Coniochaeta ligniaria an Endophytic Fungus from *Baeckea frutescens* and Its Antagonistic Effects Against Plant Pathogenic Fungi

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Abstract

Coniochaeta ligniaria (KUFC 5891), an endophyte was isolated from leaves of *Baeckea frutescens* (Myrtaceae) obtained from the Phu Luang wildlife sanctuary, Loei province, Thailand. The fungus was characterized by the characteristics of its colony and the morphological features of its sexual and asexual stages. The fungus in dual culture with various plant pathogens showed strong inhibitory effects, especially to *Pythium aphanidermatum*, *Phytophthora palmivora*, *Sclerotium rolfsii*, and *Rhizoctonia oryzae*. A crude ethyl acetate extract of a rice based media, at 100 ppm yield completely suppressed *P. palmivora* with higher concentrations needed to inhibit *P. aphanidermatum* and other fungal pathogens. This organism and its crude ethyl acetate extract has potential for control of various plant diseases including some of the most important diseases caused by *P. palmivora*.

Keywords: *Coniochaeta*, endophytic fungi, antagonistic test, plant pathogenic fungi

Introduction

Endophytic fungi are microbes that colonize living internal tissues of plants without causing any immediate or negative effects (Bacon and White, 2000). These microorganisms are to be found in virtually every plant on earth (Strobel and Daisy, 2003). Endophytic fungi have been recognized as a repository of novel secondary metabolites in phamaceutical and agricultural systems (Hoffman et al., 2008; Pongcharoen et al., 2008; Strobel, 2006; Riga et al., 2008; Worapong, 2009). Endophytic fungi within plants have been known to produce plant growth regulatory, antimicrobial, antiviral or insecticidal substances to enhance the growth and competitiveness of the host in nature (Wiyakrutta et al., 2004; Kim et al., 2007) Thus, endophytic fungi are expected to be potential sources of new bioactive agents and to be useful as agents of biocontrol against plant disease.

Most endophytic fungi were reported from wild plants in the forest (Strobel, 2006). In our investigation, we collected many forest tree in Phu Luang Wildlife Santuary, Loei province the interesting endophytic fungus *Coniochaeta ligniaria* was isolated from plant where *Baeckea frutescens*, a promising plant. *Baeckea frutescens* L. (Family Myrtaceae) a small tree which is found in Southeast Asia to Australia, including southern China, Peninsular Malaysia, Sumatra, Borneo and Thailand. *B. frutescens* grows wild in Thailand, it is found on the beach forest, melaleuca forest and the mountain

tops on sandstone from Loei, Surat Thani, Pattani, Nakhon Si Thammarat, Chanthaburi, Ubon Ratchathani and Narathiwat (Smitinand, 2001). It is a medicinal plant the leaves are also used to treat impetigo. In Thailand use the leaves as a tea to reduced body aches (Parnell and Chantharanothai, 2002). Chinese people use the leaves as remedy for sunstroke and fever. In Malaysia and Indonesia, they are used as an ingredient of the traditional medicine given to mothers during confinement (Herbal Medicine Research Centre, 2002). Jantan et al. (1998) reported the constituents of the essential oil including pinenes, terpinene, cineole from leaf of *B. frutescens.* The methanol extracts of *B. frutescens* exhibited potent antibacterial activity against the cariogenic bacterium *Streptococcus mutans* (Hwang et al., 2004). Lu et al. (2008) reported a new flavanol glycoside, name 6,8-dimethylkaempferol-3-O-alpha-L-rhamnoside from Chinese herb *B. frutescens.*

