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Antifungal Activity of Endophytic Fungi from Palm Trees Against Coffee Anthracnose Caused by *Colletotrichum Coffeanum*

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Abstract Endophytic fungi are able to produce mixture of volatile organic compounds that are lethal to human and plant pathogenic fungi and bacteria. Endophytic fungi *Nigrospora sphaerica* isolated from *Chrysalidocarpus lotescens* and *Fusarium falciforme* isolated from *Mascarena Lagencuulis* were identified by morphological characters and molecular phylogeny on the basis of ITS1-5.8S-ITS4 ribosomal gene sequence acquisition and analyses. Crude hexane, ethyl acetate and methanol of these two endophytic fungi were yielded and examined for bioactivity test against *Colletotrichum coffeanum* causing coffee anthracnose. The results showed that crude methanol from *Nigrospora sphaerica* and *Fusarium falciforme* gave the highest inhibition of colony growth of *C. coffeanum*, which were 54.00% and 63.00%, respectively at concertration of 1000, 500 ppm, respectively. Methanol crude extract from *Fusarium falciforme* showed significantly highest inhibition for the spore production of *C. coffeanum* as 76.74% at the concentration of 500 ppm with effective dose (ED_{50}) for spore production inhibition at concentration 161.14 ppm. The research findings are reported that the metabolites from *Nigrospora sphaerica* and *Fusarium falciforme* acts as new antagonist against *C. coffeanum* causing coffee anthracnose.

Keywords: endophytic fungi; bioactivity test; coffee; *Colletotrichum coffeanum*

Introduction

Endophytes are microorganisms that live within plants for at least a part of their life cycle without causing any visible manifestation of disease (Bacon and white, 2000). A wide range of plants have now been examined for endophytes, and endophytes have been found in nearly all of them, including trees, grass, algae and herbaceous plants. An enormous number of different fungi can be isolated from plants growing in their native habitat. Most of the fungi are uncommon and narrowly distributed, taxonomically and geographically. However a few fungi are widely distributed with the host, suggesting a long standing, close and mutually beneficial interaction. Endophytic fungi have attracted great attention in the past few decades

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due to its ability to produce novel secondary metabolites for medical, agricultural and industrial use. And they are also considered as an outstanding source of bioactive compounds due to its ability to occupy any plants at any environments (Strobel and Daisy, 2003). Endophytic fungi are asymptomatic and may be mutualistic; plants protect and feed endophytes, which produce plant-growth-regulatory, antimicrobial, antiviral or insecticidal substances to enhance the growth and competitiveness of the host in nature (Carroll, 1988). Some endophytic fungi are known as reliable sources of bioactive substances with agricultural and/or pharmaceutical potential, as exemplified by taxol (Stierle *et al*., 1993; Wang *et al*., 2000), subglutinol A and B (Lee *et al*., 1995), and peptide leucinostatin A (Stroble and Hess, 1997). Endophytic fungi are thus expected to be potential sources of new bioactive agents.

Several studies on the use of bioactive compounds from endophytic fungi have been reported. Endophytic fungi are able to produce antimicrobial, anticancer such as Taxol (Walker and Croteau, 2001) and antimalarial activities (Wiyakrutta *et al.*, 2004). Study done by Woropong *et al.* (2001) showed that isolated endophytic fungi are able to produce mixture of volatile organic compounds that are lethal to human and plant pathogenic fungi and bacteria. Antifungal products are vastly produced by majority of the endophytes. Griseofulvin-producing endophyte was first reported in fungus from *Abies holophylla* and was evaluated *in vivo* antifungal activity against plant pathogenic fungi. (Park *et al.*, 2004).The natural and biological control of pests and diseases affecting cultivated plants has gained much attention in the past decades as a way of reducing the use of chemical products in agriculture. Vega *et al.*, (2008) studied fungal endophyte - mediated plant defense as a novel biological control mechanism against the coffee berry borer the most devastating pest of coffee throughout the world, A survey of fungal endophytes in coffee plants has revealed the presence of various genera of fungal entomopathogens including *Acremonium, Beauveria, Cladosporium, Clonostachys,* and *Paecilomyces*. Two of these *B. bassiana* and *Clonostachys rosea* were tested against the coffee berry borer and were shown to be pathogenic.

