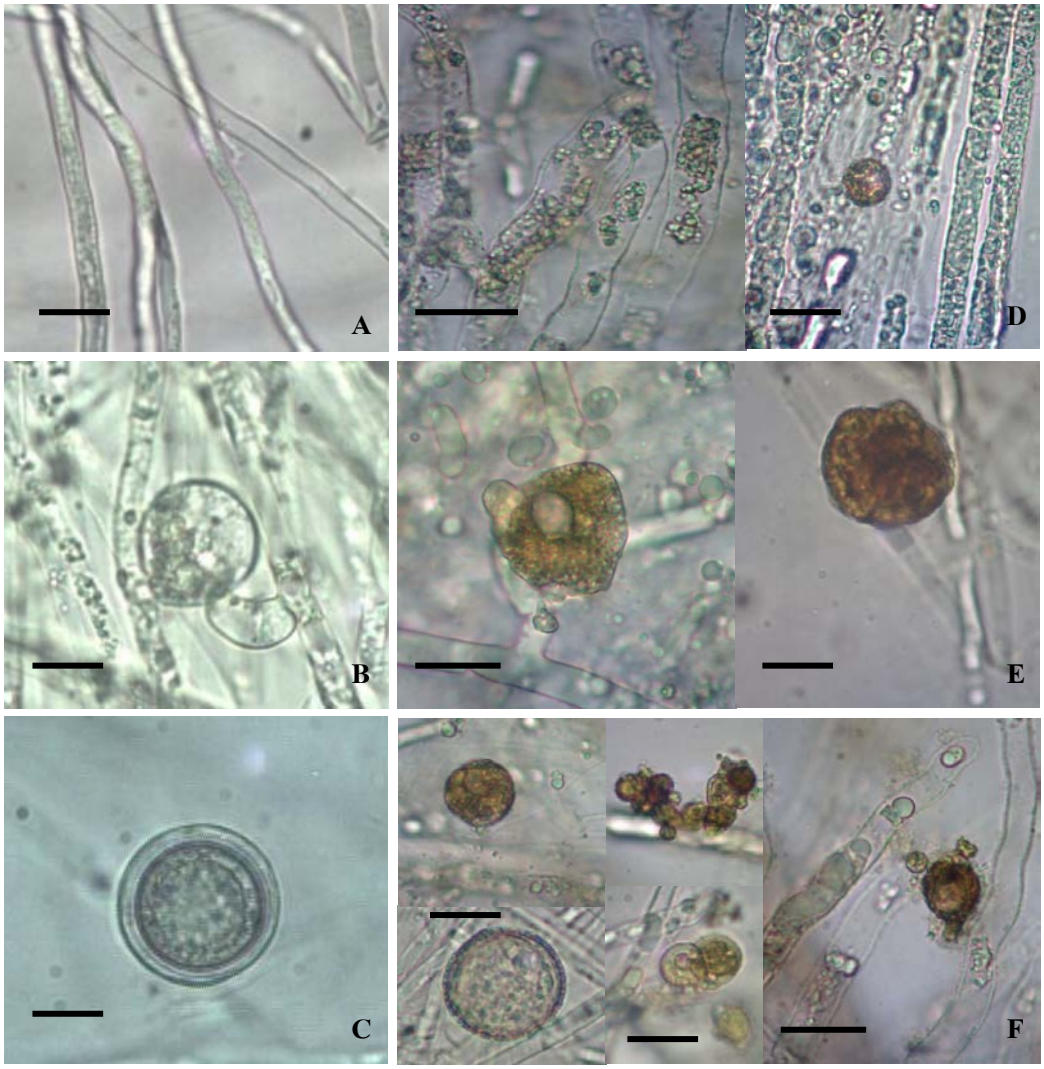

Fig. 2. Comparison of normal and abnormal mycelia, oogonia and oospores of *Pythium aphanidermatum* RY803 on PDA added with crude EtOAc extract from *Chaetomium cochliodes* RY301. A, B and C showed normal mycelia, oogonium and oospore, respectively. D, E and F showed abnormal mycelia, oogonia and oospores, respectively. Bars. = 10 µm.

Crude hexane, EtOAc and MeOH extracts from 10 promising antagonistic fungi *C. aureum* MB601, *C bostrychodes* PR101, *C. cochliodes* RY301, *C. cupreum* NB201, *C. cupreum* RY202, *G. catenulatum* RY102, *G. catenulatum* RY111, *T. harzianum* RY 101, *T. harzianum* RY 104 and *T. harzianum* RY 112 were tested to find out the most efficient antagonist for controlling pineapple root rot caused by *P. aphanidermatum* RY803. *C. cupreum*, *G. catenulatum* and *T. harzianum* have well known to be antagonist for plant disease control (Soytong,

1992a, 1992b; Biren *et al*.; 1999; Ezziyyani *et al*., 2007). *C. cupreum* has been reported to reduce leaf spot disease of corn caused by *Curvularia lunata*, rice blast caused by *Pyricularia oryzae*, sheath blight of rice caused by *Rhizoctonia oryzae* and tomato wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* (Soytong, 1992a; 1992b). *G. catenulatum* was a mycoparasite of several fungal genera including *Aspergillus flavus* and *Sclerotium cepivorum* (Biren *et al*., 1999; Tsigbey *et al*., 1999). *T. harzianum* has been reported as biocontrol agent for controlling *Phytophthora capsici* (Ezziyyani *et al*., 2007) and *Pythium ultimum* (Naseby *et al*., 2000). In this study *C. cochliodes* RY301 was found to be the greatest antagonist against *P. aphanidermatum* RY803 causing root rot of pineapple. The results implied that the antagonistic mechanism of *C. cochliodes* RY301 was lysis and antibiosis due to it produce some metabolites that could inhibit mycelial growth, oospore formation and formed abnormal of hyphae, oogonia and oospores. This result was supported by previous report (Phonkerd *et al*., 2008) that *C. cochliodes* VTh01 and *C. cochliodes* CTh05 could produce four new dimeric spiro-azaplilones, cochliodones A-D, two new azaphiliones, chaetoviridines E and F, a new epi-chaetoviridin A, and known compounds, chaetoviridin A, ergosterol, chaetochalasin A. Chaetoviridines E and chaetochalasin A exhibited antimalarial activity against *Plasmodium falcipulum* while cochliodones C, chaetoviridines E and F, chaetochalasin A expressed antimycobacterial activity against *Mycobacterium tuberculosis*. Furthermore, *C. cochliodes* VTh01 and *C. cochliodes* CTh05 were reported to be antagonistic to *Fusarium oxysporum* f. sp. *lycopersici* causing tomato wilt (Phonkerd *et al*., 2008). Therefore it was possible that *C. cochliodes* RY301 might have the similar active compounds that could be antagonist against *P. aphanidermatum* RY803. In this study, it was pointed out that *C. cochliodes* RY301 could exhibit inhibitory activity against *P. aphanidermatum* RY803.

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