The fungus, *Coniochaeta* (Sacc) Cook was originally introduced by Saccardo in 1882 as a subgenus of *Rosellina* (Xylariaceae). Most species of the genus differ from the xylariaceous genus *Roseilinia* by their non-stromatic ascomata and lack of an amyloid apical apparatus in the ascus (Garcia et al., 2006). The species of *Coniochaeta* and their anamorphs occur on dung, wood or bark of trees, soil, leaves and leaf litter and rarely in non- woody host plants like Gramineae (Lopez-Archilla et al. 2004, Asgari et al. 2007), In addition some species, such as *Lecythophora haffmannii* (teleomorph *Coniochaeta ligniaria*) and *L. mutabilis* are also known as human pathogens involved and septic shock (de Hoog et al., 2000; Drees et al., 2007; Taniguchi et al., 2009). They have also been isolated from food, such as butter (Samson et al., 2004). On the other hand, *Coniochaeta ellipsoidea* (DSM13856) have been found to exhibit useful biochemical properties the antibiotic coniosetin, which has a pronounced antibacterial and antifungal action, inhibiting even drug-resistant strains of *Staphylococcus aureus* (Segeth et al., 2003)

The aims of this study were 1) to study the morphology of *Coniochaeta ligniaria*, an endophytic fungi isolated from *Baeckea frutescens*, and 2) to test for antagonistic activity of *Coniochaeta ligniaria* against some plant pathogenic fungi *in vitro*.

Materials and Methods

Fungal Isolation

The twigs and leaves of *Baeckea frutescens* (Myrtaceae) were collected from Phu Luang wildlife sanctuary, Loei province, Thailand altitude 1,200 m, 17° 8' 24" N 101° 39' 54" E 101 (Figure 1). Plant samples were placed in plastic bags in ice box and brought to the laboratory. After isolation, the samples were kept in the herbarium at Kasetsart University. The surface sterilize method was employed to isolate endophytic fungi. (Li et al., 2005; Radu and Chen, 2002). A random sample from each plant consisting of an asymptomatic leaf was taken. Leaf portions were thoroughtly washed in running tap water, after which they were surface sterilized by submerging in 70% ethanol for 2 min. After drying, each leaf was divided into four segments and placed on water agar (WA) supplemented with 50 mg L^{-1} streptomycin to suppress bacterial growth. All the plates were incubated at room temperature for 3-4 weeks. Emerging fungi were transferred to fresh potato dextrose agar (PDA) plates, incubated for 1 week, and periodically checked for purity.

Figure 1 Healthy plant sample *Baeckea frutescens* collected from Phu Luang wildlife Sanctuary, Loei province.

Induction of Spores and Ascospores

Endophytic fungi isolate KUFC5891 can not produce conidia and ascospores. To induce sporulation, the fungus KUFC 5891 was cultivated on PDA. Double-autoclaved grapevine wood pieces were placed onto the surface of synthetic nutrient-poor agar medium (SNA; Nirenberg 1976) and then the hyphal tip from culture was transferred to the SNA incubated at 28°C for 1 week (anamorphs) and 3-4 weeks (teleomorphs) (Damm et al., 2010).

Morphological Studies

Macroscopic features were studied including colony growth pattern, color, texture, and fungal growth rate was measured on PDA. Colony characteristics and pigment production were noted after 7 days of growth (Crous et al., 2009) incubation at 28°C. Colony colors were rated according to Rayner (1970). Conidia, ascomata, asci and ascospores were determined from the inducing grapvine on SNA. Microscopic preparations were made in water, with 30 measurements per structure, and morphological characteristics were examined under stereo and light microscopes (Olympus BH-2 with Normaski Interference Contrast). Camera lucida drawings were employed of conidiophores, conidia, ascomata, asci and ascospores.

Preparation of Crude Extract

The endophytic isolate KUFC 5891 was cultured on autoclaved rice in 1,000 ml flasks (200 g of rice in 200 ml of water) and incubated at room temperature (28ºC). After incubation for 30 days, 800 ml of ethyl acetate was added to the culture for 3 days. The organic phase was evaporated to dryness under reduced pressure with a rotary evaporater. A dark brown viscous mass of crude ethyl acetate extract was collected and kept at room temperature (28ºC) until used (Dethoup et al., 2007).