Coffee is a tropical crop and according to FAO statistics production of green beans is around 4 million tons annually with sales of 6 to 12 billion dollars (González, 2000). The main important factors for poor quality of coffee are disease and insect pests, especially coffee anthracnose caused by *Colletotrichum coffeanum*. Use of chemical for control plant disease is one of the most commonly used strategy usually what farmers followed, but nowadays people are more aware of various side effects caused by pesticide residues present in food and water, and also may lead to environmental pollution. There is needed to research biological product for control plant disease.

The aim of this research focused on preliminary test for bioactivity

substances from endophytic fungi to inhibit *C. coffeanum* causing anthracnose of coffee.

Material and methods

Isolation and identification of endophytic fungi

Endophytic fungi were isolated from palm trees, *Mascarena Lagencuulis* and *Chrysalidocarpus lotescens* at King Mongkut's Institute of Technology Ladkrabang (KMITL), Ladkrabang, Bangkok, 10520, Thailand. The isolates were identified based on the morphological characters of the fungal pure culture by following the standard mycological manuals (e.g. M.B. Ellis, 1971) and molecular phylogeny on the basis of ITS1-5.8S-ITS4 ribosomal gene sequence acquisition and analyses (Soytong and Poeaim, 2014). The fungal mycelia were freeze-dried and the genomic DNA were extracted by the modified CTAB (Cetyl trimethyl ammonium bromide) method (Ausubel *et al.*, 1994). The ITS regions of the fungi were amplified with the universal ITS primers, ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3'), using the polymerase chain reaction (PCR). The amplified products were sequenced and aligned with the sequences in the GenBank by basic local alignment search tool (BLAST) analysis (Altschul *et al.*, 1997) in the National Center for Biotechnology Information (NCBI) databases to find out the sequence homology with closely related organisms. Sequences from the closely related organisms were downloaded to construct the phylogenetic trees. The closely related sequences obtained were aligned through CLUSTALW using MEGA version 6.0 software (Tamura, K. *et al.*, 2007) and a phylogenetic tree was constructed by neighbor-joining method using the same software.

Isolation of pathogen and pathogenicity test

The plant pathogen *C. coffee* causing coffee anthracnose were isolated by tissue transplanting technique from coffee leaves with obvious symptoms and performed pathogenicity test followed Koch's Postulate by Song Jiaojiao *et al.* (2015a).

Crude Extraction of Bioactivity Substances

The bioactive compounds were extracted from endophytic fungi as crude extracts. The extraction was performed using the method of Kanomedhakul *et al*. (2003) by Song Jiaojiao *et al.* (2015b).

Bioactivity against C. capsici

The crude extracts were tested for inhibition of *C. coffeanum.* The experiment was conducted by using 3x6 factorials in Completely Randomized Design (CRD) with four replications. Factor A represented crude extracts which consisted of crude hexane, crude ethyl acetate and crude methanol and factor B represented concentrations 0, 10, 50, 100, 500, and 1,000 ppm. Each crude extract was dissolved in one drop 2% dimethyl sulphite (DMSO), mixed into 30 ml potato dextrose agar (PDA) before autoclaving at 121C , 15 p for 30 minutes. The tested pathogen were cultured on PDA and incubated at room temperature for 7 days, and then colony margin was cut by 3 mm diameter sterilized cork borer. The agar plug of pathogen was transferred to the middle of PDA(amending with each crude extracts) plate (5.0 cm diameter) in each concentration and incubated at room temperature (28°C-30°C) until the pathogen on the control plates growing full. Data were collected as colony diameter and the number of conidia. Percentage inhibition of pathogen colony growth and conidia production were calculated using the following formula:

% inhibition = (A-B) / A ×100

Where, A is the diameter of colony or number of conidia produced by the pathogen in control plates and B is the diameter of colony or number of conidia produced by the pathogen in treatment plates.

Data were statistically computed analysis of variance and treatment means were compared using Duncan Multiple's Range Test (DMRT) at P = 0.05 and 0.01. The effective dose (ED_{50}) will be calculated using probit analysis.

Results and Discussion

Isolation and identification of endophytic fungi

Two isolates of endophytic fungi were selected by screening for antagonistic activity in vitro against coffee anthracnose causing by *Colletotrichum coffeanum.* They were *Nigrospora sphaerica* (17-6) from *Chrysalidocarpus lotescens* and *Fusarium falciforme* (5r-1) from *Mascarena Lagencuulis* (Bottle palm).

***Description of Nigrospora sphaerica* (17-6)**

The white woolly colonies grow fairly rapidly. Colonies at first white with small, shining black conidia easily visible under a lower-power dissecting microscope, later brown when sporulation is abundant. Mycelium all immersed or partly superficial. Stroma none. Setae and hyphopodia absent. Conidiophores semi-macronematous, branched, flexuous, colourless to brown, smooth. Conidia solitary, with a violent discharge mechanism, acrogenous, simple, spherical or ellipsoidal, compressed dorsiventrally.

Black, shining, smooth, 0-septate (Fig. 1). The morphology seems to be *Nigrospora* spp. (17-6). And then, Phylogeny tree of *Nigrospora* spp. (17-6) was reconfirmed species based on ITS1-5.8S-ITS4 ribosomal gene sequence using Neighbor-joining method (Fig. 2) and *Nigrospora sphaerica* (17-6) was confirmed identification by morphological characters and molecular technique.

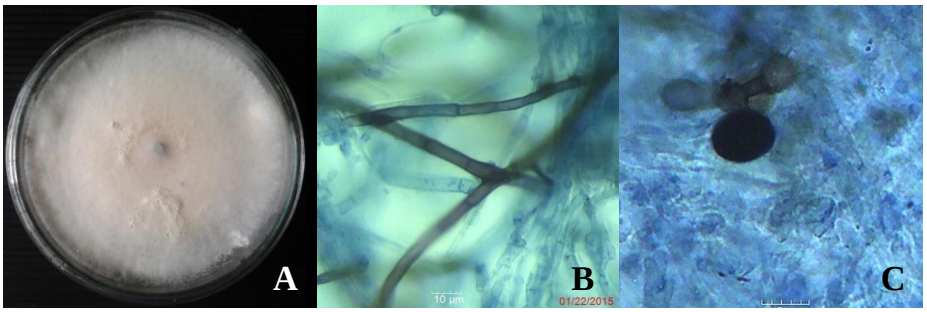

Figure 1. *Nigrospora sphaerica* **(17-6)** **A**: The colony culture on PDA 30 days **B:** mycelia (40X) C: Spores (40X)

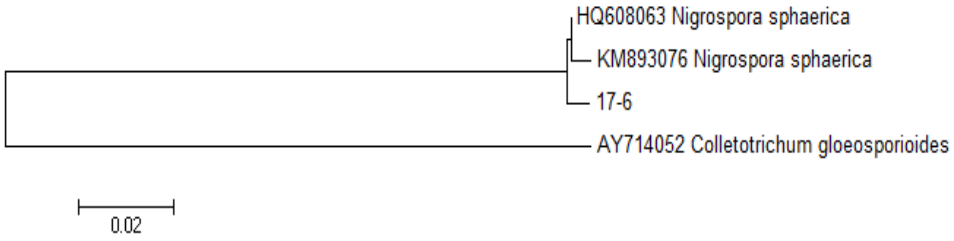

Figure 2. Phylogeny tree of *Nigrospora sphaerica* (17-6) based internal transcribed spacer (ITS) rDNA sequence using Neighbor-joining, bootstrap = 1000.