Antagonistic Tests of KUFC 5891 *in vitro*

Dual Culture Tests

Young mycelium of the endophytic fungus isolate KUFC5891 was cut from the colony margin with sterile Pasture pipette and placed in the center of a petri dish with PDA and cultivated for 14 days at room temperature. The specific plant pathogenic fungi were cut and placed near the colony margin of the fungus at the distances 0.5, 1.0 and 1.5 cm. All petri dishes were incubated at room temperature (28^{o}C) and the colony diameters were recorded 24, 48, 72 hr after incubation. The inhibition levels were calculated by using the formula: G_1-G_2/G_1x 100, when G_1 indicated colony radius of plant pathogenic fungi in control and G_2 indicated colony radius of plant pathogenic fungi in the dual culture test (Intana et al., 2003). Each treatment was performed with two replicates.

Crude Extract Test

One gram of dark brown crude extract of KUFC5891 was dissolved in 10 ml of ethyl acetate (1,000,000 ppm). Then the stock solution was serially diluted to four concentrations (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}). Each concentration of crude extract was added to 9 mL of warm PDA, mixed, and poured into petri dishes. The young mycelia of the various plant pathogenic fungi (Table 1) were transferred to the PDA plates containing various concentrations of the crude extract solution. All petri dishes were incubated at 28°C. The colony diameters were recorded 24, 48, 72 h after incubation. The inhibition levels were calculated by comparison with a control.

Results and Discussion

Identification of the Endophyte

Culture characteristic: colonies of KUFC 5891 were slow growing on PDA, reaching 1.5-2.0 cm in diameter after 7 days at 28^{o}C no sporulation on PDA (Figure 3A) Conidiophores and conidia were found on SNA surface grapevine twig after 7 days incubation. B. Anamorph state were similar to the genus *Lecythophora* described by Webber (2002) but our isolation size 3.5-4.5 x 1.5-2.0 µm (Figures 2A and 2B).

Lecythophora anamorph: The fungus produced conidia on SNA in 7 days. Colony on SNA smooth, lacking aerial mycelium; colours white to salmon, pink isabelline; reverse stronger colours (Rayner, 1970). Conidiogenous cells enteroblastic, phialidic, hyaline (Figures 2A and 2B). Conidia ellipsoidal to cylindrical, curved, hyaline, one-celled, smooth-walled, aggregated in heads, biguttlate, 2.5-7 x 0.7-1.5 µm Conidiophores hyaline, cylindrical (Webber, 2002).

Table 1 Species of plant pathogenic fungi from various diseased fruits and vegetables used for the antagonistic activity test.

Plant pathogenic fungi	Host plant	Diseases
Alternaria alternata	*Pyrus pyrifolia* (pear)	Fruit rot
Curvularia lunata	*Zea mays* (corn)	Leaf spot
Fusarium oxysporum	*Musa sapientum* (banana)	Fusarium wilt
Lasiodiplodia theobromae	*Citrus maxima* (pomelo)	Fruit rot
Pythium aphanidermatum	*Brassica albogaba* (chinese vegetable)	Damping-off
Phytophthora palmivola	*Durio zibethinus* (durian)	Root rot
Rhizoctonia oryzae	*Oryza sativa* (rice)	Sheath rot
Sclerotium rolfsii	*Solanum tuberosum* (potato)	Southern blight