Description of Fusarium falciforme (5r-1)

The colonies fast growing with discrete sporodochia and white-ochraceous colour. Aerial mycelium floccose. Macro-conidia abundant and more-celled, slightly curved or bent at the pointed ends; central part straight, cylindrical, typically canoe-shaped. Phialides bearing micro-conidia very long (Fig. 3). The morphology seems to be *Fusarium* spp. (5r-1). And then, Phylogeny tree of *Fusarium* spp. (5r-1) is reconfirmed species based on ITS1-5.8S-ITS4 ribosomal gene sequence using Neighbor-joining method (Fig. 4) and *Fusarium falciforme* (5r-1) was confirmed identification by morphological characters and molecular technique.

Figure 3. *Fusarium* sp1. **A**: The colony culture on PDA 30 days **B:** mycelia and chlamydospores(40X) C: Macro-conidia and micro-conidia (40X)

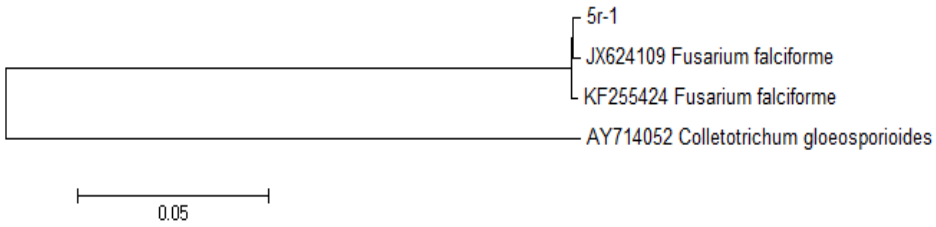

Figure 4. Phylogeny tree of *Fusarium falciforme* (5r-1) based internal transcribed spacer (ITS) rDNA sequence using Neighbor-joining, bootstrap = 1000.

Isolation of pathogen and pathogenicity test

Colletotrichum coffeeanum were isolated from anthracnose of coffee leaf with obvious symptom (Fig.5). The isolate was confirmed pathogenic isolate from pathogenecity test (Fig. 6). The result showed that pathogenic isolate could be infected in coffee leaf and caused symptom with the same symptom caused by *C. coffeannum* causing fruit anthracnose on coffee.

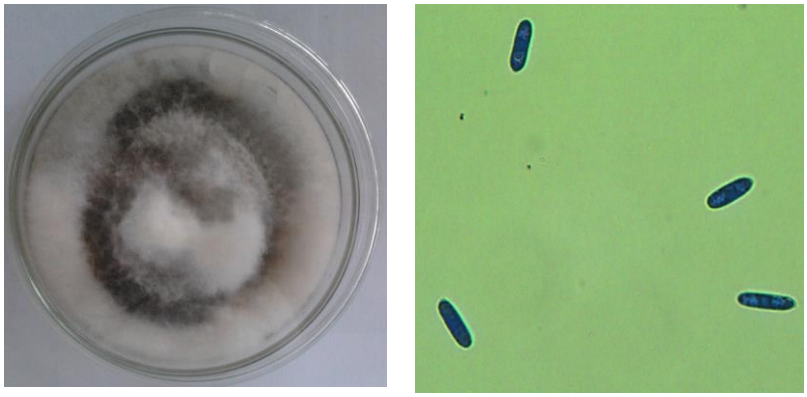

Figure 5. *Colletotrichum coffeanum* pure culture and spores

Inoculated one Non-inoculated control

Figure 6. Pathogenicity test on coffee leaf

Bioactivity against C. coffeanum

Endophytic fungi *Nigrospora sphaerica* and *Fusarium falciforme* were selected to yield metabolite as crude extracts and examined for bioactivity test against anthracnose of coffee caused by *C. coffeanum*.