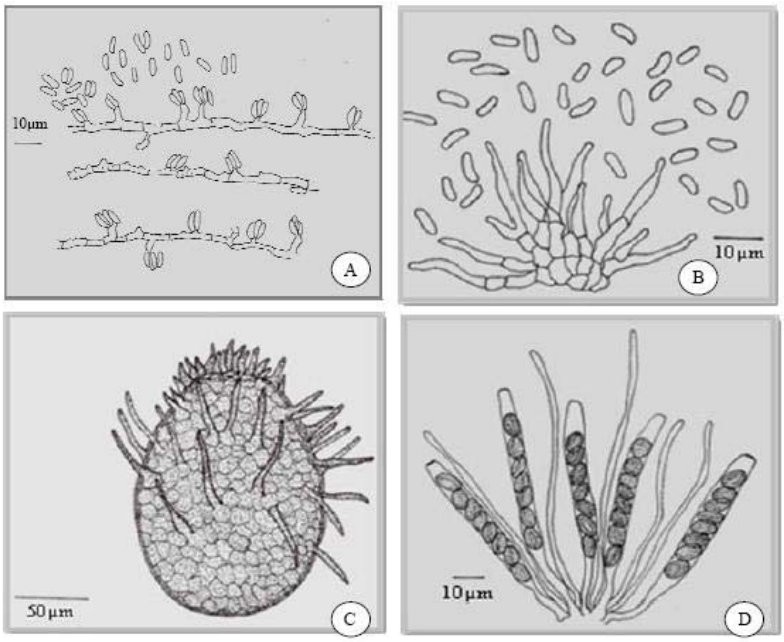

Figure 2 Camera lucida drawing of *Coniochaeta ligniaria* (Grev.) (KUFC5891). (A) *Lecythophora* anamorph on SNA 2 weeks; (B) *Lecythophora* anamorph on SNA 4 weeks; (C) Perithecium with a central ostiole and setose; (D) Asci, ascospores and paraphyses.

Coniochaeta ligniaria teleomorph: The organism produced perithecia that are solitary, superficial on grapevine wood pieces, and superficial or immersed in SNA, subglobose; dark brown, obviously setose, up to 227 x 175 µm. Setose dark brown, cylindrical, tapering to a round tip, smooth-walled, 2.5-3 µm wide, up to 50 µm long. Asci cylindrical, with a truncate to rounded apex and amyloid apical ring, 8 ascospores, thin walled. Paraphyses numerous, hyaline, filiform, non septate. Ascospores narrowly ellipsoidal, lenticular, one celled, smooth, dark brown, germ slit straight, longitudinal, 11 x 6 µm (Figures 2C, 2D, 3A, 3F). Endophytic fungi isolate KUFC5891 have been nearly morphology with genus Lecythophora (Weber, 2002) in Table 2.

Table 2 Morphological study of the endophytic fungus isolate KUFC 5891 compare with *Coniochaeta ligniaria* (Weber, 2002).

Morphology	*Coniochaeta ligniaria*	Endophytic fungi KUFC5891
Colony on PDA	White to Yellowish or orange, salmon, pink or isabelline	Yellowish to orange
Odour	Not	Not
Vegetative hyphae	2.4 µm wide, hyaline, septate, smooth walled	2 µm, hyaline, septate, smooth walled
Chlamydospores	Absent	Absent
Conidiogenous cell	Phialidic, mainly adelophialides, more rarely ventricose discrete phialide	Enteroblastic phialide, cylindrical hyaline aerial mycelium whitish, sometimes sparse or lacking
Conidia	Ellipsoidal to cylindrical, often somewhat curved, hyaline, 1 cell, smooth walled, aggregated in heads, mostly biguttulate 3.5-6 x 1.5-2.5 µm	Conidia aggregated in heads, hyaline, 1-celled, smooth-walled, cylindrical sometimes slightly curved, occasionally biguttulate, 3.5 - 4.5 x 1.5-2 µm
Perithecia	Black with setose	Dark brown with setose
Ascospores	Almond-or lemon-shaped, brown, with longitudinal germ slit 11-17 x 6-8 µm	Almond shaped, brown, with longitudinal germslit, 11 x 6 µm
Setae	2-4 µm wide, 25-65 µm long, thick wall, smooth not branched	2.5-3 µm wide, up to 55 µm long straight, smooth-walled
Anamorph	*Lecythophora*	*Lecythophora*

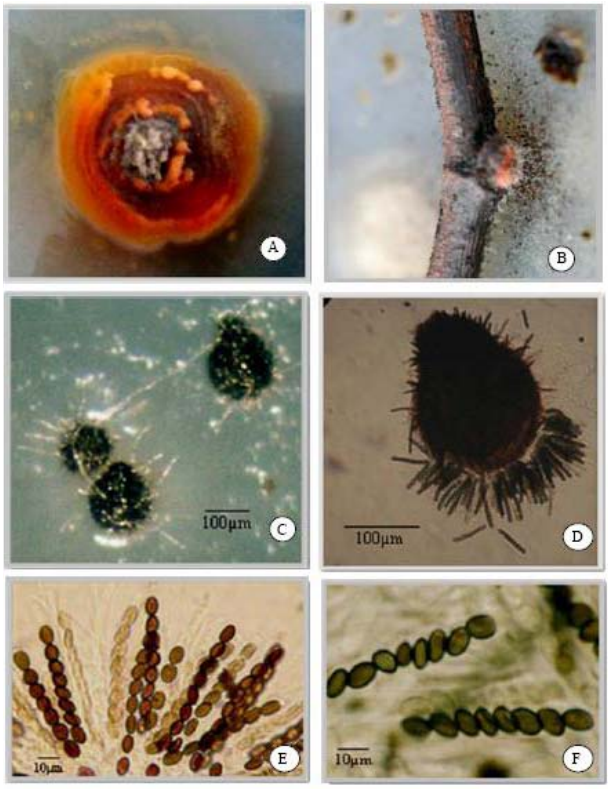

Figure 3 *Coniochaeta ligniaria* (Grev.) Massee. (KUFC5891) (A) Colony on PDA 7 days; (B) Perithecia and conidial mass on SNA and grapevine wood under stereomicroscope; (C) Perithecia under stereomicroscope; (D) Perithecium, asci and ascospores; (E-F) Asci, ascospores and paraphyses.

This isolate KUFC5891 produced anamorph and teleomorph on SNA media that was incubated at room temperature. This is different from the report of Damm (2010) reported *Coniochaeta* (anamorph: *Lecythophora*) that is pathogen of wood hosts and can also cause opportunistic human infections. The enhance sporulation, double-autoclaved pine needles or grapevine wood pieces were placed onto the SNA media and incubated in dark for 2 weeks for anamorphs or 2-3 months for teleomorphs.

Coniochaeta ligniaria is effective in biological detoxification of lignocellulosic biomass and can potentially be used to convert it in to fuels and chemicals (Lopez et al., 2004). Colonisation of torrefied grass fibres with the same fungus resulted in reduced phytotoxicity and increased plant growth (Trifonova et al., 2009). *Coniochaeta* and associated anamorphs have been isolated from asymptomatic dormant buds and young plants of *Vitis vinifera* (Dugan et al., 2002; Casieri et al., 2009). Kim et al. (2007) reported *C. ligniaria* is an endophytic fungus isolated from vegetable crops and it could inhibit *Phytophthora infestans* in tomato plants in Korea.

Antagonistic activity of *Coniochaeta ligniaria* (KUFC5891)

The dual culture test of *Coniochaeta ligniaria* (KUFC5891) with eight plant pathogenic fungi showed that the fungus could completely inhibit mycelial growth of *P. aphanidermatum*, *P. palmivora*, *S. rolfsii*, *R. oryzae* and *F. oxysporum* at 24 h of incubation. However, the inhibition percentages of the later three fungal pathogens were reduced when incubated up to 72 h. *Coniochaeta ligniaria* (KUFC5891) provided the lowest inhibition percentage (45%) against mycelia growth of *L. theobromae* when incubated to 72 h. For *C. lunata* the percent inhibition was increased from 35% at 24 h of inhibition to 75% when incubated for 72 h (Table 3).