The results showed that crude methanol from *Nigrospora sphaerica* gave highest inhibition of *C. coffeanum* colony growth which was 54.00% at the concentration of 1,000 ppm when compared to the control (Table 1). Methanol crude extract from *Nigrospora sphaerica* showed significantly highest inhibition for the spore production of *C. coffeanum* as 72.18% at the concentration of 1,000 ppm, and the ED_{50} inhibited *C. coffeanum* spore production at concentration 143.65 ppm. Followed by crude ethyl acetate gave 69.99% inhibition at concentration of 1,000 ppm, and ED_{50} at concentration of 404.53 ppm. Crude hexane showed 51.63% inhibition at concentration of 1,000 ppm, and ED_{50} at concentration of 1092.44 ppm (Table 2). This result was similar with Zhao J. H. *et al.* (2012) who reported that four antifungal secondary metabolites were isolated from endophytic fungi *Nigrospora sp.* and antifungal assay showed clear inhibition of the growth of 8 plant pathogenic fungi in vitro.

Methanol cude extract from *Fusarium falciforme* gave significantly highest inhibition of *C. coffeanum* colony growth which was 63.00% at the concentration of 500 ppm when compared to the control (Table 3). Methanol crude extract from *Fusarium falciforme* showed significantly highest inhibition for the spore production of *C. coffeanum* as 76.74% at the concentration of 500 ppm, and the ED_{50} inhibited *C. coffeanum* spore production at concentration 161.14 ppm. Crude ethyl acetate gave 68.83% inhibition at concentration of 1,000 ppm, and ED_{50} at concentration of 262.01 ppm; crude hexane showed 52.41% inhibition at concentration of 1,000 ppm, and ED_{50} at concentration of 1054.88 ppm (Table4). Meca *et al.* (2010) reported that some strains of *Fusarium tricinctum* are known to

produce different enniatins which have strong biological activities including antifungal properties. This study were similar to the study of Masroor Qadri *et al.* (2013) who reported that endophytic fungus, *Fusarium tricinctum* inhibited several phytopathogens significantly.

Table 1. Crude extracts of *Nigrospora sphaerica* testing for growth inhibition of *Colletotrichum coffeanum* at 7 days

Crude extracts	**Concentration (ppm)**	**Colony diameter (cm)[/1]**	**Growth inhibition(%)[/2,3]**
Crude Hexane	0	5.00[a]	-
	10	4.97[a]	0.50[i]
	50	4.91[ab]	1.75[hi]
	100	4.77[cd]	4.50[fg]
	500	3.63[h]	27.25[b]
	1000	4.26[fg]	14.75[cd]
Crude EtOAc	0	5.00[a]	-
	10	4.85[bc]	3.00[gh]
	50	4.75[d]	5.00[f]
	100	4.57[e]	8.50[e]
	500	4.33[f]	13.25[d]
	1000	4.19[g]	16.00[c]
Crude MeOH	0	5.00[a]	-
	10	4.98[a]	0.25[i]
	50	4.87[b]	2.50[h]
	100	4.63[e]	7.25[e]
	500	3.58[h]	28.25[b]
	1000	2.29[i]	54.00[a]
C.V.(%)		1.35	10.65

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

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

[3]/Inhibition(%)=R1-R2/R1x100 where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in treated plates.

Table 2. Spore production inhibition of crude extracts from *Nigrospora sphaerica* to *Colletotrichum coffeanum* at 30 days and effective dose (ED_{50}) values

Crude extracts	**Concentration (ppm)**	**Number of spores /1 (10^6)**	**inhibition(%)/2,3**	**ED_{50}**
Crude Hexane	0	5.56 a	-	1092.44
	10	5.25 ab	5.42 i	
	50	4.62 bc	16.66 g	
	100	4.12 cd	25.46 f	
	500	3.25 ef	41.39 d	
	1000	2.68 fg	51.63 c	
Crude EtOAc	0	5.56 a	-	404.53
	10	5.00 ab	10.05 h	
	50	4.06 cd	26.91 f	
	100	3.62 de	34.77 e	
	500	2.75 f	50.44 c	
	1000	1.68 h	69.99 a	
Crude MeOH	0	5.56 a	-	143.65
	10	4.18 cd	24.53 f	
	50	3.68 de	33.58 e	
	100	2.68 fg	51.69 c	
	500	2.00 gh	64.02 b	
	1000	1.56 h	72.18 a	
C.V.(%)		9.73	7.98	

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

2/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.01.