The efficacy of crude extract of *Coniochaeta ligniaria* (KUFC5891) against eight plant pathogenic fungi on PDA revealed that this crude extract completely inhibited *P. palmivora* (100%) at 100 ppm, *P. aphanidermatum* (100%) at 1,000 ppm, *S. rolfsii* (100%) at 10,000 ppm., whereas the inhibition against *R. oryzae* was over 80% at 10,000 ppm. (Table 4, Figures 3, 4).

Table 3 Percent inhibition on mycelial growth of plant pathogenic fungi by *Coniochaeta ligniaria* on PDA as dual culture at 28˚C for 24, 48 and 72 hours.

Plant pathogenic fungi	Inhibition (%)		
	24 h	48 h	72 h
Pythium aphanidermatum	100	100	100
Phytophthora palmivora	100	100	100
Sclerotium rolfsii	100	98	95
Rhizoctonia oryzae	100	85	80
Lasiodiplodia theobromae	70	65	45
Alternaria alternata	65	60	54
Fusarium oxysporum	100	70	68
Curvularia lunata	35	68	75

Table 4 Efficacy of a crude extract of *Coniochaeta ligniaria* (KUFC 5891) in the inhibition of mycelia growth of plant pathogenic fungi on PDA at 28˚C for 24, 48, and 72 hours.

Concentration (ppm)	Inhibition (%)											
	Rhizoctonia oryzae			*Sclerotium rolfsii*			*Phytophthora palmivora*			*Pythium aphanidermatum*		
	24	48	72	24	48	72	24	48	72	24	48	72
10	3	0	0	38.9	46	43.5	61.5	77.8	80	17.5	0	0
100	9.1	0	0	72.2	82	84.7	100	100	100	62.5	63.3	61
1,000	3.3	5.6	0	100	88	89.4	100	100	100	100	100	100
10,000	100	81.3	82.2	100	100	100	100	100	100	100	100	100
	Curvularia lunata			*Fusarium oxysporum*			*Alternaria alternata*			*Lasiodiplodia theobromae*		
10	0	0	0	10	4.5	10	6.4	5.6	0	0	6.3	4
100	13	21.2	21.1	20	31.8	26.3	29.8	36.7	16.7	0	43.8	36
1,000	33	54.5	46.2	30	45.5	52.6	46.8	42.2	22.2	0	50	44
10,000	33	69.7	71.2	100	68.2	73.6	68.9	70	44.4	0	62.5	52