3/Inhibition (%) = R1-R2/R1x100 where R1was number of pathogen spores in control and R2 was number of pathogen spore in treated plate which number of spores are less than that in control .

Table 3. Crude extracts of *Fusarium falciforme* testing for growth inhibition of *Colletotrichum coffeanum* at 7 days

Crude extracts	**Concentration (ppm)**	**Colony diameter (cm)[/1]**	**Growth inhibition(%)[/2,3]**
Crude Hexane	0	5.00[a]	-
	10	3.70[d]	26.00[g]
	50	3.45[ef]	31.00[ef]
	100	3.41[f]	31.75[e]
	500	3.44[ef]	31.00[ef]
	1000	3.87[c]	22.50[h]
Crude EtOAc	0	5.00[a]	-
	10	3.48[ef]	30.25[ef]
	50	3.22[g]	35.50[d]
	100	3.21[g]	35.75[d]
	500	4.04[b]	19.00[i]
	1000	2.21[i]	55.75[b]
Crude MeOH	0	5.00[a]	-
	10	1.89[j]	62.00[a]
	50	2.71[h]	45.75[c]
	100	2.19[i]	56.00[b]
	500	1.84[j]	63.00[a]
	1000	3.61[de]	27.75[fg]
C.V.(%)		2.43	4.75

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

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

[3]/Inhibition(%)=R1-R2/R1x100 where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in treated plates.

Table 4. Spore production inhibition of crude extracts from *Fusarium falciforme* to *Colletotrichum coffeanum* at 30 days and effective dose (ED_{50}) values

Crude extracts	**Concentration (ppm)**	**Number of spores /1 (10^6)**	**inhibition(%) /2,3**	**ED_{50}**
Crude Hexane	0	8.00[a]	-	1054.88
	10	6.93[ab]	13.15[j]	
	50	6.68[bc]	16.09[j]	
	100	6.12[bcd]	23.38[i]	
	500	4.75[efg]	40.85[g]	
	1000	3.81[gh]	52.41[d]	
Crude EtOAc	0	8.00[a]	-	262.01
	10	5.75[cde]	27.91[hi]	
	50	5.37[def]	32.51[h]	
	100	4.31[fg]	46.41[ef]	
	500	4.00[gh]	50.26[de]	
	1000	2.50[ij]	68.83[b]	
Crude MeOH	0	8.00[a]	-	161.14
	10	4.93[efg]	38.31[g]	
	50	4.81[efg]	39.96[g]	
	100	3.12[hi]	61.21[c]	
	500	1.87[j]	76.74[a]	
	1000	4.62[efg]	42.35[fg]	
C.V.(%)		14.02	8.47	

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

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

[3]/Inhibition (%) = R1-R2/R1x100 where R1was number of pathogen spores in control and R2 was number of pathogen spore in treated plate which number of spores are less than that in control .

Conclusion

As results, it showed that endophutic fungi isolates *Nigrospora sphaerica* (17-6) from *Chrysalidocarpus lotescens* and *Fusarium falciforme* (5r-1) from *Mascarena Lagencuulis* were identified based on based on morphology and molecular phylogeny. *Nigrospora sphaerica* and *Fusarium falciforme* also were proved biological activity against *C. coffeanum* causing coffee anthracnose. The results demonstrated that crude methanol of *Fusarium falciforme* showed the best inhibition of colony growth and spore production of *C. coffeanum* at concentration of 500 ppm with effective dose (ED_{50}) for spore production inhibition at concentration 161.14 ppm.

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