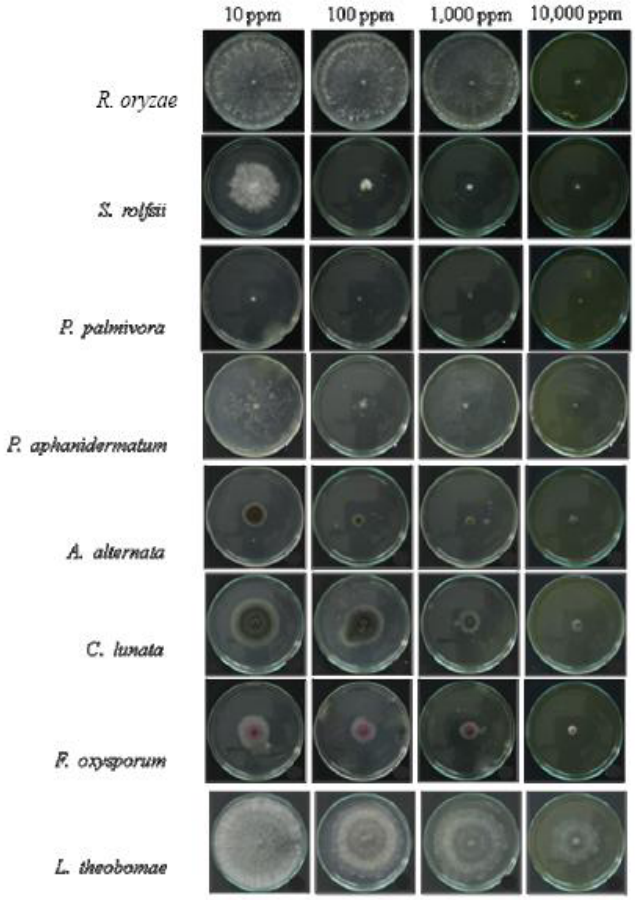

Figure 4 Antagonistic activity tests of four concentrations of crude extract from *Coniochaeta ligniaria* (KUFC5891) against eight species of plant pathogenic fungi incubated on PDA, at 28°C for 27 h.

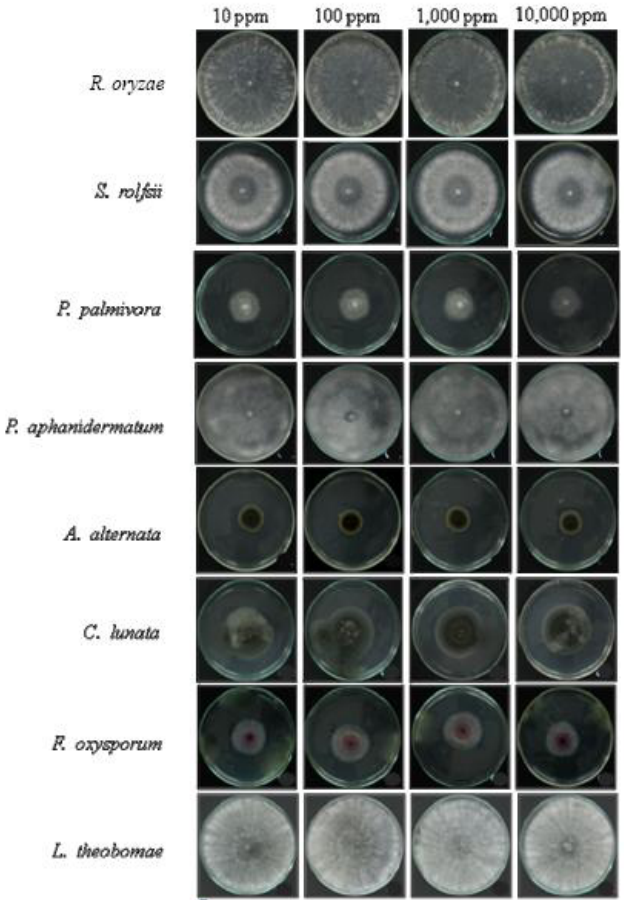

Figure 5 Mycelial growth of eight species of plant pathogenic fungi on PDA amended with each of four concentrations of ethyl acetate, incubated on PDA at 28°C for 72 h.

Conclusions

Coniochaeta ligniaria (KUFC 5891) was a single isolate from leaf of *Baeckea frutescens* (Myrtaceae) collected from Phu Luang wildlife sanctuary, Loei province, Thailand. Morphological study showed this endophytic fungus can produced ascomata on SNA media in 2 weeks and divided in sordariomycetes genus *Coniochaeta ligniaria* that has the anamorph is *Lecythophora*, which are known as pathogens of woody hosts.

The efficacy of a crude extract of the endophytic fungi, *Coniochaeta ligniaria* (KUFC5891), was tested with eight plant pathogenic fungi on PDA. The results showed that the crude extract at 100 ppm suppressed 100% mycelia growth of *P. palmivora*, whereas the same suppression of *P. aphanidermatum* was observed at 1,000 ppm. This crude extract at 10,000 ppm suppressed mycelia growth of *S. rolfsii* and *R. oryzae* by 100 and over 80%, respectively.

This crude extract is fungistatic, inhibiting mycelia growth of plant pathogenic fungi especially class Oomycetes genus *P. palmivora*, and *P. aphanidermatum*.

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